UNIVERZITA PALACKÉHO V OLOMOUCI

Lékařská fakulta

MUDr. Vít Perlík

Patofyziologie endotoxinem indukované horečky

DISERTAČNÍ PRÁCE

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Prohlašuji, že jsem disertační práci zpracoval samostatně s použitím citované literatury.

V Olomouci dne: …… ……

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1. Úvod

U zdravých jedinců se tělesná teplota mění v závislosti na velkém počtu environmentálních a biologických faktorů, jako jsou denní doba, místo měření tělesné teploty, úroveň fyzické aktivity, věk (Florez-Duquet & McDonald, 1998), pohlaví, a rasa a řada dalších. (Lim et al., 2007). Navzdory proměnlivosti těchto faktorů je teplota těla regulována v poměrně konstantním rozsahu, označovaném jako tzv. bod tepelné rovnováhy (thermal balance point), (Romanovsky, 2007), procesem nazývaným termoregulace.

1.1. Termoregulace

Termoregulace jako schopnost regulovat vnitřní teplotu organismu je jedním ze základních vývojových kroků umožňujících větší nezávislost organismů na prostředí, ve kterém žijí. Poikilotermní organismy (studenokrevní) jsou do značné míry limitovány ve své aktivitě teplotou okolního prostředí (Romanovsky et al., 2000). Oproti tomu homoiotermní organismy (teplokrevní), vzhledem ke své schopnosti udržovat stálou tělesnou teplotu, a tím i relativně konstantní rychlosti metabolických procesů, jsou svou aktivitou na vnějším prostředí téměř nezávislí. Termoregulační systém umožňuje udržovat teplotu teplokrevných živočichů v požadovaném rozmezí prostřednictvím souhry řady specializovaných buněk, jež tvoří vlastní architekturu celého termoregulačního systému (Romanovsky, 2007). Jedná se o: termoreceptory, aferentní dráhy, termoregulační centrum v CNS, eferentní dráhy a efektorové orgány. Na základě souhry periferie a centra je tak zajištěna rovnováha mezi tvorbou nebo ztrátou tepla v organismu vedením, vyzařováním, sdílením a evaporací (Weller, 2005).

1.2. Termosenzory

Kožní termosenzory v souhře s vnitřními termosenzory zprostředkovávají termoregulačnímu centru přesný obraz o teplotním stavu tělesného jádra a povrchu těla. Tato teplotní čidla lze rozdělit podle jejich topiky a způsobu přenosu informací.

1.2.1. Periferní termosenzory

Kůže má dva anatomicky odlišné typy receptorů pro teplo a chlad, jež pro hypotalamické centrum regulace tepla zprostředkovávají informaci o teplotě prostředí, ve kterém se organismus nachází (Charkoudian, 2003; Morrison et al., 2008). Tepelné receptory stále zvyšují svou základní aktivitu, a to od 30 °C až do teplot kůže 44 - 46 °C. Chladové receptory na druhou stranu zvyšují svou základní aktivitu s poklesem teplot od 40 °C až k hodnotám 24 – 28 °C. Oba typy senzorů reagují jen na dynamickou komponentu (změny teploty), v případě ustáleného stavu se rychle adaptují. Taková odpověď umožňuje organismu rychle reagovat na změny prostředí (Romanovsky et al., 2006). Vnímání okolní teploty se tak děje v podstatě prostřednictvím primárních aferentních senzorických neuronů ganglií zadních kořenů míšních a trigeminálních ganglií. Existují důkazy, že za tento mechanismus teplotní citlivosti mohou zodpovídat někteří zástupci rodiny TRP (transient receptor potential) kanálů, jež pokrývají teplotní spektrum od chladu pod 8 ºC až po teplo nad 52 ºC, kdy už dochází k denaturaci proteinů. Jedná se o 4 receptory z podrodiny TRPV (vanilloid) receptorů detektujících teplo: TRPV4 >25ºC, TRPV3 >31ºC, TRPV1 >43ºC, TRPV2 >52ºC a dva zástupce podrodin TRPM (melastatin) a TRPA (ankyrin), které detekují chlad: TRPM8 <28ºC a TRPA1<18ºC (Okazawa et al., 2002; Charkoudian, 2003; Story et al., 2003; Dhaka et al., 2006; Romanovsky et al., 2006; Bandell et al., 2007; Morrison et al.,

2008). Přesný mechanismus teplotní aktivace TRP receptorů zůstává stále nejasný a je dosud předmětem četných studií. V úvahu připadají tři možnosti vedoucí k otevření kanálů. Jednak produkce a vazba ligandů, které následně otevírají kanál se změnou teploty, dále teplotně závislé strukturní změny proteinového komplexu iontového kanálu, které by přímo vedly k aktivaci iontového kanálu, a nakonec aktivace strukturními, teplotně závislými změnami lipidové dvojvrstvy, jež by kanál mechanicky otevřely. Nejlépe prozkoumaný chladový receptor TRPM8, mezi jehož ligandy patří mimo jiné chladivá látka mentol (Reid, 2005), se aktivuje nejen teplotami pod 28 ºC (McKemy et al., 2002), ale také depolarizačním napětím. Pro aktivaci a regulaci TRPA1 receptoru mají zásadní význam vápenaté ionty. Extracelulární vápenaté ionty zesilují proudy vyvolané alylisothiokyanátem (AITC), indukují desenzitaci a tachyfylaxi po opakovaných aplikacích. Bez jejich přítomnosti k plné aktivaci kanálu nedojde (Fernandez et al., 2011; Jordt et al., 2004; Nagata et al., 2005). Chladové signály z periferie jsou zprostředkovány tenkými myelinizovanými A-δ vlákny. Méně běžné senzory snímající vyšší teploty jsou umístěny o něco hlouběji do podkoží, jejich signály přenášejí nemyelinizovaná C vlákna (Lumpkin a Caterina, 2007; Romanovsky et al., 2006).

1.2.2. Centrální termosenzory

Termoregulační reakce může být iniciována i z oblasti centrálních termoreceptorů, nacházejících se v různých částech CNS (mozkový kmen, spinální mícha). Zásadní význam však mají termoreceptory umístěné v preoptické oblasti hypotalamu (POA), kde byl zjištěn velký počet na teplo reagujících neuronů. Neurony POA jsou považovány za nejdůležitější pro spuštění autonomní termo-efektorní odpovědi (Romanovskyet al., 2006).

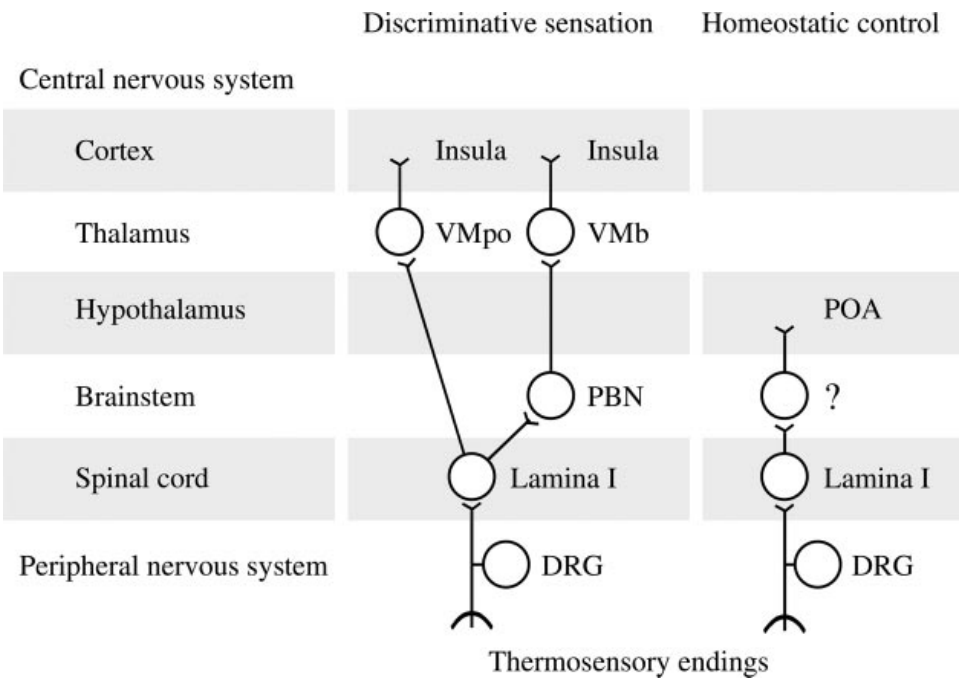
Vnitřní receptory jsou i mimo CNS, v zadní stěně břišní dutiny a podél velkých cév (Campbell, 2011).

1.3. Dráhy termoregulace

1.3.1. Aferentní dráhy termoregulace

Aferentní dráhy začínají primárními termosenzory. Jedná se o bipolární neurony, jejichž těla jsou v gangliích zadních kořenů míšních. Centrální axony těchto neuronů vytvářejí synapse v oblasti zadních rohů míšních (především v lamina I) se sekundárními neurony. Axony sekundárních neuronů kříží střední čáru a tvoří ascendentní část funikulus lateralis spinální míchy. Dlouhou dobu se předpokládalo, že sekundární neurony projikují přímo ipsilaterální ventrobazální komplex thalamu, z něhož jsou signály třetím neuronem přenášeny do somatosenzorické korové oblasti - gyrus postcentralis. Dle Craiga (2003) se tato dráha účastní taktilního čití, není však dráhou termoregulace. Signály o teplotě přenášejí do kůry (insula Reil) neurony lamina-I s přepojením v posterolaterálním thalamu (konkrétně zadní části ventromediálního jádra) nebo přes dvojí přepojení v parabrachiálních jádrech a bazální části ventromediálního jádra thalamu. Tyto dvě větve spino-thalamo-kortikální dráhy se podílejí na diskriminační percepci teploty (zadní insulární korový region), zatímco korelace se subjektivním hodnocením se nachází v orbitofrontální části insulární kůry (Nomoto et al., 2004), (Obr. č. 1). Dráha vykazuje topické uspořádání (Hua et al., 2005). Odpovědi termoefektorů se spouštějí na podkladě dostatečně silné teplotní expozice, která je schopna způsobit teplotní výměnu mezi organismem a prostředím.

Obrázek č. 1: Schematické znázornění termoregulačních aferentních drah *(převzato z: Romanovsky A: Thermoregulation: some concepts have changed. Functional architecture of the thermoregulatory system. Am J Physiol Regul Integr Comp Physiol. 2007, 292:R37-R46).*

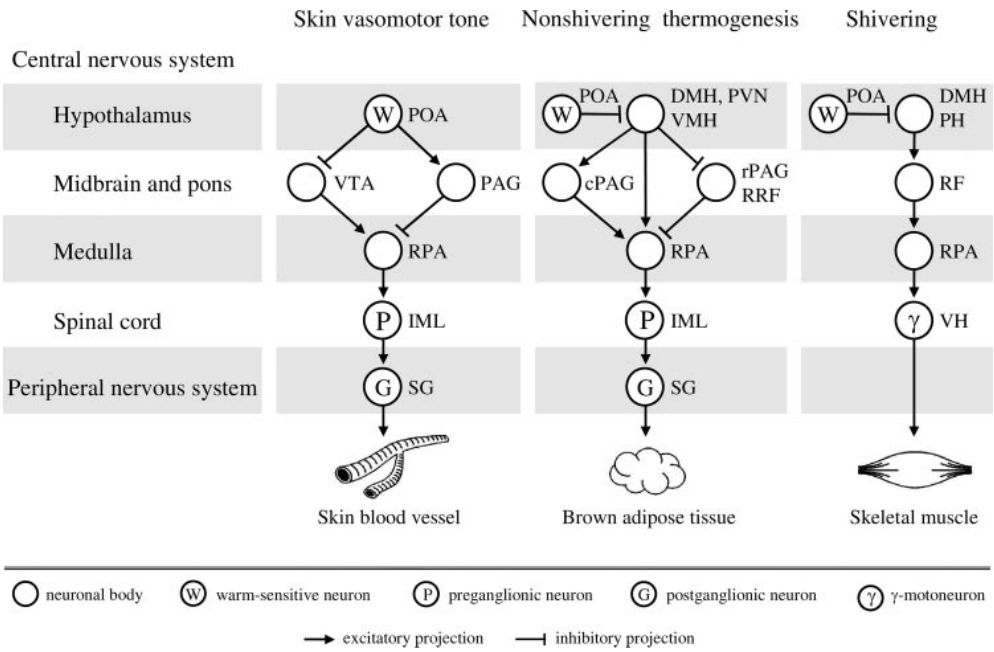


Vysvětlivky zkratek: DRG, dorsal root ganglion; PBN, parabrachial nucleus; POA, preoptic anterior hypothalamus; VMb, part basalis nucleus ventromedial thalami VMpo, pars posterior nucleus ventromedial thalami.

1.3.2. Eferentní dráhy termoregulace

Eferentní termoregulační dráhy směřují k periferním cévám a termogenním orgánům. Vaskulatura hnědé tukové tkáně a kůže je na periferii přímo ovlivněna gangliemi sympatiku (SG). Pregangliové neurony (P) jsou lokalizovány v intermediolaterálním sloupci spinální míchy (IML). Míšní neurony přijímají přímé vstupy z buněk, které se nacházejí především v raphé /peripyramidální oblasti medully (RPA). Tyto míšní buňky jsou pod kontrolou dorsomediálních a paraventrikulárních jader hypotalamu, středního mozku (oblast periaqueduktální šedé hmoty, retrobulbární oblast a ventrální tegmentální area) a pontinních neuronů z oblasti locus ceruleus se superpozicí nadřazeného preoptického centra (POA) (Bamshad et al., 1999; Morrison et al., 2008). I přes značnou podobu eferentních drah směřujících ke kožním vasomotorům a do BAT nejsou tyto dráhy totožné. Významné odlišnosti představuje pak dráha k příčně pruhovaným svalům, zajišťující třesovou termogenezi. Třesová termogeneze je mediována cestou α- a γ- ventrálních motoneuronů (VH). Tyto motoneurony přijímají přímé i nepřímé impulzy ze středního mozku a mozkového kmene včetně raphé/peripyramidální arei (RPA), traktem retikulospinálním a rubrospinálním. Neurony středního mozku jsou pod kontrolou neuronů dorsomediálního a zadního hypotalamu (DMH, PH) a jsou inhibovány z oblasti termosenzitivních neuronů preoptické oblasti (POA), (Obrázek č. 2).

Obrázek č. 2: Schematické znázornění eferentních drah termoregulace. *(převzato z: Romanovsky A: Thermoregulation: some concepts have changed. Functional architecture of the thermoregulatory system. Am J Physiol Regul Integr Comp Physiol. 2007, 292:R37-R46).*



Vysvětlivky zkratek: DMH, dorsomedial hypotalamus, IML, intermediolateral column; PAG periaqueductal gray matter; cPAG, caudal PAG; rPAG, rostral PAG; PH, posterior hypothalamus; PVN, paraventricular nucleus; RF, reticular formation; RPA, raphe´/peripyramidal area; RRF, retrorubral field; SG, sympathetic ganglia; VH, ventral horn; VMH, ventromedial hypothalamus; VTA, ventral tegmental area.

Z výše uvedeného lze tedy shrnout, že centrum termoregulace je v preoptické oblasti předního hypotalamu (POA). Dochází zde k integraci informací z periferních senzorických neuronů a centrálních termoreceptorů s řízením efektorových systémů, které prostřednictvím autonomního nervového systému regulují předávání tepla do okolí nebo jeho produkci organismem v případě potřeby (Boulant, 2000; McAllen, 2004; Egan et al., 2005; DiMicco & Zaretsky, 2006; Mahmood & Zweifler, 2007; Nakamura & Morrison, 2008a,b). Novější experimentální práce (DiMicco & Zaretsky, 2006) dokládají, že významnou roli v termoregulaci mají i neurony v oblasti dorsomediálního hypotalamu (DMH). Zaretskiaia a kol. (2002) poskytla jasnou informaci o tom, že aktivace neuronů v oblasti DMH vede k dramatické změně tělesné teploty klasickou termoregulací.

1.4. Termogeneze

Procesy termogeneze mohou v podstatě probíhat různou měrou ve většině tkání, protože tvorba tepla souvisí s tvorbou a využíváním ATP. Nicméně cílené termogenní procesy se spouštějí obvykle jako odpověď na působení chladu prostředí, pokles teploty jádra, nebo jako odpověď na působení pyrogenních cytokinů ve třech orgánech: a) v hnědé tukové tkáni (brown adipose tissue – BAT), b) v srdečním svalu, c) v příčně pruhovaných svalech. Integrální složkou těchto dějů je i regulace vasomotorická.

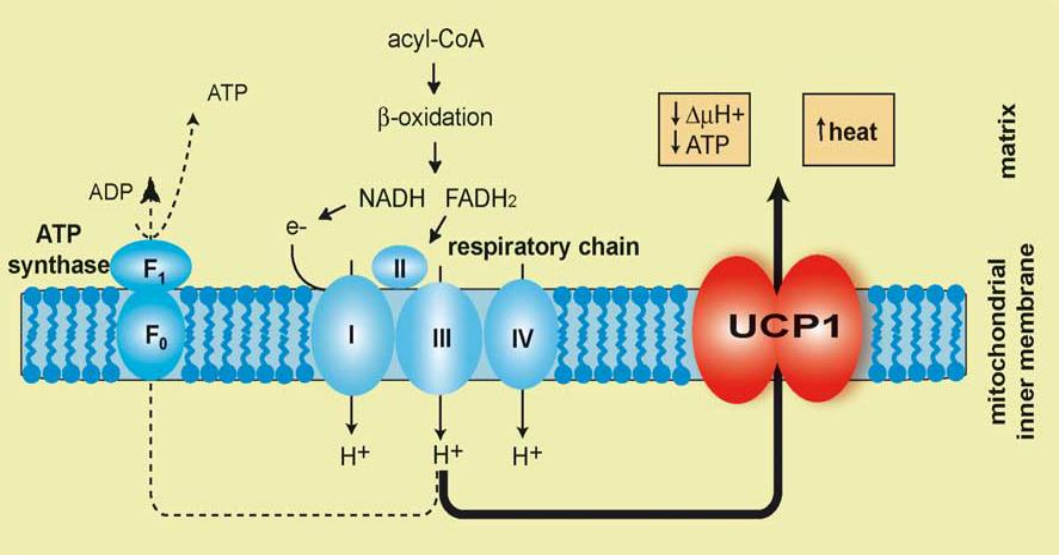
1.4.1. Metabolická termogeneze

Tvorbu energie spojenou s oxidací živin tvoří dvě hlavní složky: produkce ATP a tvorb tepla (zvýšení tělesné teploty = termogeneze). Úplná oxidace energetických substrátů jako je glukóza, mastné kyseliny, za vzniku konečných metabolitů oxidu uhličitého a vody, se odehrává na vnitřní mitochondriální membráně v procesu buněčné respirace. Děje se tak transportem a transferem protonů (H^+), kaskádou přes čtyři proteinové komplexy dýchacího řetězce katalyzující redukci kyslíku. V důsledku toho vzniká protonový gradient na vnitřní mitochondriální membráně (Masopust, 2005). Metabolická energie, která se tímto mechanismem tvoří, je ATP-synthasou inkorporována do ATP, jako zdroje energie pro veškeré energeticky dependentní reakce. Pokud nedojde k zachycení protonů při přechodu mitochondriální membránou proteinovým komplexem ATP-synthasy, uniklá energie se mění v teplo, které zvyšuje tělesnou teplotu (termogeneze) a je vyzařováno do okolí. Říká se tomu rozpojení (uncoupling proces) fosforylace od oxidace. Regulace rozpojovacího procesu se děje pomocí rodiny tzv. uncoupling proteinů, (UCPs, rozpojovací /odpřahovací proteiny), (Garlid et al., 2000). Nejlépe je zatím

charakterizován rozpojovací protein-1(UCP-1), vyskytující se ve velkém množství v tzv. hnědé tukové tkáni. Hnědá tuková tkáň se vyvinula jako základní termoregulační efektor obrany proti chladu u hlodavců a jiných malých savců (Trayhurn, 1993; Golozoubova et al., 2006), včetně lidských novorozenců (Cinar & Filiz, 2006). Velký podíl hnědé tukové tkáně na tělesné hmotnosti u těchto jedinců umožňuje udržení dostatečné tělesné teploty v chladném prostředí metabolickou cestou.

Mechanismus účinku této termogenní reakce spočívá v tom, že na zvýšenou aktivitu sympatiku (Bamshad et al., 1999), navozenou např. chladem, reaguje hnědá tuková tkáň vzestupem lipolýzy, a tím uvolněním neesterifikovaných kyselin, které následně stimulují tvorbu UCP-1 (Cannon & Nedergaard, 2004; Nakamura & Morrison, 2007). Rozpojovací proteiny fungují jako iontové kanály. Jejich otevření snižuje mitochondriální membránový potenciál. Důsledkem je větší únik protonů vnitřní mitochondriální membránou a zvýšený výdej energie ve formě termogeneze (Obrázek č. 3). U dospělého člověka bylo nalezeno jen velmi malé množství hnědé tukové tkáně. Musí zde být tedy ještě i jiné rozpojovací proteiny než UCP-1. Bylo prokázáno že „UCP-like“ proteiny jsou evolučně velmi staré a pro život nezbytné; vyskytují se v určité formě už u rostlin, protozoí, a pak v evoluci dále až po člověka. U dospělých lidí byly objeveny dva rozpojovací proteiny s označením UCP-2 a UCP-3.

Obrázek č. 3: Schematické znázornění vzniku tepla při metabolické termogenezi *(převzato z: Sell H., Deshaies Y., Richard D.: The brown adipocyte: update on its metabolic role. The International Journal of Biochemistry & Cell Biology. 2004, 36:2098-2104).*



1.4.2. Třesová termogeneze

Při třesové termogenezi se energie uvolňuje mimovolními stahy příčně pruhované svaloviny, jež nevykonávají žádnou svalovou práci (GLOSSARY-IUPS, 2001). K těmto dějům řadíme svalový třes a svalový tonus. Takto uvolněná tepelná energie má u většiny živočichů primární termoregulační význam. Zahájení třesové termogeneze vyžaduje chladový podnět (Eyolfson et al., 2001), který obvykle pochází z kožních receptorů, případně z jiných míst (orální a urogenitální mukóza, vnitřní orgány, hypotalamus, střední mozek, mozkový kmen nebo mícha).

Po vystavení organismu chladu nejprve dochází ke svalovému tonu a při dalším zvětšování chladu nastupuje svalový třes. Zvětšená intenzita ochlazování vede k tomu, že stahy svalů nabývají na intenzitě a že dochází k synchronizaci jednotlivých stahů do tzv. výbuchů o frekvenci 10 – 35 Hz. Mechanismus koordinace různých motorických jednotek během tohoto děje je vyvolán periodickými inhibicemi impulzů v dostředivých nervových vláknech (tj. oscilačními ději v aferentní složce) a ne centrální mozkovou synchronizací těchto podnětů. Třesová termogeneze je tak v podstatě reflexní děj zprostředkovaný somatickými nervy, během něhož kosterní svaly uvolňují značné množství tepla jako vedlejší produkt (Tanaka et al., 2006). Zdrojem energie jsou karbohydráty a lipidy, proteiny se podílí jen málo. (Haman, 2006). Termogeneze ve svalu je zahájena α-motorickou stimulací s následnou depolarizací membrány svalových buněk a uvolňováním iontů vápníku ze sarkoplazmatického retikula. Zvýšená hladina Ca^{2+} vede k aktivaci myosinové ATP-ázy se vznikem ADP, AMP a adenosinu. Nárůst ADP urychluje mitochondriální oxidační fosforylaci, zvyšuje spalování paliv. Postupnou degradací makroergních fosfátových vazeb vzniká adenosin s vasodilatačním účinkem, který vede k vyššímu prokrvení a zvýšení metabolismu.

1.4.3. Regulace průtoku

Mezi termo-efektorové systémy patří i regulace průtoku krve kůží prostřednictvím sympatiku a řízení vazokonstrikce hladkého svalstva arteriol a sympatikem regulovaných potních žláz (Charkoudian, 2003; Hodges et al., 2008; Kurz, 2008; Johnson and Kellogg, 2010). Vasodilatační a vasokonstrikční nervy sympatiku inervují všechny oblasti ochlupené kůže, zatímco oblasti lysé kůže (dlaně, chodidla, rty) jsou inervovány jen sympatickými vazokonstrikčními nervy. Dalším důležitým rozdílem mezi lysou a ochlupenou kůží je existence arteriovenózních anastomóz (AVA), které jsou silnostěnné, s nízkým odporem vedení umožňujícím vysoké průtoky přímo z tepének do žil. V lysých kožních oblastech jsou anastomózy četné, bohatě inervované sympatickými vazokonstrikční nervy. Otevření nebo zavření anastomóz v těchto oblastech může proto způsobit podstatné změny v průtoku krve kůží (Lossius et al., 1993). V kontrastu s tím ochlupená kůže obsahuje málo anastomóz a její cévy jsou inervovány jak vasokonstrikčními, tak i vasodilatačními nervy.

Sympatické vazokonstrikční nervy uvolňují noradrenalin působící prostřednictvím vazby na postsynaptické α-receptory kožních arteriol a AVA. Kromě toho noradrenergní vazokonstrikční nervy uvolňují co-transmittery, které také působí vasokonstrikční reakci (Stephens et al., 2001; Stephens et al., 2002).

Potní žlázy jsou inervovány cholinergně ze sympatického nervového systému. Jedná se o vyjímku, kdy je acetylcholin postgangliovým neurotransmiterem sympatiku (Guiton and Hall, 2006).

1.5. Hormonální ovlivnění termoregulace

Kromě hormonů, které regulují termoregulaci v rámci již popsaných dějů, se na termoregulaci podílejí i některé další.

Klíčový význam v odpovědi na chlad nese štítná žláza. Její funkce je při vystavení chladu ovlivňována působením hypotalamických thyrotropin-releasing hormonů, které zvyšují produkci thyroxinu a trijodothyroninu. V experimentu bylo prokázáno, že po odstranění štítné žlázy u laboratorních potkanů docházelo u zvířat vystavených působení chladu k úhynu do šesti dnů, pokud nebyla prováděna suplementace thyroxinem. Přesný mechanismus působení není znám, předpokládá se, že trijodotyronin je schopen zvýšit expresi UCP, což vede k využívání protonů ke tvorbě tepla, nebo působí jako druhý posel a zvyšuje oxidativní fosforylaci v mitochondriích (Wheeler, 2006; Campbell, 2011).

V termoregulaci významný je i melatonin, hormon vylučovaný epifýzou, který je zodpovědný za denní kolísání hladin ostatních hormonů. Je prokázáno, že narušením jeho sekrece (např. změnou spánkového rytmu) se mění i regulace tělesné teploty. Exogenní podání melatoninu v experimentálních podmínkách způsobuje pokles tělesné teploty (Wheeler, 2006).

Pohlavní hormon progesteron vykazuje termogenní aktivitu, působí teplotní změny během normálního menstruačního cyklu rozsahu cca 5°C (Wheeler, 2006).

Na termogenních metabolických pochodech se podílejí i inzulín a glukagon, nemají však primární termogenní regulační efekt (Silva, 2006).

2. Horečka

Horečka jako významný rys řady především infekčních nemocí, byla předmětem pozornosti již od starověku (Blatteis, 1986). Febrilní reakce je řízena centrální nervovou soustavou přes endokrinní, neurologické, imunologické a behaviorální mechanismy. Horečku, na rozdíl od jiných typů změn tělesné teploty, často doprovázejí různé poruchy chování a změny. Jak ty metabolické, tak změny funkcí systémů a změny v imunitní odpovědi. Horečka a febrilní reakce významně přispívají k patogenezi, klinickému obrazu a průběhu mnoha nemocí a chorob.

Oproti hypertermii horečka představuje regulovaný vzestup teploty vyvolaný exogenními či endogenními pyrogeny syntetizovanými jako reakce na infekční případně jiné onemocnění (Cannon, 2011).

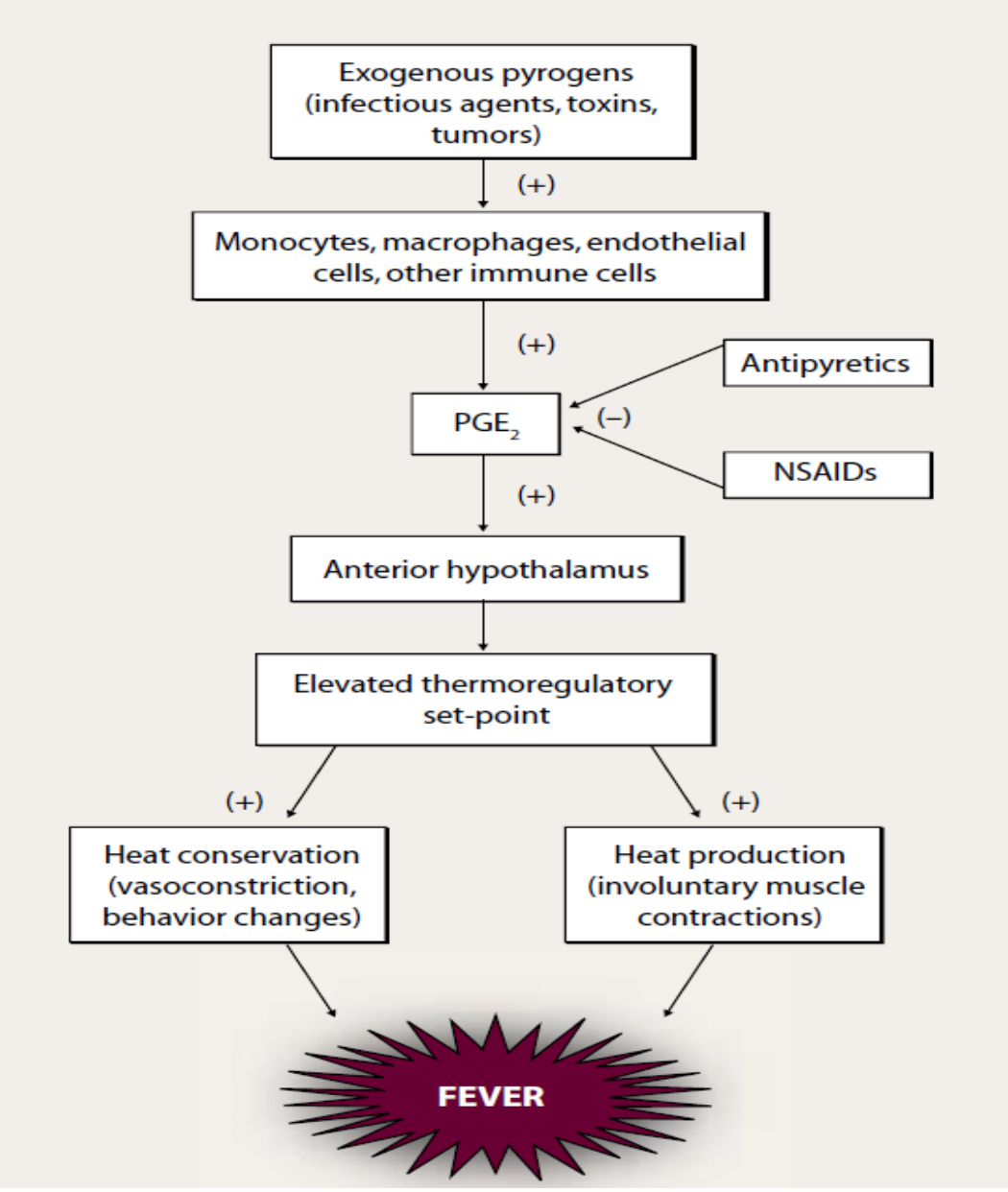
Horečka je tedy jedním ze základních obranných mechanismů organismu proti infekčním patogenům jako jsou viry, bakterie a parazité, stejně jako projevem jiných neinfekčních insultů, jako jsou nádorová onemocnění, metabolická onemocnění, alergické reakce, transfuze, CNS onemocnění embolizace, hemoragie atd.

Mezinárodní unie (International Union of Physiological Sciences Commission for Thermal Physiology - IUPS Thermal Commission) v roce 2001 definovala horečku jako stav zvýšené teploty jádra, která je často, ale ne nezbytně, součástí obranné reakce mnohobuněčných organismů vůči invazi mikroorganismů nebo neživé hmoty rozpoznané hostitelem jako patogenní (GLOSSARY, 2001).

Horečku vyvolávají zánětlivé mediátory (prostaglandiny, cytokiny), produkované buňkami imunitního systému, který je aktivován kontaktem s cizorodými molekulami (exogenní pyrogeny). Tyto mediátory indukující febrilní reakci jsou produkovány hostitelskými buňkami a nazývají se endogenní pyrogeny. Výsledné regulace tohoto jevu je dosaženo

antagonním působením dvou typů endogenních cytokinů, z nichž některé působí jako pyrogeny, ostatní jako kryogeny (antipyretika), obrázek č. 4.

Obrázek č. 4: Schematické znázornění regulace horečky *(převzato z: Dalal S. and Zhukovsky D.S.: Pathophysiology and Management of Fever. J Support Oncol. 2006, 4:009–016).*



2.1. Endogenní pyrogeny

Endogenní pyrogeny, které prezentují indikátory horečky, jsou cytokiny ze skupiny interleukinů (IL): IL-1, IL-6, IL-8, makrofágový zánětlivý protein-1 (macrophage-inflammatory protein-1, MIP-1), interferon-gama, (INF-γ) a interferon-alfa (INF-α), (Dafny, 1998).

Endogenní antipyretický účinek zajišťují v průběhu febrilních reakcí kryogeny, cytokiny, které regulují teplotní vzestup i trvání febrilní reakce. Jsou představovány především IL-10, arginin vasopresinem (AVP), α-melanocyty stimulujícím hormonem (α-MSH) a glukokortikoidy. Kryogeny realizují antipyretický účinek inhibicí syntézy pyrogenních cytokinů (glukokortikoidy), blokádou cytokinových receptorů (IL-1 receptor), zvýšením tepelných ztrát organismu, zvýšením citlivosti termoregulačních neuronů (bombesin) a některými dalšími. Tyto endogenní antipyretické systémy tak chrání hostitele před ničivými důsledky nekontrolované horečky.

Obě skupiny cytokinů prostřednictvím hypotalamického termoregulačního centra nastavují tělní termostat na vyšší/nižší hodnoty, což vede k nastartování řady behaviorálních a fyziologických pochodů s následnou změnou v produkci tepla v organismu (Luheshi, 1998; Leon, 2001).

Signály zajišťované exogenními a endogenními pyrogeny v konečném důsledku vedou k resetu termoregulační centra dvěma základními, v podstatě nezávislými cestami, humorální a nervovou aferentací (Ootsuka, 2008).

2.2. Exogenní pyrogeny

Infekci, jež vyvolává zánětlivou odpověď, způsobují různé patogenní podněty, které vstupují do těla hostitele. Mohou to být mikroorganismy (viry, bakterie, mykobakterie, plísně a parazité), non-mikrobiální agens (různé antigeny, zánětlivé látky, alkaloidy), nebo syntetické produkty (polynukleotidy, nádorové látky, immunoadjuvants), produkty hostitelských látek (zničena tkáň, antigen-protilátka, aktivované fragmenty komplementu, metabolity a produkty lymfocytů), (Silbernagl and Lang, 1998).

Všechny tyto patogenní podněty mohou vyvolat zánětlivou reakci vedoucí k horečce. Proto jsou považovány za hlavní induktory horečky - pyrogeny. Vzhledem k tomu, že jsou většinou odvozeny z externího prostředí, nazývají se exogenní pyrogeny. V organismu jsou uvolňovány po poranění tkání mechanickou, chemickou nebo fyzikální noxou (extrémní teploty, radiace, toxiny), nebo se dostanou do těla přes porušenou kůži, respirační trakt, GIT nebo urogenitálním systémem. Mikroorganismy musí atakovat, či alespoň adherovat na hostitelskou buňku, nebo buňky obsadí (viry), aby byly schopny se množit a šířit v těle, a tím vyvolat infekci. Infekce viry indukuje syntézu interferonů (INF) v infikovaných buňkách. RNA viry jsou silnější induktory interferonu než DNA viry. Mohou být produkovány i aktivovanými buňkami imuno-systému: INF-α je syntetizován převážně leukocyty, INF-β hlavně fibroblasty a INF-γ pouze lymfocyty. Interferony vykazují silnou antivirovou aktivitu syntézou proteinů, které inhibují replikaci virů, ovlivňují také humorální a buněčnou imunitu a růstovou aktivitu mikroorganismů. INF byly nalezeny i v mozku, kde mohou působit jako neuromodulátory (Dafny, 1998). Většina patogenních bakterií produkuje toxiny jedné ze dvou skupin: *exotoxiny* a *endotoxiny*.

2.2.1. Exotoxiny

Exotoxiny jsou vylučovány Gram-pozitivními i Gram-negativními bakteriemi během jejich růstové fáze nebo v důsledku lytických pochodů, způsobených aktivní obranou organismu, případně působením antibiotik. Jsou to velmi toxické, silně antigenní polypeptidy s molekulovou hmotností 10 - 900 kD, relativně nestabilní. Toxicitu ztrácejí při teplotách nad 60°C (tetanus, botulo-toxin, cholera-toxin, Diphteria-toxin). Exotoxiny těchto typů obvykle neindukují horečnatou reakci. Dle biologických efektů se obvykle dělí na neurotoxiny - produkty např. klostridií (botulotoxin, tetanospasmin), cytotoxiny - *Corynebacterium diphteriae* (difterický toxin) a enterotoxiny stimulující hypersekreci vody a elektrolytů z intestinálního epitelu a působící vodnaté průjmy (*Vibrio cholerae, Shigely, E.coli*).

2.2.2. Endotoxiny

Endotoxiny jako toxiny patogenních mikrobů - lipopolysacharidy (LPS) jsou složkou vnější membrány u G-bakterií (Reitschel et al., 1994; Sutherland & Kennedy, 1986; Steiner et al., 2006b). Mají výrazný vliv na lidský organismus. Ovlivňují imunitní aparát, zodpovědný za řadu příznaků a poškození vyvolaných bakteriálními infekcemi (nespecifická odpověď) jako jsou: produkce interleukinů makrofágy, horečka, průjem, zvracení, zvýšení permeability cév, vasodilatace (pokles tlaku), srážení krve, oběhová selhání (septický šok). Na rozdíl od exotoxinů se uvolňují až po zániku bakterie (přirozená lýza, desintegrace in vitro). Ve srovnání s exotoxiny jsou tepelně stabilní, s molekulovou hmotností v rozmezí 5 - 9000 kD. Jsou méně toxické, mohou být řadou metod získány ze suspenzí Gram-negativních bakterií (Derveau et al., 1983). Savci se s endotoxiny běžně setkávají v důsledku přítomnosti Gram-negativních bakterií v GIT, které představují součást střevní flory. Za normálních okolností se

endotoxin z bakterií rezidentní flory tlustého střeva vstřebává a stimuluje imunitní systém. Toxicita endotoxinu se uplatňuje v případech systémových onemocněních, při bakteriémii nebo v tkáních, nikoliv v lumen GIT.

LPS jsou zakotveny ve vnější membráně lipidem A - kovalentně navázaný cukr s 8 atomy uhlíku a ketodeoxyoktonát (vázaný na oligosacharidy), vytváří vysoce variabilní strukturu O-antigenu G-bakterií (Mayer et al., 1989). Lipopolysacharidy mají strukturální oblasti - specifický polysacharid (O-řetězec), který je spojen s dřeňovou oblastí (core) a ta je napojena na lipid A, polysacharidová část je tvořena O-specifickým řetězcem (z oligosacharidů, nese antigenní determinanty, určuje sérologickou specifitu bakteriálního druhu, chybí u *Neisseria meningitidis, N. gonorrhoeae, Haemophilus influenzae, Bordetella pertusis*. Dřeňový polysacharid je vázán kovalentně na lipid A, jehož kostra je složena ze dvou molekul glukosaminu spojených fosfátovými můstky - hydroxylové skupiny jsou esterifikovány vyššími MK, lipid A je zodpovědný za toxicitu a antigenitu.

LPS aktivují komplement alternativní cestou. Podstatná část účinku je způsobena lipidem-A, který je spolu s LPS silným aktivátorem makrofágů (indukce řady cytokinů regulujících imunitní a zánětlivou odpověď). Biologická aktivita LPS se projeví pouze po uvolnění z vnější membrány vazbou na plasmatický protein, který se podílí na vazbě LPS na CD14 receptor (monocytomakrofágový systém), (Liu et al., 1998; Romanovsky et al., 1998; Ivanov et al., 2003; Feleder et al., 2007).

2.3. Humorální cesty aktivace horečky

2.3.1. První humorální cesta

První humorální cestou jsou signály zajištěny komponentami mikrobních produktů tzv. patogen associated molecular patterns (PAMPS) nebo pyrogenními cytokiny. Cirkulující PAMPS mikroorganismů jsou prezentované gramnegativními LPS, známé jako toll-like receptors 4 (TLR-4) na různých buňkách. Vazbou a aktivací TLR-4 lokalizovaných na fenestrovaných kapilárách dochází k uvolnění prostaglandinu E_2 (PGE_2). Malá molekula prostaglandinu E_2 snadno proniká přes hematoencefalickou bariéru, váže se na EP_3 receptor v preoptické arei, čímž aktivuje a přelaďuje na vyšší hodnotu termální neurony v předním hypotalamu (Steiner et al., 2006a, 2006b; Romanovsky et al., 2006). Febrilní odpověď se vyznačuje rychlou ranou fází se zpožděním pozdní fáze. Na základě studie provedené na zvířecích modelech s LPS evokovanou horečkou, první fáze této reakce je závislá na PGE_2, syntetizovaném v játrech a plicích před migrací do mozku, a představuje zahájení febrilní reakce. Druhá fáze je mediovaná centrálně syntetizovaným PGE_2 a podílí se na prolongaci reakce (Gross, 2006; Steiner et al., 2006b).

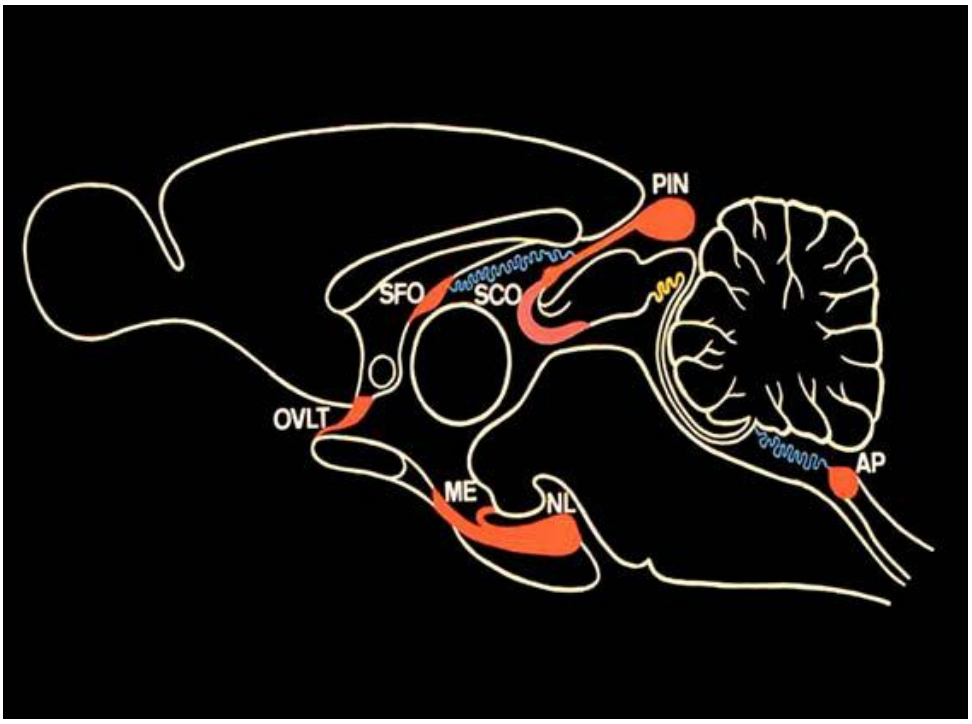
2.3.2. Druhá humorální cesta

Druhá humorální cesta je řízena v oběhu cirkulujícími pyrogenními cytokiny. Ty přenášejí febrilní signály přímo i nepřímo. V nepřímé cestě pyrogenní cytokiny působí mimo mozek vazbou a aktivací receptorů cytokinů na fenestrovaných kapilárách circumventrikulárních orgánů, (Takanashi et al., 1997; Roth et al., 2006) což vede k uvolňování PGE_2.

Circumventrikulární orgány (CVOs) jsou struktury CNS, které mají buňky v kontaktu s cerebroventrikularním systémem. Mají husté prokrvení a méně kompaktní hematoencefalickou bariéru. Podskupina CVOs se nazývá senzorická CVOs a zahrnuje cévní orgán laminae terminalis (OVLT), subfornical orgán (SFO) a oblast area postrema (AP). Tyto mozkové struktury mají kapiláry s fenestrovanými endoteliemi obklopené perivaskulárními prostory. Vzhledem k nekompaktní hematoencefalické bariéře jsou buňky těchto oblastí přímo vystaveny působení cirkulujícím signálním molekulám, a signály pak vnímají prostřednictvím specifických receptorů. OVLT a SFO se nacházejí v přední stěně třetí komory - lamina terminalis. AP je součástí hřbetu vagového motorického komplexu, hlavního viscerosensorického a autonomního centra v prodloužené míše. Umístění smyslové (a jiné) CVOs jsou znázorněny schematicky na obrázku č. 5.

Přímou cestou cirkulující cytokiny penetrují hematoencefalickou bariéru a působí na cytokinové receptory exprimované na cévních, gliových a neuronálních strukturách mozku. Aktivace těchto centrálních receptorů stimuluje další syntézu PGE_2 a podporuje de-novo syntézu cytokinů v mozku. I když PGE_2 představuje základní regulátor horečnaté reakce, některé cytokiny a mnoho dalších mediátorů mohou aktivovat febrilní odpověď nezávisle na PGE_2 (Roth et al., 2001). Patří k nim například bradykinin, kortikotropin uvolňující hormon, oxid dusnatý, MIP-1, IL-6 a IL-8, předem pyrogenní faktory (PFPF), substance P a endotelin-1 (Roth et al., 2001).

Obrázek č. 5: Schematický diagram sagitálního řezu mozku laboratorního potkana. Místa označená červeně představují oblasti s vyšší propustností hematoencefalické bariéry *(převzato z: Roth et al., Molecular Aspects of Feverand Hyperthermia. Neurol Clin. 2006, 24:421-439).*



ME- eminencia medialis; NL- neural lobe of the pituitary; PIN- pineal organ; SCO- subcomissural organ.

2.4. Neuronální cesta aktivace horečky

Na periferii vznikající febrilní signály mohou komunikovat s CNS pomocí periferních nervů, jako jsou kožní smyslové nervy a nervus vagus. Aktivace nervové dráhy je alternativní cesta, kterou může být velmi rychle spuštěna febrilní reakce (Roth et al., 2001, Hopkins, 2007).

Tvorba PGE_2 v místech zánětu přispívá ke vzniku febrilní odpovědi aktivací chladových kožních receptorů (nervů), které převádějí signály do oblastí v mozku, odpovědných za febrilní reakci (Rummel et al., 2005). Přenos signálů prostřednictvím nervu vagu představuje komplexnější cestu. Cirkulující pyrogeny jako např. LPS aktivují komplement, jehož produkty stimulují Kupfferovy buňky v játrech k produkci endogenních mediátorů včetně pyrogenních cytokinů (Zeisberger, 1999). Tyto cytokiny dráždí hepatální větve nervu vagu. Nervus vagus následně přenáší febrilní signály do nucleus tractus solitarius (NTS). Z NTS jsou signály transmitovány do preoptické a hypotalamické oblasti cestou ventrálního svazku a vedou k uvolňování noradrenalinu v intrapreoptické oblasti (Roth & de Souza, 2001).

Noradrenalin zprostředkovává dvojím způsobem zřetelné zvýšení teploty jádra. Prvním z nich je α_1-adrenoceptor (AR) mediované, je rychlejší ve svém nástupu a nezávislé na PGE_2, druhá reakce je zprostředkovaná α_2-AR, je opožděná a závislá na PGE_2 (Roth & de Souza, 2001).

Role vagových aferentních drah v patogenezi horečky byla definována na základě experimentálních studií na laboratorních potkanech, které ukázaly, že chirurgická vagotomie vede k oslabení nebo úplné ztrátě febrilních reakcí na pyrogenní signály (Watkins et al., 1994; Watkins et al., 1995; Romanovsky et al., 2000; Roth & de Souza, 2001; Hopkins, 2007). Nicméně, některé nedávné studie přisuzují chybějící febrilní reakce na pyrogenní signály vedlejším účinkům vagotomie, jako je podvýživa

(Romanovsky et al., 2005). Pokud se podaří zabránit těmto vedlejším účinkům, pak experimentální studie na potkanech naznačují, že úplná nebo částečná vagotomie neeliminuje febrilní reakce na pyrogenní signály, jako je nitrožilní podání PGE_2 (Ootsuka et al., 2008).

Reference

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3. Cíl práce

Cílem práce je přispět k porozumění patofyziologie endotoxinem vyvolané horečky se zaměřením na periferní rozpoznání endotoxinu, následné předání imunitního signálu a jeho zpracování v termoregulačním centru – preoptické oblasti hypotalamu. Základní hypotézou pro vznik horečky je generování endogenních pyrogenů (IL-1beta, IL-6, TNF-alfa) v reakci na exogenní stimulus. Již dlouhá léta se ovšem k léčbě horečky využívá nesteroidních antirevmatik, jejichž mechanismus účinku tkví ve více či méně selektivní inhibici cyklooxygenáz, enzymů esenciálních pro tvorbu prostaglandinu E_2.

V následujících částech práce proto budou komentovány naše jednotlivé původní vědecké práce zaměřené na ozřejmění role produkce prostaglandinu E_2 (PGE_2) ve vzniku horečky, ozřejmění jeho periferní a centrální role a identifikace buněk zodpovědných za jeho produkci a regulaci a centrální aferentaci.

Diskutována budou tři následující témata:

- Význam Kupfferových buněk a makrofágů při periferní signalizaci endotoxinem mediované horečky zejména pokud jde o roli endogenních mediátorů horečky a průkaz zásadní role komplementu a prostaglandinu E_2 v iniciálních fázích vzniku horečky u experimentálního modelu endotoxinem spouštěné horečky.
- Význam sleziny v regulaci horečky
- Centrální mediace signálu v preoptické oblasti hypotalamu

4. Metodika, výsledky a diskuse

4.1. Význam Kupfferových buněk při periferní signalizaci horečky.

V souvislosti se sledováním této problematiky jsme provedli experimentální práci v autorském kolektivu Perlík V, Li Z, Goohra S et al., *LPS activated complement, not LPS per se, triggers the early PGE_2 by Kupffer cells*, která byla prezentována v *American Journal of Physiology* - Regulatory, Integrative, and Comparative Physiolgy v roce 2005, a dále práci v autorském kolektivu Li Z, Perlik V, Feleder C et al., *Kupffer cell-generated PGE_2 triggers the febrile response of guinea pigs to intravenously injected LPS.* Práce byla otištěna ve stejném periodiku v roce 2006.

Cílem původní práce „LPS activated complement, not LPS per se, triggers the early PGE_2 by Kupffer cells“, bylo potvrdit předpoklad, že lipopolysacharidem indukovaná horečka u morčat může být spouštěna prostřednictvím prostaglandinu E_2 (PGE_2). Součástí testované hypotézy bylo také ozřejmit vliv komplementu a dalších faktorů ovlivňujících rychlý nárůst hladin prostaglandinu E_2 a tělesné teploty po podání lipopolysacharidu. Pro ověření uvedených cílů jsme morčatům v anestezii aplikovali do portální žíly dvě různé dávky LPS (2 nebo 8 μg/kg), a to dohromady s kobřím jedem anebo bez něj. (CVF, 25U/zvíře). Kobří jed je známý okamžitý aktivátor celé kaskády komplementu a v důsledku jeho stálé aktivace tímto jedem dojde postupně k jeho vyčerpání. Vliv komplementové kaskády by tímto měl být v následných patofyziologických procesech imuno-neuro modulace zásadně omezen (Cochrane, 1970; Fink et al., 1989, Van den Berg et al., 1991; Vogel et al., 1996). Dále jsme sledovali odpověď organismu v podobě měření plasmatických hladin prostaglandinu E_2, tumor necrosis faktoru alfa, interleukinu – 1beta (IL-1beta) a interleukinu – 6 (IL-6) v dolní duté žíle 60 a 180 minut po aplikaci PGE_2 a CVF. V druhé části experimentu jsme ověřili produkci prostaglandinu E_2 in vitro na izolovaných Kupfferových buňkách po přidání shodných látek do kultivačního media tak, jako při in vivo části experimentu.

Zjistili jsme, že aplikace LPS a kobřího jedu spouští okamžitou produkci prostaglandinu E_2 během prvních 5 minut. Zatímco zvýšené hladiny PGE_2 se stabilizují po 15 minutách a přetrvávají zvýšené po léčbě lipopolysacharidem po celou dobu trvání experimentu (60min), zvýšené hladiny prostaglandinu E_2 způsobené aplikací CVF se vrátí ke svým hodnotám před léčbou po 15 minutách. Aplikace lipopolysacharidu 3 hodiny po předcházející léčbě kobřím jedem způsobila zpoždění odpovědi tvorby prostaglandinu E_2. Ke srovnání hladin PGE_2 s pozitivní kontrolní skupinou léčenou pouze LPS dojde až 30-45 minut po léčbě. Uvedené

pozorování, že komplement hraje významnou roli v produkci prostaglandinu a že jeho deplece vede k redukci této odpovědi, je v souladu s předcházejícími pozorováními (Fink et al., 1989; Pestel et al., 2003; Puschel et al., 1993; Schieferdecker et al., 2001; Schlaf et al., 2003). Nový poznatek je rozlišení rychlé, komplement dependentní, a pomalé, pravděpodobně na cytokinech závislé produkci prostaglandinu E2 iniciované podáním lipopolysacharidu, která byla již dříve postulována (Perera et al., 2001; Rhee et al., 2000).

Nárůst hladin TNF-alfa, IL-1beta a IL-6 ve srovnání s prostaglandinem E_2 nastupuje po léčbě lipopolysacharidem a CVF později, přibližně za 30-45 minut. Navíc není nikterak alterován po předcházející eliminaci komplementového systému prostřednictvím působení kobřího jedu, a to ani v podobě nástupu hladin cytokinů, ani v podobě míry odpovědi vlastní produkce cytokinů.

Obdobné výsledky byly potvrzeny v in vitro experimentech na primárních Kupfferových buňkách, kde samotné přidání LPS do media nevyvolalo žádnou odpověď v podobě zvýšené produkce prostaglandinu E_2, zatímco přidání faktorů komplementu vedlo k okamžité produkci prostaglandinu Kupfferovými buňkami. Plně v souladu s identifikací komplementu jako jednoho z faktorů časné periferní signalizace, pak byla identifikována specifická část komplementové kaskády spolupodílející se na signalizaci LPS navozené horečky a to C5a (Li et al., 2005; Li et al., 2002).

Pokud zvážíme časovou souslednost tvorby prostaglandinu E_2 zejména v iniciálních fázích s časovou sousledností zvýšení tělesné teploty po intravenózním podání LPS, nabízí se zde alternativní koncept k cytokiny mediovanému přenosu imunitního signálu. Tato alternativní signalizace se uskutečňuje prostřednictvím imuno-neuro humorální odpovědi zprostředkované komplementem a časně syntetizovaným PGE_2, zejména v iniciálních fázích vzniku horečky u tohoto experimentálního modelu.

Uvedená skutečnost byla také později potvrzena v našich dalších pracích (Li et al., 2006, Li et al., 2005).

Roli cyklooxygenázy 1 a 2 (COX-1 a COX-2) při tvorbě LPS mediované horečky pak dále upřesnil Steiner et al, který prokázal zásadní podíl COX-2 i pro iniciální fázi horečky prostřednictvím aplikace LPS u myší s genetickou delecí COX-1 a nebo COX-2 a postuloval, že konstitutivní COX-2 nebo její rychlá upregulace může být zodpovědná za časnou produkci PGE_2 a vznik horečky (Stainer et al., 2005).

Vliv časné a na cytokinech nezávislé produkce PGE_2 byl také demonstrován u aktivace hypothalamo-pituitární osy a tvorby kortikosteronu jako další součásti reakce akutní fáze způsobené při experimentálním, intraperitoneálním podání Escherichia coli u laboratorních potkanů (Mouihate et al., 2010; Zimomra et al., 2011). Podobný závěr, podtrhující vliv prostaglandinů v experimentálním modelu sub-pyrogneického LPS vyvolaného zánětu, byl konstatován u myší, a to v souvislosti se změnami chování laboratorních myší souvisejícími se „sickness behavior“, kde změnám chování zabránilo podání indomethacin, a to i přes zvýšené hladiny cytokinů IL-1β, IL-6 and TNF-α (Teeling et al., 2007).

Na druhou stranu vliv cytokinů na pomalejší produkci PGE_2 a pozdní fázi horečky nebyl potvrzen, a to prostřednictvím práce zkoumající cytokinovou upregulaci v souvislosti s podáním LPS u laboratorních myší s delecí genu pro mikrosomalní prostaglandin E synthasu-1 (mPGES-1), která vedla k aberaci produkce prostaglandinu E_2 a febrilní reakce. U zvířat s chybějící mPGES-1 totiž nedošlo k narušení produkce prozánětlivých cytokinů, interleukinu (IL)-1β, IL-6 a tumour necrosis factoru (TNF), a to i přesto, že došlo k oslabení febrilní reakce na LSP u mPGES-1 defektních zvířat (Nilsberth et al., 2009).

Komplementem mediovaná časná upregulace prostaglandinu E_2 tak představuje životaschopnou alternativu ke zcela zásadní roli cytokinů pro patofyziologii horečky, tak jak je shrnuto v řadě review článků shrnujících patofyziologii horečky. Kontroverzní naopak zůstává mechanismus působení PGE_2 a aferentace signálu během časné odpovědi organismu na periferii ať už přímou vazbou na volná zakončení nervus vagus v játrech či dalších orgánech, nebo jeho zapojení do humorální odpovědi prostřednictvím signalizace cytokinové, či přímým působením v CNS (Blatteis, 2006; Blatteis, 2007; Hopkins, 2007; Steinman, 2008).

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LPS-activated complement, not LPS per se, triggers the early release of PGE_2 by Kupffer cells

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Perlik, Vit, Zhongua Li, Sarita Goorha, Leslie R. Ballou, and Clark M. Blatteis. LPS-activated complement, not LPS per se, triggers the early release of PGE_2 by Kupffer cells. *Am J Physiol Regul Integr Comp Physiol* 289: R332–R339, 2005. First published March 31, 2005; doi:10.1152/ajpregu.00567.2004.—The intravenous injection of LPS rapidly evokes fever. We have hypothesized that its onset is mediated by prostaglandin (PG)E_2 quickly released by Kupffer cells (Kc). LPS, however, does not stimulate PGE_2 production by Kc as rapidly as it induces fever; but complement (C) activated by LPS could be the exciting agent. To test this hypothesis, we injected LPS (2 or 8 μg/kg) or cobra venom factor (CVF, an immediate activator of the C cascade that depletes its substrate, ultimately causing hypocomplementemia; 25 U/animal) into the portal vein of anesthetized guinea pigs and measured the appearance of PGE_2, TNF-α, IL-1β, and IL-6 in the inferior vena cava (IVC) over the following 60 min. LPS (at both doses) and CVF induced similar rises in PGE_2 within the first 5 min after treatment; the rises in PGE_2 due to CVF returned to control in 15 min, whereas PGE_2 rises due to LPS increased further, then stabilized. LPS given 3 h after CVF to the same animals also elevated PGE_2, but after a 30- to 45-min delay. CVF per se did not alter basal PGE_2 and cytokine levels and their responses to LPS. These in vivo effects were substantiated by the in vitro responses of primary Kc from guinea pigs to C (0.116 U/ml) and LPS (200 ng/ml). These results indicate that LPS-activated C rather than LPS itself triggers the early release of PGE_2 by Kc.

liver; fever; portal vein cannulation; cobra venom factor; pyrogenic cytokines

FEVER DEVELOPS QUICKLY AFTER the intravenous bolus administration of a pyrogenic dose of LPS to conscious guinea pigs, rats, and other species, but the afferent mechanism that induces this response is controversial. It is generally thought that it is mediated by pyrogenic cytokines produced secondarily in response to the LPS challenge, rather than by its direct action. Tumor necrosis factor-α (TNF-α), IL-1β, and IL-6 are the major cytokines implicated in this response (9). There is, however, a temporal disconnect between the first appearance of these cytokines and the onset of the febrile response to intravenous LPS; that is, fever appears within 10–15 min (13, 51) whereas TNF-α, the first cytokine to appear, is not detectable until 30 min after LPS treatment (18, 23, 24, 39). This temporal discrepancy is less evident after the intraperitoneal administration of low to moderate doses of LPS, when the latency of fever onset is ~60 min but becomes evident also when higher doses are administered, when the onset latency approaches that after intravenous LPS (7).

We have shown previously that the onsets of the febrile responses to intravenous and intraperitoneal LPS are correlated with the appearance of LPS in the liver's Kupffer cells (Kc) (31), the body's principal clearinghouse of LPS (16, 34, 45) and source of pyrogenic cytokines (10). As cytokines are not constitutively expressed by Kc and their de novo production occurs after some delay (32), we and others have proposed, to account for the promptness of the febrile response to intravenous LPS, that the peripheral pyrogenic signal could be transmitted to the preoptic-anterior hypothalamic area (POA, the presumptive locus of the febrigenic controller) via a neuronal rather than a humoral mechanism (6). Thus evidence was adduced by us and others (12, 49, 59) that the vagus and its hepatic branch, in particular (52), may convey the pyrogenic signal to the brain. Indeed, IL-1β injected into the portal vein of anesthetized rats had been shown earlier to increase the electrical activity of the vagus (36). But because this cytokine lags behind the onset of fever induced by intravenous LPS, it also seems unlikely that it could be the direct, peripheral mediator responsible for vagal activation.

We (6, 50) and others (12, 44) have suggested that, alternatively, PGE_2 could be that mediator. It is synthesized by all macrophages, including Kc, in response to LPS (46), and its levels in plasma are correlated with the febrile course (35). PGE_2 receptors are also present on peripheral sensory neurons, e.g., in kidneys (26), lungs (27), stomach (54), and jejunum (19), as well as on nodose ganglion vagal sensory neurons receiving information from thoracic and abdominal compartments (12), and on cervical vagal afferents (25). It has also been proposed that, alternatively, in lieu of stimulating vagal terminals, peripherally synthesized PGE_2 could trigger fever by being transported to the brain as an albumin-bound complex (22, 43). Paradoxically, however, the elevation of plasma PGE_2 after LPS challenge is faster in vivo (22, 35) than the ability of macrophages to synthesize PGE_2 in vitro (33, 37). It would seem, therefore, that LPS per se could not be the stimulus for the rapid production of PGE_2 in vivo. Hence, presumably, another factor rapidly evoked by LPS should drive this response. The complement (C) cascade is immediately activated in vivo by the presence of LPS (58), and we have recently reported that the anaphylatoxic complement component 5a

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(C5a) is an essential mediator of the febrile response to LPS (5, 30). It was shown earlier that the in vitro production of PGE_2 by Kc is stimulated by C5a, whereas C depletion limits this production (15, 41, 48); Kc express abundant C5a receptors (14, 48, 61).

We have hypothesized, therefore, that LPS-activated C5a may trigger PGE_2 production by Kc. The purpose of this study was to test this hypothesis, using two different approaches: the first was to determine whether the rapid production of PGE_2 by the liver induced by LPS in vivo is indeed C-dependent; cobra venom factor (CVF) was used to address this question because it immediately activates the C cascade, thereby adding all the C components to the circulation, and because, furthermore, it ultimately depletes the C substrate for the cascade, thereby causing hypocomplementemia (8, 15, 56, 57; see also Discussion). The second was to verify whether Kc, in fact, secretes PGE_2 more rapidly in response to C than to LPS in vivo and in vitro. A corollary was to substantiate that cytokines are not liberated as early as PGE_2 in response to LPS.

MATERIALS AND METHODS

Animals

Male, pathogen-free, Hartley guinea pigs were used in these experiments. The guinea pigs (550–650 g; Charles River Laboratories, Wilmington, MA) were quarantined for 1 wk, three to a cage, in the vivarium of our Department of Comparative Medicine before any experimental use. Tap water and food (Agway Prolab guinea pig) were available ad libitum. The ambient temperature (T_a) in the animal rooms was 23 ± 1°C; light and darkness alternated, with light on from 0600 to 1800. All animal protocols were approved by the University of Tennessee Health Science Center Animal Care and Use Committee and fully conform with the standards established by the U. S. Animal Welfare Act and by the document entitled "Guiding Principles for Research Involving Animals and Human Beings" (3).

Drugs

For the in vivo guinea pig studies, the solvent of most drugs was sterile, nonpyrogenic, isotonic (0.9%) NaCl solution (pyrogen-free saline, PFS; Abbott Laboratories, Chicago, IL); for the in vitro studies, it was its PBS analog. Heparin was purchased from Elkins-Sinn (Cherry Hill, NJ). LPS was *Salmonella enteritidis* LPS B (lot #651628; Difco Laboratories, Detroit, MI), suspended in PFS. Cobra venom factor (CVF; cat #233552) was from Calbiochem-Novabiochem (San Diego, CA). Murine serum C was purchased from Sigma-Aldrich (cat. #S3269), and recombinant murine (rm)IL-1β was from R&D Systems (cat. #401-ML, lot #BN021021; Minneapolis, MN).

Cannulation of the Portal Vein and Inferior Vena Cava of Guinea Pigs

The guinea pigs were anesthetized with ketamine (35 mg/kg im) and xylazine (5 mg/kg im) and prepared for surgery. Under aseptic conditions, a midline laparotomy upward from the umbilicus, ~3 cm long, was performed. The ileum and mesentery were exposed and the vascular arcades in the mesentery visualized. Two connecting tributary mesenteric veins were identified and back-ligated to stop inflow to these veins. The junction just proximal to the two veins was carefully incised, and a sterilized, RenaSil silicon cannula (ID 0.012 in., OD 0.025 in.; Braintree Scientific, Braintree, MA) prefilled with heparinized (50 IU/ml) PFS was inserted and directed toward the portal vein. Another two ligatures were placed around the proximal end of the cannula to secure it in place. The laparotomy incision was closed.

Immediately thereafter, a sterilized, HelixMark silicon cannula (ID 0.020 in., OD 0.037 in.; Baxter Healthcare, McGraw Park, IL) prefilled with heparinized PFS was inserted into the left jugular vein and guided toward the inferior vena cava (IVC). The incision was sutured. The animals remained under surgical anesthesia and were maintained on heating pads set at 30°C for the experimental tests.

Isolation of Guinea Pig Kupffer Cells

This method was modified from Do et al. (11). Briefly, under deep ketamine (50 mg/kg ip) xylazine (50 mg/kg ip) anesthesia, the guinea pigs were laparotomized, their portal vein isolated, and their liver perfused in situ through the portal vein with, successively, 15–20 ml of HBSS and 30 ml of liver perfusion medium (Gibco/Invitrogen, Carlsbad, CA); drainage was through the IVC. The liver was removed and submerged in ice-cold serum-free RPMI 1640 medium. To obtain Kc, the gall bladder was removed and the liver was minced with a sterile razor. The minces were digested in 50 ml of liver digest medium (Gibco/Invitrogen) at 37°C, with occasional shaking. The resulting cell suspension was filtered through a 75-μm mesh, and the filtrate was centrifuged at 50 g for 5 min, to pellet the hepatocytes. The supernatant was removed and pelleted by centrifugation at 600 g for 10 min. The cells were next washed once with HBSS, layered over a 1,038 mg/ml solution of Percoll (Pharmacia-Amersham, Piscataway, NJ), and centrifuged at 400 g for 20 min. The resulting debris-containing upper layer was removed. The lower layer was diluted threefold with PBS and centrifuged at 600 *g* for 10 min, to pellet the cells. The washed cell pellets from the Percoll centrifugation were suspended in PBS, 0.5% BSA, and 2 mM EDTA. They were then isolated by anti-CD-11b-conjugated magnetic beads cell sorting over MS + MiniMACS separation columns (Miltenyi, Auburn, CA), according to the instructions provided by the manufacturer. Approximately 2–4 × 10^6 cells were collected from the livers of four animals.

Assays

LPS. LPS in the plasma of guinea pigs was evaluated using a chromogenic Limulus amebocyte lysate assay (Pyrochrome; Associates of Cape Cod, Woods Hole, MA), according to the supplier's instructions. The detection limit of this assay was 0.005 endotoxin unit (EU). The First International Standard for Endotoxin (84/650, World Health Organization) was used as the reference. Endotoxin concentrations are expressed as international EUs per milliliter.

PGE_2. PGE_2 in guinea pig plasma was analyzed using an enzyme immunoassay (EIA) kit (high sensitivity prostaglandin E_2 EIA Kit #931–001; Assay Designs, Ann Arbor, MI), according to the manufacturer's instructions; the prostaglandin synthetase inhibitor indomethacin (10 μg/ml) was added to all the blood samples immediately after collection. All of the samples were diluted before analysis in the assay buffer supplied, according to the manufacturer's instructions. The detection limit of this assay was 8.26 pg/ml.

The levels of PGE_2 in the incubation media of guinea pigs Kc were determined by our radioimmunoassay (RIA), as previously described (4). Briefly, it is based on the competition of PGE_2 in the test samples with ^{3}H-labeled PGE_2 for binding to anti-PGE_2 antibody. A 100-μl aliquot of culture medium was added to the RIA assay buffer (0.1 mM phosphate buffer, pH 7.4, containing 0.9% sodium chloride, 0.1% sodium azide, and 0.1% gelatin), mixed with appropriate amounts of labeled PGE_2 and reconstituted antiserum, and incubated overnight at 4°C. The assay tubes were then placed on ice, and 1.0 ml of cold charcoal-dextran suspension was added. Fifteen minutes later, the tubes were centrifuged at 2,200 *g* for 10 min at 4°C, and the supernatants decanted into scintillation vials. Radioactivity was determined by scintillation spectrometry (Packard Tricarb 200CA). Percent binding was compared with a standard curve and the amounts of PGE_2 in the samples calculated.

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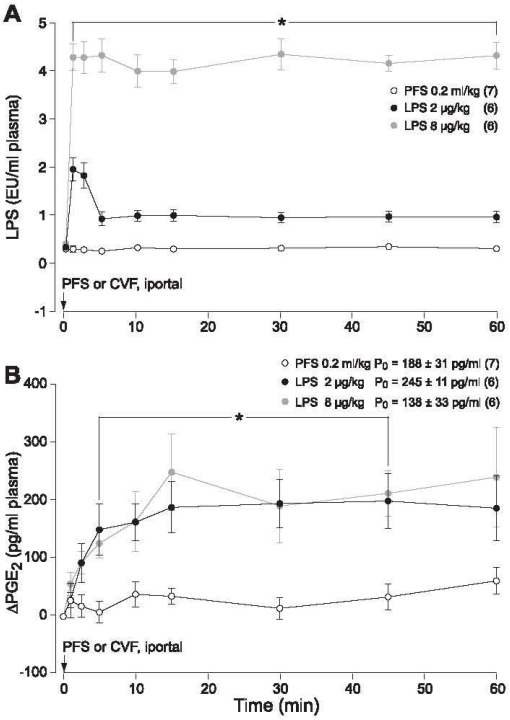

Fig. 1. The levels of LPS (*A*) and ΔPGE_2 (*B*) in the plasma of the inferior vena cava (IVC) of anesthetized guinea pigs in response to the intraportal vein (iportal) injection of LPS (2 or 8 μg/kg) or pyrogen-free saline (PFS; 0.2 ml/kg). Values are means ± SE; (*n*) = number of animals. $^*P < 0.05$, both LPS treatments relative to PFS.

Cytokines. The determination of TNF-α in the plasma of guinea pigs was performed by a bioassay based on the cytotoxic effect of TNF-α on the mouse fibrosarcoma cell line WEHI 164 subclone 13(c). The assay was performed using sterile, 96-well microtiter plates. Serial dilutions of biological samples or different concentration of TNF-α standard (code 88/532, National Institute for Biological Standards and Control, South Mimms, UK) were incubated for 24 h in wells that had been seeded with 50,000 actinomycin D-treated WEHI 164 cells. The number of surviving cells after 24 h was measured by use of the dimethylthiazol-diphenyl tetrazolium bromide (MTT) colorimetric assay. Plasma IL-1β and IL-6 were determined by bioassays based on the dose-dependent growth stimulation of the D10 and B9 hybridoma cell lines, respectively. These assays were performed also using sterile, 96-well microtiter plates. In each well, 5,000 D10 or B9 cells were incubated for 72 h with serial dilutions of biological samples or with different concentrations of IL-1β or IL-6 standards (code 86/680 and 89/548, National Institute for Biological Standards and Control). The number of cells in each well was measured using the MTT assay.

Complement. Five microliters of plasma were added to wells placed in agarose gel containing standard sheep erythrocytes sensitized with hemolysin (Kit RC001; The Binding Site, San Diego, CA). Plates were incubated for 18 h at 4°C and then for 1 h at 37°C. The diameters of the zones of hemolysis around each well (radial immunodiffusion) were measured. These values were converted to the percentage activity of complement in the samples by interpolating from calibration curves plotted using the manufacturer's standard, diluted according to the manufacturer's directions. The detection limit of this assay was 32 CH_{100} units/ml. The results are expressed as the percentage of C activity remaining after CVF treatment compared with the pretreatment level.

Experimental Design

Experiment 1. To determine initially the efficacy of the clearance of LPS by the liver as directly as possible and to correlate this with the production of PGE_2 by the liver, we injected two doses of LPS (2 and 8 μg/kg) directly into the portal vein (iportal) of anesthetized guinea pigs and measured their appearance in the IVC at 0, 1, 2.5, 5, 10, 15, 30, 45, and 60 min after their administration. The volume of each blood collection was 0.4 ml; it was immediately replaced by an equal volume of PFS. PFS (0.2 ml/kg) was also the control solution for this experiment.

Experiment 2. To verify whether the rapid production by the liver of LPS-induced PGE_2 is C-dependent, we injected CVF (25 U/animal) into the portal vein of anesthetized guinea pigs. CVF causes the immediate activation of the alternative pathway of the C cascade (8, 56, 57); the consequently formed C components then interact with their cognate receptors on cells and activate them. PFS (0.2 ml/kg) was the control solution. Blood samples were collected from the IVC at 0, 1, 2.5, 5, 10, 15, 30, 45, and 60 min after this treatment and analyzed for their PGE_2, TNF-α, IL-1β, and IL-6 levels.

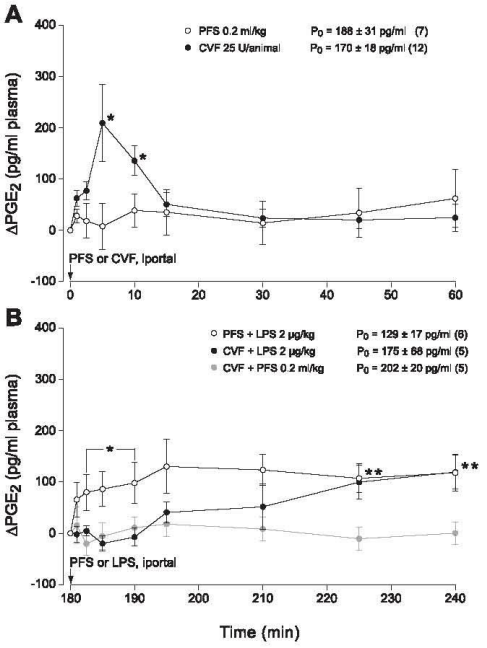

Fig. 2. *A*: effects of iportal injected CVF (25 U/animal) or PFS (0.2 ml/kg) on the IVC plasma levels of PGE_2 in anesthetized guinea pigs. $^*P < 0.05$, cobra venom factor (CVF) relative to PFS. *B*: ΔPGE_2 levels in the IVC plasma of the same animals after the iportal injection of PFS or LPS (2 μg/kg) 3 h after PFS or CVF pretreatment. The complement (C) remaining was ~9% of its control level (see text). Abbreviations and conventions as in Fig. 1. $^*P < 0.05$, LPS after PFS relative to LPS after CVF. $^{**}P < 0.05$, both LPS treatments relative to PFS.

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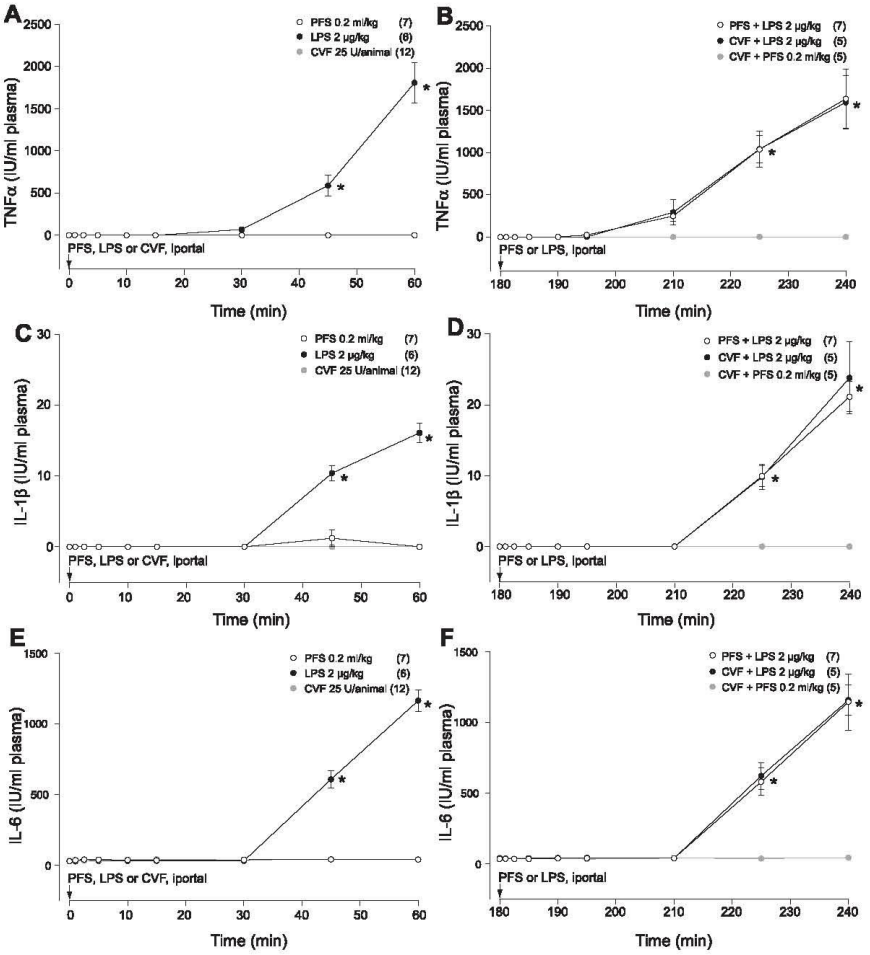

Fig. 3. IVC plasma levels of TNF-α (*A*), IL-1β (*C*), and IL-6 (*E*) following the iportal injection of LPS (2 µg/kg), CVF (25 U/animal) or PFS (0.2 ml/kg). Effects of 91% plasma C reduction (*B*, *D*, *F*) on the same variables in the same animals 3 h later. The subjects in Figs. 2 and 3 are the same. Abbreviations and conventions as in Fig. 1. *$P < 0.05$, LPS relative to PFS. *A* and *E*: the CVF data are hidden under the PFS data, both of which they are identical.

The activation of C induced by CVF, however, continues unabated, so that, ultimately, C becomes significantly reduced (8, 15, 56, 57; see also DISCUSSION). To determine, therefore, the effect of hypocomplementation on LPS-induced PGE_2 and cytokine production, we injected into the portal vein of these CVF-treated animals a second dose of LPS (2 µg/kg) or PFS (0.2 ml/kg) 180 min after CVF. The guinea pigs previously treated with PFS received LPS (2 µg/kg). A PFS+PFS control was considered redundant in this design and was, therefore, not performed. Plasma samples were collected from the IVC and analyzed as before. The level of hypocomplementemia was determined by analyzing plasma C levels before and 180 min after CVF administration.

Experiment 3. To compare the effects of C and LPS on Kc PGE_2 production directly, the Kc from guinea pigs were seeded at 1×10^5 cells/well in 24-well tissue culture microplates (0.5 ml/well) in RPMI 1640 medium with L-glutamine containing 20% heat-inactivated FBS and incubated overnight at 37°C. The next morning, the medium was replaced with fresh RPMI 1640 medium containing 1% FBS, and the following test agents were added: none (NA; 0.5 ml of medium), murine serum C (0.116 U/ml), *S. enteritidis* LPS (200 ng/ml), rmIL-1β (1 ng/ml), and C + these factors. These doses were based on preliminary dose-response studies. Samples of culture media were collected at 0, 2.5, 10, 30, and 60 min after these treatments, and their PGE_2 contents analyzed.

Statistical Analysis

The results are reported here as means ± SE. The data were evaluated by a repeated-measures analysis of variance model, where factor 1 was the between-group factor (the experimental treatment)

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and factor 2 the within-subject factor (the different sampling periods). The analyses were performed using Instat 3 (GraphPad software; Instant Biostatistics, San Diego, CA). Each variable was considered to be independent. The 5% level of probability was accepted as statistically significant.

RESULTS

Experiment 1

LPS at both doses (2 and 8 μg/kg) injected into the portal vein appeared in IVC plasma virtually immediately (1 min); the level of the lower dose was reduced by half in 5 min, and then stabilized over the remainder of the 60-min experimental period. The plasma level of the higher dose, however, remained at its high, initial level over the duration of this experiment (Fig. 1*A*). The LPS level of the PFS-treated controls remained stable throughout the hour, although it was slightly elevated (i.e., above 0) due presumably to the surgery-associated manipulation of the animals' intestinal tract (60).

IVC plasma PGE_2 levels were not significantly elevated by the administration of PFS, but they rose within 2.5 min to approximately half of their maximal value after the administration of both LPS doses, then continued to rise more slowly over the next 25 min. They then both stabilized at ~200 pg/ml above their basal level for the remainder of the hour, irrespective of the LPS dose (Fig. 1*B*).

Experiment 2

IVC plasma PGE_2 levels increased within 2.5 min, peaked in 5 min, and then returned to control levels in 15 min following the administration of CVF (Fig. 2*A*.). PFS administration had no significant effect on PGE_2 levels.

The iportal injection of LPS 3 h after PFS induced the same pattern of IVC PGE_2 elevation as was observed when injected at time 0, albeit that PGE_2 levels increased only approximately half as much (PFS+LPS, Fig. 1*B*). But LPS given 3 h after CVF pretreatment, when plasma C levels were reduced to ~9% relative to their basal levels (the basal level of C was 393 ± 68 CH_{100} units/ml; 3 h after CVF treatment, it was reduced to 34 ± 14 CH_{100} units/ml; data from 6 animals), did not significantly raise PGE_2 levels until 30–45 min after its injection (CVF+LPS, Fig. 2*B*). The injection of PFS into these hypocomplementemic guinea pigs (CVF+PFS, Fig. 2*B*) had no effect on PGE_2 levels; these were not significantly different from those of PFS injected into untreated animals (Fig. 2*A*).

TNF-α (Fig. 3*A*), IL-1β (Fig. 3*C*), and IL-6 (Fig. 3*E*) became detectable in IVC plasma 30 and 45 min after LPS treatment, respectively. They were not elevated by acute CVF or PFS per se. Hypocomplementemia (Figs. 3*B*, *D*, *F*) also did not affect the appearance, rate of rise, or magnitude of the LPS-induced elevations of these cytokines.

Experiment 3

IL-1β alone induced the release of PGE_2 by primary, freshly isolated Kc from guinea pigs between 30 and 60 min after their addition, but LPS was without effect throughout the 60-min incubation period. On the other hand, C alone and C+LPS or IL-1β very quickly (2.5–10 min) triggered PGE_2 increases of similar, apparently maximal magnitudes (Fig. 4).

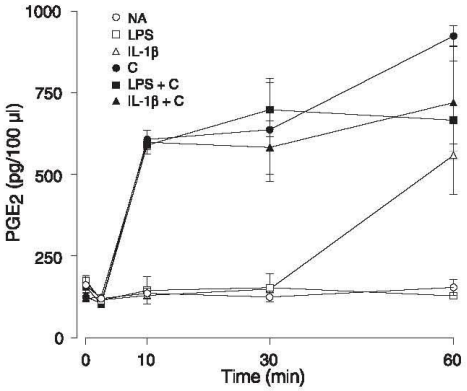

Fig. 4. The course of the production of PGE_2 by primary, freshly isolated Kupffer cells from guinea pigs in response to C (0.116 U/ml), *S. enteritidis* LPS (200 ng/ml), recombinant murine (rm)IL-1β (1 ng/ml), or serum C in combination with each of these factors. NA, no additions (0.5 ml).

DISCUSSION

The present results show that IVC PGE_2 rose rapidly and to similar levels within 5 min after the iportal administration of CVF or LPS. But, whereas the elevation caused by CVF was reversed over the following 10 min, that due to LPS continued to a higher level over the same interval, then plateaued (Figs. 1*B* and 2*A*). On the other hand, 3 h after CVF treatment, when ~91% of the original C level was depleted, PGE_2 levels did not increase significantly until 45 min after the administration of LPS (Fig. 2*B*). Both CVF and LPS cause the immediate activation of the alternative pathway of the C cascade, but that due to CVF continues unabated, reducing C and, hence, limiting its effects (8, 15, 56, 57; see also below). Moreover, whereas no PGE_2 was detectable within 60 min after the addition of LPS to freshly isolated Kc, the addition of C alone or in combination with it induced the generation of PGE_2 between 2.5 and 10 min (Fig. 4). Pyrogenic cytokines were not detectable in vivo until 30–45 min after the iportal injection of LPS (Fig. 3); neither the activation of C nor its reduction affected these in vivo responses.

We infer from these data that the almost immediate appearance of PGE_2 in IVC plasma after the iportal injection of LPS supports the notion, advanced previously by us (5, 6, 28, 30), that it may be mediated by C5a rapidly induced by LPS. On the other hand, its continued production in these animals, as manifested by its further, slower rise to a higher plateau at 15 min, its delayed elevation in the C-insufficient, LPS-treated guinea pigs (Fig. 2*B*), as well as the late production of cytokines in all the animals under all the experimental conditions (Fig. 3) are probably all the result of the direct, LPS binding protein (LBP)-cluster designation 14 (CD14)-Toll-like receptor 4 (TLR4)-myeloid differentiation protein 2 (MD2)-mediated activation of Kc by LPS (53).

Like LPS, CVF activates virtually immediately C3. The activation of C3 by LPS yields a derivative, C3b, which forms a complex with factor B, C3bBb, a convertase that cleaves the α chain of C3 and thereby enables the further production of its downstream components (58); C3bBb is very labile ($t_{1/2}$ = 1.5

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min at 37°C). By contrast, CVF itself forms a complex with factor B, CVFBb, that is functionally analogous to C3bBb, but much more stable ($t_{1/2}$ = 7 h) due to its resistance to the control mechanisms that limit the activity of the normal C3 convertase. Consequently, C3 activation continues unabated, depleting C3 and all its downstream products (8, 56). The present finding that C triggered the immediate production of PGE_2 by Kc, whereas its depletion limited this production thus substantiates similar, earlier findings by others (15, 40, 41, 47, 48). But our further in vivo finding that the similarly quick release of PGE_2 evoked by LPS at time 0 was abrogated when C was reduced 3 h later, though anticipated, is novel. It confirms that the initial, very early, LPS-induced PGE_2 rise was indeed C- rather than LPS-mediated. On the basis of the duration of the PGE_2 response to the acute effect of CVF, that is, the rapid activation, then reduction of C3, it was therefore brief. The similarities in the onset, magnitude, and duration of the PGE_2 response to LPS, irrespective of the dose administered (Fig. 1*B*), further reinforce the notion that the initial, quick, LPS-induced release of PGE_2 is C-mediated. On the other hand, the subsequent rise of IVC PGE_2 to higher levels at 15 min and the 15-min delay in its appearance when C was reduced indicate that a secondary, slower, C-independent mechanism also underlay the LPS-induced Kc production of PGE_2; this mechanism was, presumably, the LPS-TLR4 signaling pathway (38, 42). To our best knowledge, this is the first in vivo demonstration of a two-part modulation of the PGE_2 response to the iportal injection of LPS.

Although not specifically demonstrated in the in vivo portions of this study, the in vitro results of *experiment 3* strongly suggest that the targets of C5a, the anaphylatoxin specifically implicated in the febrile responses to intravenous and intraperitoneal LPS (28, 30), are in all probability the Kc. Although hepatic stellate, sinusoidal endothelial, and mast cells also express its principal receptor, $C5aR_1$, it is most abundant in Kc (48). These cells are critically linked to the onset of the febrile response to LPS (31). Mast cells are not involved (5), and there is no evidence that the other cell types are implicated. $C5aR_1$ is a G-protein-linked receptor that acts by increasing intracellular inositol-1,4,5-triphosphate and Ca^{2+} (47), rapidly activating cyclooxygenase (COX)-1-catalyzed PGE_2 production (2, 40, 46, 47). COX-1 is constitutive, functionally coupled mainly with cytosolic PGE_2 synthase (PGES) and therefore prepared to quickly synthesize PGE_2 (55). On the other hand, it is generally agreed that the production of PGE_2 induced by LPS per se is initiated by the LBP-mediated transfer of LPS to the receptor complex CD14/TLR4/MD2 and is associated with the upregulation of, specifically, COX-2 and microsomal PGES-1 (1, 20); in rats, both enzymes are induced in the liver ~30 min after LPS challenge (21). This delay in their biosynthesis presumably accounts for the lateness of the secondary appearance of PGE_2 observed in the present study. The cytokines, then also present in the blood, may further contribute to this late rise (Fig. 4). Indeed, as in previous studies (17, 18, 23, 24), these became evident in IVC plasma around 30–45 min after the iportal administration of LPS (Fig. 3), a delay that reaffirms that they probably have no role in the initiation of the febrile response to iv LPS. It is generally agreed that the LPS-TLR4 complex is the system that induces the production of pyrogenic cytokines (53). But, because the guinea pigs were anesthetized and maintained on heating pads during the present experiments (anesthesia impairs thermoregulatory responses; their body temperatures were monitored therefore only to insure their stability), the concurrence of the observed cytokines and PGE_2 responses with the normal febrile course of LPS-treated animals could not be verified. They do concord, however, with those reported in other, previous studies in conscious animals (5, 9, 18, 22–24, 29, 51).

In summary, because the very early appearance of PGE_2 in IVC plasma coincides temporally with the onset of fever, these findings would support the notion that PGE_2 quickly elaborated by Kc stimulated by LPS-activated C could be the factor that stimulates vagal terminals in the liver or circulates to the brain and, hence, may be responsible for the prompt initiation of the febrile response to intravenous LPS, as postulated previously (6, 22, 43, 49, 50). This interpretation may also help to clarify the correlation between its elevation in plasma and the febrile course (35).

In conclusion, the present data suggest that LPS injected into the portal vein (and, by inference, circulating LPS arriving in the liver) causes the very rapid appearance of PGE_2 in the blood via a two-part effect, one very rapid, but brief, exerted on $C5aR_1$-expressing Kc consequent to the virtually immediate activation by LPS of the alternative pathway of the C cascade, and another slower, but more prolonged, induced by the recognition of LPS by the TLR4 signaling complex. These results are compatible with the hypothesis that LPS-activated C rather than LPS itself rapidly triggers the release of PGE_2 by Kc.

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Dalším krokem k popisu patofyziologie horečky a ověření postulované role prostaglandinu E_2 pro periferní signalizaci imunitního signálu v podobě lipopolysacharidu bylo ověřit jeho možné zdroje in vivo a asociaci s nárůstem tělesné teploty, PGE_2 a plasmatických hladin cytokinů (IL-1β, IL-6 a TNF-α). Hladiny LPS, PGE_2 a pro-zánětlivých cytokinů byly sledovány v bodech očekávaného nárůstu tělesné teploty laboratorních morčat před podáním 2 μg/kg intravenózně a dále pak 15, 30, 60, 90, a 120 minut po podání LPS. K potvrzení základní role Kupfferových buněk, postulované v naší předcházející práci v in vitro experimentech (Perlik et al., 2005), laboratorní morčata byla předléčena gadoliniem, vzácným kovem, který se v medicíně používá nejen jako kontrastní látka, ale také při výzkumu, jako látka eliminující hepatické, splenické, plicní a ostatní makrofágy (Blatteis 1997; Hardonk et al., 1992; Li and Blatteis, 2004; Sehic, 1997). K repopulaci jednotlivých typů makrofágů dochází postupně, s Kupfferovými buňkami jako posledními, a to čtyři dny po podání gadolinia (Hardonk et al., 1992; Li and Blatteis, 2004). Z tohoto důvodu byly další experimenty prováděny třetí den po podání gadolinia, kdy byly Kupfferovy buňky ještě vyřazeny ze signalizace a zprostředkování imunitní odpovědi.

Výsledky ukázaly, že současná elevace PGE_2 spojená s nárůstem teploty tělesného jádra po intravenosním podání LPS je narušena u skupiny zvířat předléčených gadoliniem, tedy u skupiny s deplecí Kupfferových buněk. Došlo také k významnému zpomalení clearance LPS cirkulujícího v systémovém oběhu. Produkce cytokinů na léčbu samotným gadoliniem nebyla nikterak ovlivněna, stejně tak i jejich produkce po léčbě LPS, s výjimkou TNF-α, jehož hladiny byly dvojnásobné u skupiny zvířat předléčených gadoliniem a následně stimulovaných intravenosním podáním lipopolysacharidu. Vliv prostaglandinu E_2 v iniciální fázi vzniku horečky byl potvrzen také podáním králičího PGE_2 antiséra s minimální zkříženou reaktivitou k ostatním prostaglandinům, jež oslabilo horečku po

IV. podání LPS. Je třeba ovšem poznamenat, že kontrolní skupiny s vehikulem této protilátky, azidem sodným, který sám vykazuje hypotermický efekt, tak i samotné podání PGE_2 protilátky, mají termální efekt, který znesnadňuje jednoznačnou interpretaci experimentu.

Níže uvedená práce podtrhla a potvrdila význam časně produkovaného PGE_2 Kupfferovými buňkami pro iniciální fáze vzniku horečky vzhledem k tomu, že produkované hladiny prozánětlivých cytokinů, zvaných také endogenní pyrogeny jako např. IL-1β, IL-6 a TNF-α, časově nekorelují s nástupem teploty tělesného jádra.

Zásadní role PGE_2 byla také potvrzena v ostatních experimentech, při nichž byla podána nesteroidní antirevmatika blokující jeho produkci, a tím došlo také k oslabení febrilní odpovědi (Romanovsky et al., 2005; Roth and de Souza; 2001; Zeisberger 1999). Z těchto experimentů však není zřejmé, zdali se jedná o perifení či centrální efekt těchto léků, vhledem k tomu, že prochází hematoencefalickou bariérou. Naše práce naznačila, že se jedná o vliv periferního PGE_2 vzhledem k jeho asociaci s Kupfferovými buňkami a vzhledem k efektu PGE_2 protilátky, která vedla ke snížení febrilní odpovědi a která by hematoencefalickou bariérou procházet neměla. V souladu s naším pozorováním je rovněž experiment Stainera et al, který ukázal kruciální vliv delece genu kódujícího cyklooxygenázu 2 u myší pro zachování febrilní odpovědi po intravenózním podání LPS (Stainer et al., 2005). Ootsuka et al. (2008) pak u laboratorního potkana potvrdili, že periferní podání PGE_2 vyvolává zvýšení teploty laboratorních zvířat, což jednoznačně dokazuje klíčový vliv periferního PGE_2 jako mediátoru akutní reakce a vzniku horečky (Ootsuka et al., 2008). Jeho pozorování je plně v souladu s předchozími experimenty (Abu let al., 1997; Dascombe and Milton, 1979; Romanovsky et al., 1997; Skarneset al., 1981).

Zásadní role prostaglandinu E_2 pro signalizaci lipopolysacharidem navozené horečky po jeho intraperitoneálním podání u myší byla dokázána prostřednictvím delece genu pro mikrosomalní prostaglandin E synthasu-1 (mPGES-1), která vedla k aberaci produkce prostaglandinu E_2 a febrilní reakce, nezpůsobila však narušení produkce prozánětlivých cytokinů, interleukinu IL-1β, IL-6 a tumour necrosis factoru (TNF) (Nilsberth et al., 2009). Důležitým pozorováním této práce, kromě disociace produkce těchto cytokinů a horečnaté odpovědi, je rovněž fakt, že produkce cytokinů po podání LPS je PGE_2 nezávislá.

Skutečnost, že časná produkce PGE_2, generovaná Kupfferovými buňkami hraje klíčovou roli, byla prezentována na dalších modelech akutní zánětlivé odpovědi diskutovaných dříve (Zimomra et al., 2011). Zimomra et al., navíc u experimentálního modelu používajícího *E. coli* naznačili možný vliv PGE_2 na kontrole produkce pro-zánětlivých cytokinů, jakožto faktoru, který dále upřesňuje celkovou odpověď organismu (Zimomra et al., 2011). Krall et al., následně rozšířili roli prostaglandinu E_2 a D_2 v hypertermické a hypotermické reakci laboratorních potkanů na různě vysoké dávky LSP. V experimentu prokázali, že deprivace příjmu potravy nevede k hypotermii podmíněné nedostatečným signálem na úrovni PGE_2, a to vzhledem k tomu, že skupina laboratorních potkanů deprivovaných nedostatečným příjmem potravy je schopná normální hypertermické reakce asociované se zvýšením hladin PGE_2. Hypotermická reakce je pak pravděpodobně důsledkem hypotermického působení PGD_2 (Krall et al., 2010).

Můžeme tedy uzavřít, že se nám podařilo in vivo potvrdit předchozí pozorování asociace časného nárůstu plasmatických hladin PGE_2 a vzniku horečky po předchozím podání LPS. Jako buněčný typ zodpovědný za jeho produkci jsme identifikovali Kupfferovy buňky a zároveň jsme prokázali disociaci horečnaté odpovědi a up-regulace produkce tzv. endogenních cytokinů. Identifikace periferní signalizace je významným

krokem ke klinické aplikaci vzhledem k tomu, že asociace vysoké horečky a zvýšené mortality u kriticky nemocných pacientů byla již dříve prokázána. Nicméně praktická aplikace ve smyslu jasného algoritmu léčby horečky v klinické praxi u takto závažně nemocných pacientů chybí (Niven 2011). V oblasti klinického výzkumu je tedy prostor pro identifikace biomarkerů (např. hladina PGE_2, cytokinů atd.), stejně tak jako pro randomizované studie porovnávající farmakologické, fyzikální či kombinované intervence.

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Kupffer cell-generated PGE_2 triggers the febrile response of guinea pigs to intravenously injected LPS

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Li, Zhonghua, Vit Perlik, Carlos Feleder, Ying Tang, and Clark M. Blatteis. Kupffer cell-generated PGE_2 triggers the febrile response of guinea pigs to intravenously injected LPS. *Am J Physiol Regul Integr Comp Physiol* 290: R1262–R1270, 2006. First published January 12, 2006; doi:10.1152/ajpregu.00724.2005.—Because the onset of fever induced by intravenously (iv) injected bacterial endotoxic lipopolysaccharides (LPS) precedes the appearance in the bloodstream of pyrogenic cytokines, the presumptive peripheral triggers of the febrile response, we have postulated previously that, in their stead, PGE_2 could be the peripheral fever trigger because it appears in blood coincidentally with the initial body core temperature (T_c) rise. To test this hypothesis, we injected *Salmonella enteritidis* LPS (2 μg/kg body wt iv) into conscious guinea pigs and measured their plasma levels of LPS, PGE_2, TNF-α, IL-1β, and IL-6 before and 15, 30, 60, 90, and 120 min after LPS administration; T_c was monitored continuously. The animals were untreated or Kupffer cell (KC) depleted; the essential involvement of KCs in LPS fever was shown previously. LPS very promptly (<10 min) induced a rise of T_c that was temporally correlated with the elevation of plasma PGE_2. KC depletion prevented the T_c and plasma PGE_2 rises and slowed the clearance of LPS from the blood. TNF-α was not detectable in plasma until 30 min and in IL-1β and IL-6 until 60 min after LPS injection. KC depletion did not alter the times of appearance or magnitudes of rises of these cytokines, except TNF-α, the maximal level of which was increased approximately twofold in the KC-depleted animals. In a follow-up experiment, PGE_2 antiserum administered iv 10 min before LPS significantly attenuated the febrile response to LPS. Together, these results support the view that, in guinea pigs, PGE_2 rather than pyrogenic cytokines is generated by KCs in immediate response to iv LPS and triggers the febrile response.

fever; tumor necrosis factor-α; interleukin-1β; interleukin-6; liver; complement

IT IS GENERALLY CONSIDERED that the febrile response to systemic infectious pathogens is mediated by the pyrogenic cytokines TNF-α, IL-1β, and IL-6, elaborated by mononuclear phagocytes activated by the infectious noxa, and that their messages are transmitted to the fever-controlling center in the ventromedial preoptic-anterior hypothalamic area (POA) either neurally or humorally (for reviews, see Refs. 3, 6, 14, 66, 70, and 90). The neural pathway has been postulated to account for the rapid initiation of fever after, e.g., a bolus, intravenous (iv), low-to-moderate dose injection of bacterial endotoxic LPS; the afferent vagus serves as its link (69, 75, 87). The humoral pathway, on the other hand, depends on the bloodstream for the delivery of these mediators; it is, consequently, slower than the neural pathway (38). However, although cytokines, in particular IL-6, have been demonstrated in plasma correlatively with the onset of fever induced by low-to-moderate doses of LPS administered intraperitoneally, intramuscularly, and subcutaneously (into an air pouch) (~30–60 min), they are not detectable concurrently with the induction of fever provoked by higher doses of LPS or by any dose of LPS injected iv (14). For instance, the body core temperature (T_c) of conscious guinea pigs rises significantly within 10 min after the injection of 2 μg iv of LPS/kg body wt (75, 77), whereas TNF-α, the first of the cytokines to appear in the blood of similarly LPS-challenged guinea pigs, is not detectable until at least 30 min later (42, 59; present study, Fig. 3). This delay, nevertheless, should be anticipated because these cytokines are not expressed constitutively in mononuclear phagocytes, but rather are transcribed, translated, and secreted by these cells in response to the pyrogenic stimulus. Hence, if they are not yet present in the blood, it would seem improbable that circulating cytokines could provide the signals for the very prompt induction of fever after iv LPS or high doses of intraperitoneal LPS.

The liver is the body's principal filter of circulating LPS (29, 43, 51, 72); it contains ~80%-90% of all macrophages in the body. Consequently, hepatic macrophages [Kupffer cells (KCs)] are considered to be the principal cell source of pyrogenic cytokines. Indeed, the involvement of KCs in the pathogenesis of fever is supported by a variety of findings (22, 84). In further support, we recently showed that the onset of fever in guinea pigs is linked to the first appearance of LPS in the liver (48) and that splenectomy and vinblastine-induced neutropenia both significantly increase the simultaneous febrile response to LPS and the uptake of LPS by KCs (27, 49). However, because the LPS-induced production of cytokines by KCs, like that of other macrophages, is delayed in relation to fever onset (18, 35, 42, 54), it seems unlikely that KC-generated cytokines could provide the signals that rapidly initiate iv LPS-induced fever. A factor elaborated by KCs earlier than cytokines, i.e., an almost immediate reaction to the presence of LPS, should, therefore, mediate this response.

Although its cell source and the nature of the triggering mechanism that releases it are still in dispute, PGE_2 is generally believed to be the final central mediator of the febrile response (for reviews, see Refs. 3, 10, 41, and 64). It acts on thermoregulatory neurons in the POA, and its levels increase

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and decrease in this brain region in conjunction with the febrile course. PGE_2 levels also quickly increase in the peripheral circulation after the entry of microorganisms or the systemic administration of exogenous and endogenous pyrogens (21, 71, 79). Using an in vivo model for the selective hepatic portal vein infusion of anesthetized guinea pigs, we have recently shown that PGE_2 is indeed very quickly generated by KC in response to the intraportal vein injection of LPS (59). Because the peripheral injection of exogenous PGE_2 reportedly causes T_c rises in some animals (57, 62, 68), it has been suggested that it could play its mediatory role peripherally rather than centrally (23, 55, 67).

This study was designed to verify this hypothesis in conscious, iv LPS-treated animals. Because the PGE_2 generated by KCs under these conditions spills into the inferior vena cava and, thence, into the general circulation (59), and since, moreover, systemically administered LPS invariably appears in the liver (48), we have elected in the present study to inject LPS systemically (into the superior vena cava) rather than directly into the portal vein; this approach, moreover, obviates the trauma of major abdominal surgery and its possible, attendant, inflammatory consequences. Thus we selectively depleted guinea pigs of KCs by pretreatment with gadolinium chloride ($GdCl_3$) and compared the time courses of their febrile and associated PGE_2 and pyrogenic cytokine responses to iv LPS with those of untreated controls. To our best knowledge, this is the first report of the simultaneous and coordinated changes in T_c and endogenously produced plasma PGE_2 and pyrogenic cytokine levels at close intervals over the first 2 h following iv LPS administration to conscious animals. In a follow-up experiment, we further tested the validity of our hypothesis by pretreating conscious guinea pigs iv with PGE_2 antiserum and evaluating its effect on the animals' T_c responses to iv LPS.

MATERIALS AND METHODS

Animals

Male Hartley guinea pigs (300–350 g body wt on arrival; Charles River Laboratories, Wilmington, MA) were used in these experiments. The animals were quarantined for 1 wk, three to a cage, before any experimental use. Tap water and food (Agway Prolab guinea pig diet) were available ad libitum. The ambient temperature (T_a) in the animal room was 23 ± 1°C, the housing T_a recommended by the Institute of Laboratory Animal Resources Commission on Life Sciences (ILAR) (32); light and darkness were alternated, with lights on from 0600 to 1800. After quarantine, to moderate the psychological stress associated with the experiments, the animals were trained to the experimental procedures for 1 wk (daily for 4 h) by handling and placement in individual, locally fabricated, semicircular, wire-mesh confiners designed to prevent their turning around and to minimize their forward and backward movements but without causing excessive restraint stress. All animal protocols were approved by the University of Tennessee Health Science Center Animal Care and Use Committee and fully conformed to the standards established by the U.S. Animal Welfare Act and by the documents entitled "Guiding Principles for Research Involving Animals and Human Beings" (83).

General

All glassware, plasticware, instruments, and cannulas used in these studies were sterilized by autoclaving. Electrochemical grade, high-purity water (Baxter Healthcare, Muskegan, MI) was used exclusively in the preparation of all solutions. Before use, the stock solutions were passed through a sterile 0.22-μm Miller-GS filter unit (Millipore, Bedford, MA), as an added precaution against bacterial contamination. Absence of endotoxic contamination in all fluids not containing LPS by design were verified by the *Limulus* amebocyte lysate test (Pyrochrome; Associates of Cape Cod, Falmouth, MA).

Drugs

LPS was *Salmonella enteritidis* LPS B (batch no. 651628; Difco Laboratories, Detroit, MI), the same LPS batch we have used in all of our previous studies. $GdCl_3$ hexahydrate was purchased from Sigma-Aldrich (cat. no. G-7532, lot no. 121K3655; St. Louis, MO). Heparin was purchased from Elkins-Sinn (Cherry Hill, NJ). The vehicle for these solutions was pyrogen-free saline (PFS; 0.9% NaCl, USP; Abbott Laboratories, Chicago, IL). Rabbit monoclonal PGE_2 antiserum and its vehicle, phosphate buffer containing 0.1% sodium azide (NaN_3) and bovine serum gamma globulin (BSG), were procured from Assay Designs (cat. no. 905–025, lot no. 01E073A; Ann Arbor, MI) and Sigma-Aldrich (cat. no. S2002), respectively.

Jugular Vein Cannulation

In preparation for iv injections and blood collections, all animals received the antibiotic chloramphenicol (20 mg/kg body wt sc) prophylactically 1 h before the surgical procedure. Under ketamine-xylazine (35/5 mg/kg body wt im) anesthesia and aseptic conditions, a siliconized cannula (0.020 in. ID, 0.037 in. OD; Baxter Healthcare, McGraw Park, IL), prefilled with heparinized (10 IU/ml) PFS, was inserted into the left jugular vein and gently guided into the superior vena cava of each guinea pig. The free end of the cannula was passed subcutaneously toward the head, exteriorized on the top of the head, knotted, and rolled into a coil. This coil was then placed inside a protective polypropylene shield (a centrifuge microtube with a screw cap, with its cone cut off) that was fixed to the skull with dental acrylic cement and four self-tapping, miniature stainless steel screws. The neck wound was sutured and cleansed with 10% povidone-iodine solution and treated with nitrofurazone powder. Immediately after this surgery, the animals received a bolus (10 ml PFS sc) injection and pain medication (0.05 mg/kg body wt butorphanol) and, for two more days, chloramphenicol subcutaneously. To maintain the patency of the inserted cannulas, the cannulas were flushed with 0.5 ml of heparinized (3 IU/ml) PFS every day after surgery until 3 days before an experiment, when PFS alone was used because of the confounding effect of heparin on complement activation and cytokine production (88). Experiments were performed 7 days after this surgical procedure, when the animals had recovered. Retraining was performed during the latter 4 days of this recovery period.

Temperature Recording

Beginning at 0800 of the experimental day, the T_cs of the conscious guinea pigs, loosely restrained in the individual confiners to which they had been trained, were monitored constantly and recorded at 2-min intervals for 2 h on a Macintosh Plus 1 Mb microcomputer through an analog-to-digital converter, using precalibrated copper-constantan thermocouples inserted 5 cm into the colon. The data were displayed digitally on a monitor, printed on an ImageWriter printer, and stored on a diskette. A 90-min stabilization period to achieve thermal equilibrium preceded all measurements.

Blood Collections

Blood (0.4 ml) was collected at predetermined intervals from the preinserted cannulas before and after PFS or LPS injection. PFS (~0.5 ml) was used to flush blood from the cannulas and replace plasma volume following its withdrawal. Heparin (0.05 ml, 10%) was added to all samples; they were then swirled and centrifuged (3,000 *g*, 10 min, at 4°C). Aliquots of the plasma were stored at −70°C for assay later.

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Assays

LPS. LPS concentrations in the plasma of guinea pigs were measured using a chromogenic *Limulus* amebocyte lysate assay (Pyrochrome; Associates of Cape Cod), according to the supplier's instructions. The First International Standard for Endotoxin (code 84/650: World Health Organization) was used as a reference; endotoxin concentrations were expressed as endotoxin units per milliliter (EU/ml). The detection limit of this assay was 0.005 EU/ml.

PGE_2. The PGE_2 levels in the plasma of guinea pigs were analyzed using a commercial enzyme immunoassay kit (High Sensitivity PGE_2 EIA Kit model 931–001; Assay Designs), according to the manufacturer's instructions. The PG synthetase inhibitor indomethacin (10 μg/ml) was added to all blood samples immediately after collection All of the samples were diluted before analysis in the assay buffer system provided by the manufacturer, according to the manufacturer's instructions. All of the samples were analyzed in duplicate. The detection limit of this assay was 8.26 pg/ml.

Cytokines. Cytokine levels in guinea pig plasma were assayed by established bioassay techniques. All samples were analyzed in triplicate. TNF-α was evaluated based on the cytotoxic effect of TNF-α on the mouse fibrosarcoma cell line WEHI 164 subclone 13 (26). The assay was conducted using sterile, 96-well microtiter plates. Serial dilutions of biological samples or different concentration of TNF-α standard [code 88/532; National Institute for Biological Standards and Control (NIBSC), South Mimms, UK] were incubated for 24 h in wells that had been seeded with 50,000 actinomycin-D-treated WEHI 164 cells. The number of surviving cells after 24 h was measured by use of the MTT colorimetric assay.

The determinations of IL-1β and IL-6 were performed by bioassays based on the dose-dependent growth stimulation of the D10 and B9 hybridoma cell lines, respectively (36, 37). These assays were conducted using sterile, 96-well microtiter plates. In each well, 5,000 D10 or B9 cells were incubated for 72 h with serial dilutions of biological samples or with different concentrations of IL-1β or IL-6 standards (codes 86/680 and 89/548, respectively; NIBSC). The number of cells in each well was measured by use of the MTT assay.

Experimental Design

Experiment 1. Effect of LPS on the T_c and plasma LPS, PGE_2, and cytokine levels of untreated and $GdCl_3$-pretreated guinea pigs. Seven days after surgery, the guinea pigs were randomly divided into four treatment groups: *1*) PFS (0.9 ml/kg; $n = 4$), *2*) LPS (2 μg/kg; $n = 5$), *3*) $GdCl_3$ (7.5 mg/kg) + PFS ($n = 4$), and *4*) $GdCl_3$ + LPS ($n = 5$). $GdCl_3$ was injected into the animals' precannulated jugular veins 3 days before PFS or LPS. $GdCl_3$ is a lanthanide rare earth metal that, when injected at 7.5 mg/kg body wt iv (12, 33, 48, 76), inactivates the macrophages within the vasculature, i.e., hepatic, splenic, and pulmonary intravascular phagocytes. Repopulation of splenic and pulmonary macrophages starts at *day 1* and is complete in 2–3 days; repopulation of KC begins 4 days after $GdCl_3$ injection (33, 48). Hence, the present experiments were performed on *day 3* post-$GdCl_3$, when the KC, the principal clearinghouse of LPS and source of pyrogenic mediators (see Introduction), were still nonfunctional, i.e., when their affinity for LPS was significantly reduced (48). $GdCl_3$ at this dose has no demonstrable effect on hepatic stellate and sinusoidal endothelial cells, which also release PGE_2, but in smaller quantities (73).

On the experimental day, the conscious animals were placed in their confiners and connected to the T_c recording system. After the 90-min stabilization period, 2 μg of LPS/kg body wt in 0.9 ml of PFS/kg body wt or the same volume of PFS was injected via the implanted jugular vein cannulas. T_c was monitored continuously for the following 2 h. Just before *time 0* and 15, 30, 60, 90, and 120 min after PFS or LPS administration, 0.4 ml of blood was collected, prepared, and stored as described above, for later analysis.

Experiment 2. Effect of LPS on the T_c of untreated and PGE_2 antiserum-pretreated guinea pigs. To substantiate that PGE_2, rapidly released by KC in response to LPS (59), is indeed the trigger that initiates the febrile response to LPS, conscious guinea pigs, in their confiners and after their 90-min stabilization period, received via their implanted jugular vein cannulas PGE_2 antiserum (1 ml/kg) or its vehicle, 0.1% NaN_3 (1 ml/kg body wt in PBS containing BSG), 10 min before PFS (0.9 ml/kg; $n = 6$ and 5, respectively) or LPS (2 μg/kg body wt in 0.9 ml of PFS/kg; $n = 6$ and 6, respectively) by the same route. The dose of the antiserum was based on previous data in the literature regarding its PGE_2 neutralizing activity in vivo and in vitro (31, 44, 60, 61); its solvent, NaN_3, an inhibitor of oxidative phosphorylation, is a neurotoxicant that transiently reduces T_c (15, 30). The antiserum was raised in rabbits; its cross-reactivity was 50, 1.6, and <0.1% against PGE_1, $PGF_{2\alpha}$, and PGD_2, respectively, other PGs with thermoregulatory activities. T_c was monitored continuously for the following 5 h.

Statistical Analysis

The results are reported here as means ± SE. The values of T_c are changes (ΔT_c) from basal values [T_{ci} (initial), the T_c at 2-min intervals averaged over the last 10 min of the preceding 90-min stabilization period, plotted at 6-min intervals]. The PGE_2 data are expressed as changes relative to their values before a treatment (P_0). The data were evaluated by a repeated-measures ANOVA (Instat 3, GraphPad Software; Instant Biostatistics, San Diego, CA), where *factor 1* was the between-group factor (the experimental treatment) and *factor 2* the within-subject factor (the different sampling periods). Each variable was considered to be independent. Latencies of fever onset were defined as the intervals (in minutes) between the time of LPS injection (0 min) and that of the first T_c rise greater than 0.2°C (the SD of the mean T_{ci}) that continued uninterruptedly beyond 0.5°C. The 5% level of probability was accepted as statistically significant in all experiments.

RESULTS

Experiment 1

The responses of T_c, plasma LPS, PGE_2 and cytokine levels of untreated and $GdCl_3$-pretreated guinea pigs to the iv injection of LPS are illustrated in Figs. 1–3. Figs. 1*A* and 2*A* illustrate the courses of the plasma LPS levels of all animals following LPS challenge. In all cases, the plasma LPS concentration reached its maximum 15 min after LPS administration, declined rapidly to about half its maximum by 30 min, and then slowly decreased toward its original level by 120 min ($P < 0.01$ relative to their corresponding PFS controls). $GdCl_3$ pretreatment did not affect the rise and fall of plasma LPS except during the last 30 min, when its rate of fall slowed significantly ($P < 0.05$ relative to LPS, Fig. 1*A* vs. Fig. 2*A*).

The courses of the plasma PGE_2 levels of the untreated and $GdCl_3$-pretreated guinea pigs in response to PFS or LPS are shown in Figs. 1*B* and 2*B*. In the untreated guinea pigs, the plasma PGE_2 level rose to its first maximum within 15 min after LPS administration, declined to near control at about 60 min, then rose again and reached a second maximum at 90 min ($P < 0.01$ relative to PFS, Fig. 1*B*). The LPS-induced plasma PGE_2 rise was essentially eliminated in the $GdCl_3$-pretreated guinea pigs compared with their untreated LPS controls ($P < 0.05$ relative to LPS, Fig. 1*B* vs. Fig. 2*B*). PFS injection did not significantly affect the PGE_2 levels of the untreated and $GdCl_3$-pretreated guinea pigs, although they tended to decrease a little over time.

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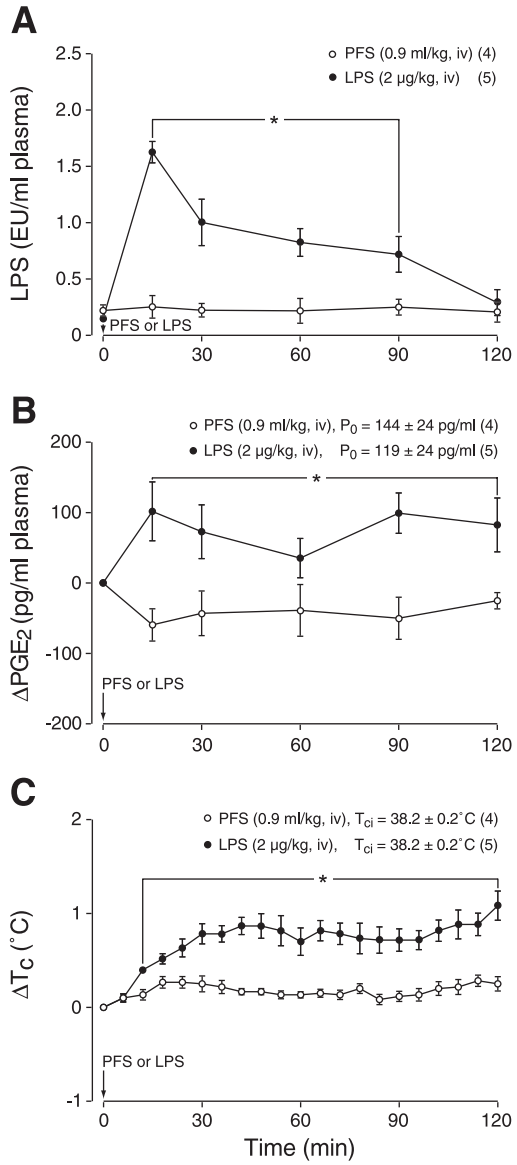

Fig. 1. The courses of the levels of plasma LPS (*A*) and PGE_2 (*B*) and of the core temperatures (T_c; *C*) of conscious guinea pigs in response to injected pyrogen-free saline (PFS; 0.9 ml/kg body wt iv) or *Salmonella enteritidis* LPS B (2 μg/kg body wt in 0.9 ml of PFS/kg), T_c and PGE_2 are expressed as different (Δ) from their respective basal values, P_0 and T_{ci} (please see *Statistical Analysis*). Values are means ± SE; number in parentheses is number of animals; $*P < 0.05$ relative to corresponding PFS. EU, endotoxin units.

The T_c of the untreated guinea pigs began to rise within 6 min after the injection of LPS, reaching its first maximum (~1.0°C) at about 48 min. It then decreased slightly over the following 10 min, then rose again toward a second peak at ~2 h post-LPS ($P < 0.01$ relative to PFS, Fig. 1*C*). $GdCl_3$ pretreatment prevented the LPS-induced T_c rise and even caused an ~0.4°C fall at ~60 min after the LPS injection ($P < 0.01$ relative to LPS, Fig. 1*C* vs. Fig. 2*C*). PFS administration had no significant effect on the T_cs of the untreated and $GdCl_3$-pretreated guinea pigs.

The plasma cytokine responses to PFS and LPS of the untreated and $GdCl_3$-pretreated guinea pigs are shown in Fig. 3. After LPS administration, TNF-α first appeared in plasma at 30 min, reached its maximum at 60 min, and then returned to its baseline at 120 min ($P < 0.01$ relative to PFS; Fig. 3*A*). KC depletion did not change the levels of plasma TNF-α during the first 30 min and 120 min after LPS administration, but it significantly increased them at 60 and 90 min ($P < 0.01$ relative to LPS, Fig. 3*A*). The plasma IL-1β (Fig. 3*B*) and IL-6 (Fig. 3*C*) were undetectable until 60 min after LPS administration when their levels began and then continued to rise during the rest of the experimental period ($P < 0.01$ relative to their corresponding PFS controls). $GdCl_3$ pretreatment did not alter the responses of these two cytokines to LPS. No pyrogenic cytokine was detectable in the plasma of the PFS-treated animals.

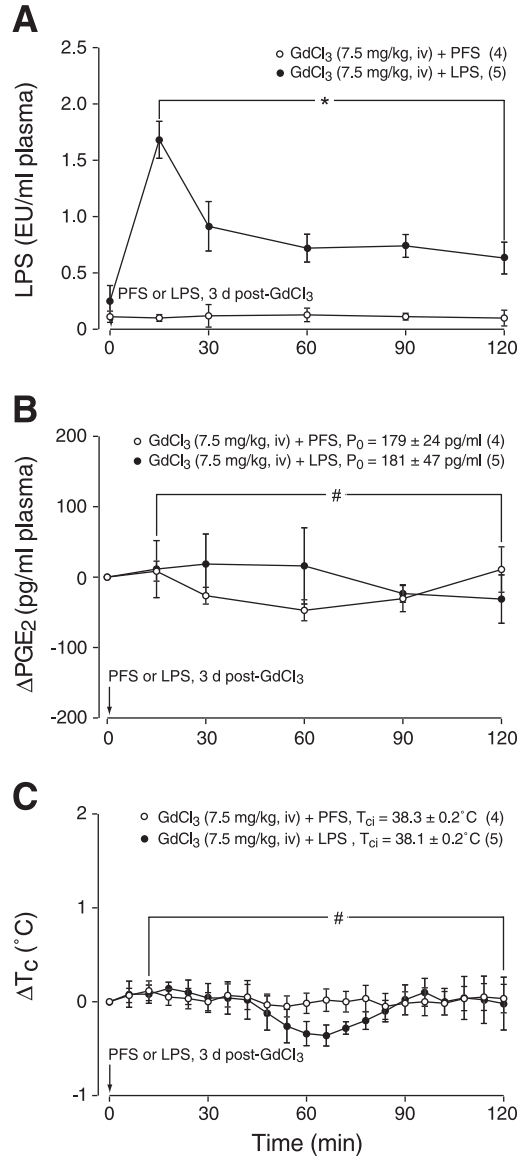

Fig. 2. The courses of the levels of plasma LPS (*A*) and PGE_2 (*B*) and of the core temperatures (T_c; *C*) of conscious guinea pigs in response to injected PFS (0.9 ml/kg body wt iv) or *S. enteriditis* LPS B (2 μg/kg body wt in 0.9 ml of PFS/kg), with gladolinium chloride ($GdCl_3$) pretreatment (7.5 mg/kg body wt iv, 3 days prior). T_c and PGE_2 are expressed as different (Δ) from their respective basal values, P_0 and T_{ci} (please see *Statistical Analysis*). Values are means ± SE; number in parentheses is number of animals; $*P < 0.05$ relative to corresponding PFS; $\#P < 0.05$ relative to $GdCl_3$-untreated LPS (Fig. 1, *A–C*).

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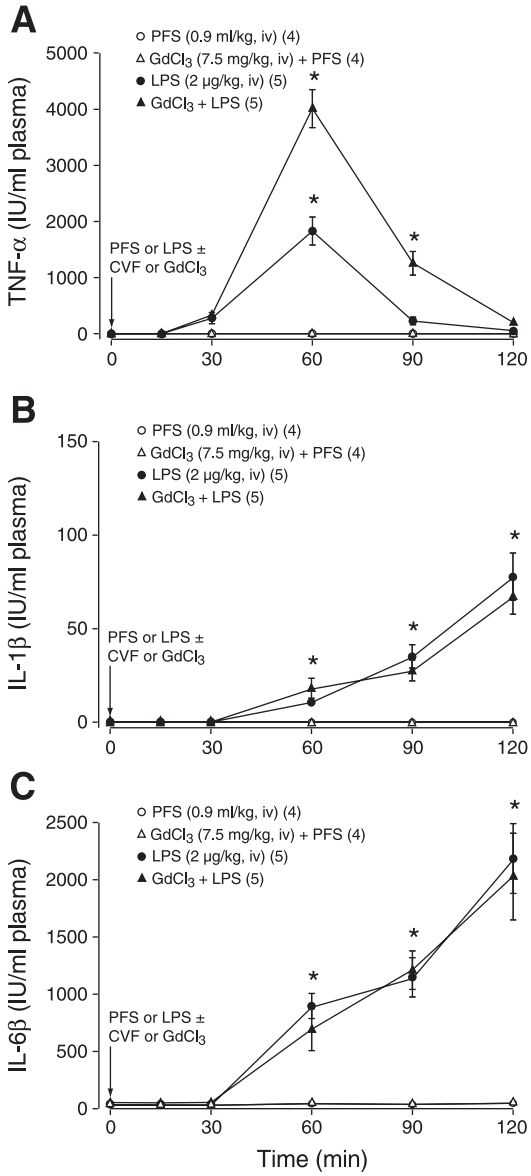

Fig. 3. Corresponding courses of the levels of TNF-α (*A*), IL-1β (*B*), and IL-6 (*C*) in the plasma of the animals depicted in Figs. 1 and 2. Values are means ± SE; number in parentheses is number of animals; $*P < 0.05$ relative to corresponding PFS ± $GdCl_3$-pretreated groups.

Experiment 2

The effects of PGE_2 antiserum and its vehicle, NaN_3, administered iv 10 min before PFS or LPS by the same route on the thermal responses to these agents are illustrated in Fig. 4. NaN_3 produced in the PFS controls an initial, transient, ~0.5°C fall in T_c that reached its nadir in ~15 min, but T_c quickly recovered thereafter, returning to its basal level by 30 min and stabilizing there for the remainder of the experimental period (Fig. 4*A*). This effect was expected (15, 30). NaN_3 caused a similar initial and transient T_c fall in the animals challenged with LPS 10 min later but did not affect the magnitude and course of the subsequent fever, which were characteristically biphasic (Fig. 4*B*). The transient hypothermic effect of the vehicle was also evident in the guinea pigs treated with PGE_2 antiserum before PFS, but, in this case, T_c "rebounded" ~0.7°C above basal by ~100 min. It decreased over the next ~100 min, then more slowly, but did not fully return to its initial value by the end of the experiment (Fig. 4*C*). The initial hypothermic effect of NaN_3 was similarly present in the animals treated with PGE_2 antiserum before LPS. However, in this group, the febrile response to LPS was abrogated (Fig. 4*D*).

DISCUSSION

To validate our hypothesis, the rise of T_c and plasma PGE_2 levels induced by iv LPS in untreated guinea pigs should occur nearly simultaneously, but not at all in KC-depleted animals; plasma cytokine levels should be irrelevant to the initiation of the febrile response. The hypothesis is well supported by the responses observed in this study. Thus the results show that the onset of fever in response to LPS injected iv into untreated guinea pigs was initiated in close coincidence with the appearance in plasma of significantly increased amounts of PGE_2, both occurring within the first 15 min after LPS administration, whereas TNF-α became detectable between 15 and 30 min and IL-1β and IL-6 after 30 min post-LPS. The iv administration of PGE_2 antiserum (which should not cross the blood-brain barrier [BBB]) before LPS significantly attenuated the subsequent LPS-induced T_c rise. Furthermore, the selective ablation of KCs by pretreatment with $GdCl_3$ prevented the rises of both T_c and plasma PGE_2, enhanced the production of TNF-α, but did not affect the rises of plasma IL-1β and IL-6; these latter results are in agreement with previous findings (63, 65). The clearance of LPS was, however, somewhat slower in this group compared with that in the untreated group, as would be expected from the inactivation of this central LPS clearinghouse (27, 48, 49). The combined effects of their inactivation on T_c and plasma PGE_2 would thus support the role of KCs as the cellular source of the LPS-induced plasma PGE_2, and the temporal separation of the appearance in plasma of endogenous PGE_2 and pyrogenic cytokines would thus implicate KC-generated PGE_2 and exclude circulating cytokines as the signaling molecules of the febrile response to iv LPS.

The role of PGE_2 as an essential mediator of fever production is well established. Thus it is generally considered that its synthesis is catalyzed by cyclooxygenase (COX)-2 and microsomal PGE_2 synthase (mPGES)-1 selectively upregulated by pyrogenic cytokines released into the circulation by mononuclear phagocytes activated by exogenous pyrogens and that it acts in the POA (for reviews, see Refs. 10 and 41). The involvement of KCs as the principal phagocytic cell source of pyrogenic cytokines is also well recognized (22, 43, 48, 76, 84); the present results again demonstrate the critical importance of these cells in LPS fever production. However, not being constitutively expressed in macrophages, the de novo production of cytokines by KCs in response to LPS involves a delay that is significantly longer than the latency of the onset of iv LPS-induced fever. Hence, as also demonstrated by the present results, KC-generated cytokines cannot account for the rapid development of the febrile response to iv LPS. This would infer, therefore, that its prompt beginning is mediated by a different, KC-derived product very quickly elaborated in response to the presence of LPS. We suggested in 1997–1998 (9, 11–13) that PGE_2 could be this mediator because it is synthesized by KCs in response to LPS (for a review, see Ref.

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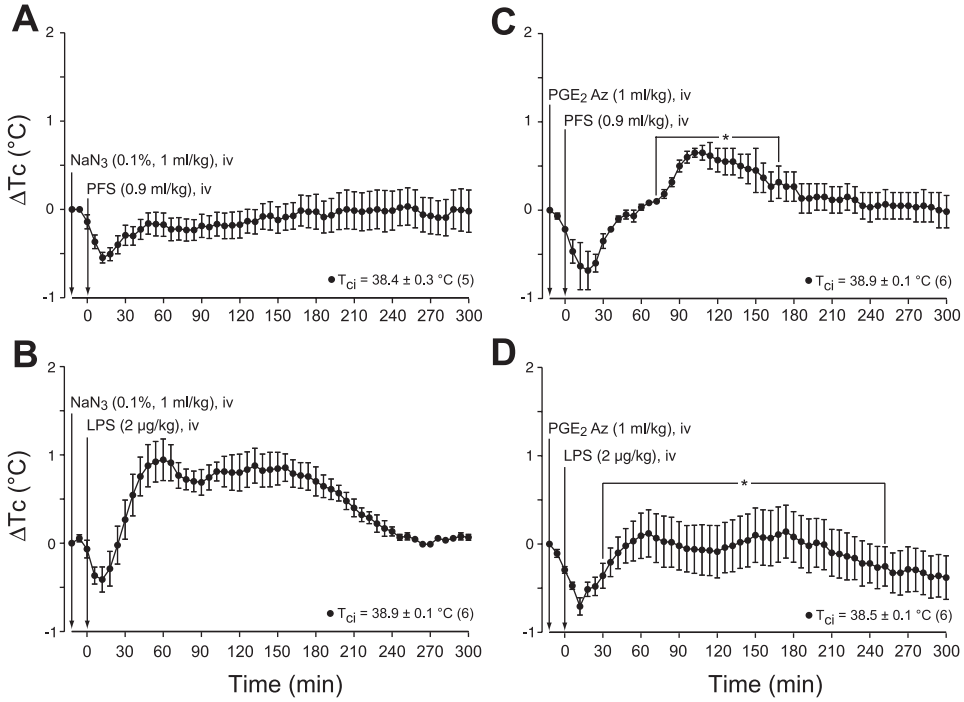

Fig. 4. The responses to injected PFS (0.9 ml/kg body wt iv; *A* and *C*) or LPS (2 μg/kg body wt in 0.9 ml PFS/kg body wt; *B* and *D*) of the T_c of conscious guinea pigs pretreated 10 min prior with PGE_2 antiserum (PGE_2Az, 1 ml/kg body wt iv; *C* and *D*) or its vehicle (0.1% NaN_3, 1 ml/kg; *A* and *B*). Values are means ± SE; number in parentheses is number of animals; $*P < 0.05$ relative to corresponding NaN_3-pretreated group.

10), and it rises in venous and, to a lesser extent, in arterial blood very quickly after the peripheral administration of both exogenous and endogenous pyrogens (21, 59, 71, 79). Moreover, the peripheral injection of exogenous PGE_2 has been reported to cause T_c rises (1, 20, 69, 79), prompting the suggestion that the PGE_2 that acts in the POA in response to peripheral pyrogenic stimuli could, in fact, be PGE_2 that enters it from the blood (20, 23, 55, 67). It is controversial, however, whether PGE_2 can actually pass from the blood into the brain and, especially, whether PGE_2 entering the brain in this way can raise T_c (57, 78). But be that as it may, the present data are, to our best knowledge, the first to show in conscious animals that endogenous PGE_2 specifically released by the liver's KCs in response to iv LPS is coincident with and critical to the appearance of the febrile response.

To our knowledge, the antipyretic effect of PGE_2 antiserum demonstrated in this study is the first report directly implicating circulating PGE_2 as a mediator of the febrile response to LPS, although, as already noted, elevations of PGE_2 in the blood following the administration of pyrogens have been described previously by various authors. However, in those studies, with few exceptions (57, 78), the pyrogenic action of plasma PGE_2 was generally ascribed to its diffusion into and action within the POA (for a review, see Ref. 10). Attenuations of fever similar to the one produced in this study have likewise been observed previously in other studies (for reviews, see Refs. 67, 70, and 90) after the iv administration of nonsteroidal anti-inflammatory drugs and selective COX-2 inhibitors, which prevent the synthesis of PGE_2. These agents, however, being lipophilic, cross the BBB, therefore leaving unclear whether it is the blockade of the peripheral or the central PGE_2 that accounts for their antipyretic action. It is improbable, by contrast, that the present PGE_2 antiserum would cross the BBB. Because it was injected directly into the jugular vein, it probably bound the majority of the PGE_2 produced by the KCs, hence reducing the circulating level of free PGE_2. Indeed, cross-reactivity results reported by the supplier indicate that this antiserum is directed almost exclusively against the PGE_2 structure (see MATERIALS AND METHODS and Ref. 16), and previous dose-response studies of a similar antagonist (56) have indicated that it neutralizes the functional activity of PGE_2 in a concentration-dependent manner. Although the levels of plasma PGE_2 were not assayed in *experiment 2*, the dose we chose had been demonstrated previously to be antagonistic to PGE_2 in vivo (31, 44, 60, 61); it clearly was effective also in this experiment. On this basis, we would postulate that its observed antipyretic effect was due to its specific neutralization of PGE_2 synthesized by the liver, and hence, that the observed attenuation of the fever resulted from the blockade of a peripheral rather than a central mode of action of PGE_2. Because, according to the neural concept of afferent pyrogenic signaling (3, 6–8), the first site of action of PGE_2 would be hepatic vagal sensory terminals, their activation would thus not occur, preventing the manifestation of fever; or alternatively, according to the humoral concept (66–68), antiserum-free PGE_2 not being available, its transport to the brain would be precluded to the same effect.

The effect on T_c of blocking the synthesis or activity of PGE_2 in the periphery has apparently not been investigated previously, presumably due to the paucity of pharmacological agents that antagonize PGE_2 selectively in vivo, but perhaps even more so due to the little incentive to consider a possible role of peripheral PGE_2 in temperature regulation. According to the conventional view of its central action, the selective, in vivo blockade of peripheral PGE_2 would not seem conceptually warranted. In view of its demonstrated antipyretic effect, the rise of T_c produced by PGE_2 antiserum (PGE_2Az) in the control animals would seem, a priori, paradoxical. We would submit, however, that it is consistent. Thus it is well established that PGE_2 inhibits the release of norepinephrine (NE)

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from sympathetic prejunctional nerve endings and, hence, modulates the response of target tissues to this neurotransmitter (24, 34, 50). Conversely, the inhibition of PGE_2 synthesis has been shown to augment NE turnover in a number of organs and tissues (50, 82). A major thermoregulatory function controlled by NE signaling in rodents is brown adipose tissue (BAT) thermogenesis, activated through β_3-adrenoceptors (for a review, see Ref. 17). We suggest that a competitive interaction between noradrenergic stimulation and PGE_2 inhibition of BAT function could underlie the observed T_c rise. That is, the elimination of circulating free PGE_2 that resulted from its specific neutralization by its antiserum caused the gradual disinhibition of the peripheral sympathetic nervous system in *experiment 2*, allowing the continual release of NE from its terminals, thus stimulating the metabolism of brown adipocytes and thereby increasing their rate of heat production, hence raising T_c (for reviews, see Refs. 17 and 89); it is pertinent in this regard that the T_c rise in this experiment developed relatively slowly and had not fully returned to control levels by the end of the experiment. One example in support of this proposition comes from earlier studies showing that PGE_2 secreted by the placenta into the circulation of cooled fetal lambs inhibits BAT thermogenesis before birth, whereas the disappearance of PGE_2 after placental separation at birth allows its initiation (32). Other peripheral thermoeffectors responsive to sympathetic stimulation, e.g., cutaneous vasculature, adrenal medulla, could also have contributed to the observed T_c rise. An interesting implication of these data, parenthetically, is that PGE_2 may be important not only for fever production, but also for the maintenance of normal T_c.

It is generally agreed that the production of PGE_2 induced by LPS is initiated by the LBP-mediated transfer of LPS to the receptor complex CD14/TLR4/MD2 (2, 19, 53). The LPS-TLR4 complex is also the system that induces the production of pyrogenic cytokines. Although, as already mentioned, it is clear that the increased biosynthesis of PGE_2 thus induced is selectively catalyzed by COX-2 and mPGES-1 (58, 80, 86), the transcription and translation of these inducible enzymes also involve a significant delay. In rats, the ex vivo expression of genes encoding COX-2 and mPGES-1 in liver is not strongly enhanced until 0.5 h after iv LPS (40, 41), i.e., after the onset of fever. This implies, therefore, that the prompt elevation of plasma PGE_2 levels in response to iv LPS cannot be accounted for by its COX-2/mPGES-1-mediated production in KCs. Therefore, again, another factor, very quickly released in response to LPS, must mediate the rapid appearance of PGE_2 into the blood before and independently of the occurrence of these PGE_2-synthesizing enzymes in the liver. We have recently demonstrated that this factor is complement (C), in particular its anaphylatoxic component 5a (C5a; for reviews, see Refs. 4, 7, 8). To wit, the C cascade is activated immediately upon contact with LPS (85). KCs express C5a receptors ($C5aR_1$) (73, 74), and the release of PGE_2 is stimulated within 0 to 2 min after C5a administration to in situ-perfused rat livers and isolated rat KCs (28, 74). The addition of C alone or in combination with LPS, but not that of LPS alone, to freshly harvested mouse and guinea pig KCs provokes the virtually immediate, near-maximal release of PGE_2 by these cells (7, 59). This response is catalyzed nondifferentially by COX-1 and COX-2 (4), which are both constitutive in KCs. Cytokines are released significantly later. C5a induced by LPS, not LPS per se, is the trigger of the early release of PGE_2 from the livers of conscious guinea pigs (59). Decomplementation abrogates all of these effects. Finally, the presence of C5a is necessary for the febrile response of guinea pigs and mice to LPS (7, 8, 45–47). An impairment of the uptake of LPS by KCs because of the insufficiency of C in those studies is excluded by subsequent findings that the uptakes of LPS by the livers of C-sufficient and C-insufficient guinea pigs are not different (Li Z and Blatteis CM, unpublished observations), consistent with an earlier report in rhesus monkeys and rabbits (52). The virtually instantaneous release of PGE_2 induced by C5a has been accounted for by its binding to $C5aR_1$, a G protein-linked receptor that acts by increasing intracellular inositol-1,4,5-trisphosphate and Ca^{2+}, rapidly activating COX-1-catalyzed PGE_2 production (for a review, see Ref. 73). COX-1 is functionally coupled mainly with cPGES and, therefore, prepared to quickly synthesize PGE_2 (81).

Taken together, therefore, these findings further substantiate the importance of KCs in initiating the febrile response of guinea pigs to iv LPS and validate the notion that the fever is triggered by peripheral PGE_2 rapidly generated by KCs presumptively stimulated by LPS-activated C5a; cytokines are not involved in this initial febrigenic process. It was not addressed in this study how the PGE_2 thus released may inform the POA. It could, indeed, be transported to it by the circulation and eventually penetrate it (23, 68). Or, alternatively, it could activate sensory vagal terminals in the liver [PGE_2 receptors are widely distributed on sensory neurons, including vagal afferents (25)]; these could transmit its signals without delay to the POA and thus account for the prompt onset of the febrile response to iv LPS (3–6).

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4.2. Význam sleziny v regulaci horečky

Význam sleziny v regulaci horečky je prezentován pracemi:

Feleder C, Li Z, Perlik V, Evans A, Blatteis CM. *The spleen modulates the febrile response of guinea pigs to LPS.* Am J Physiol Regul Integr Comp Physiol. 2003 Jun; 284(6):R1466-76. Epub 2003 Mar 6. PubMed PMID: 12623774.

Feleder C, Perlik V, Tang Y, Blatteis CM. *Putative antihyperpyretic factor induced by LPS in spleen of guinea pigs.* Am J Physiol Regul Integr Comp Physiol. 2005 Sep; 289(3):R680-7. Epub 2005 May 26. PubMed PMID: 15919737.

Asociace LPS mediované horečky a Kupfferových buněk je postulována dlouhou dobu (Dinarello et al., 1968) a přes nepřímé důkazy mnoha autory potvrzena v několika pracích týmu profesora Blatteise. Sehic et al., (1996) poprvé demonstrovali, že deplecí Kupfferových buněk pomocí gadolinia dojde k oslabení horečnaté odpovědi po léčbě LPS a k prevenci nárůstu PGE_2 v preoptické oblasti hypotalamu s tím asociované. Základní role Kupfferových buněk ve spojení s periferní produkcí PGE_2 pro iniciální fáze horečky pak byla prokázána později (Li 2004; Perlik et al., 2005; Li et al., 2006). Nicméně vliv ostatních makrofágů včetně makrofágů ve slezině na vychytávání LPS z cirkulace byl také popsán, a to u různých zvířecích modelů zahrnující LPS podání u králíka, laboratorního potkana, psa (Adams and Hamilton, 1984; Ge et al., 1997; Mathison and Ulevitch 1979; Moeniralam et al., 1997; Ruiter et al., 1981; Yoshida et al., 1995). Pro objasnění vlivu makrofágů sleziny na vznik horečky a pro objasnění možného ovlivnění funkce Kupfferových buněk, tak jak bylo postulováno dříve (Billiar et al., 1988; Marshall et al., 1993), jsme proto provedli níže uvedené experimenty (Feleder et al., 2003; Feleder et al., 2005).

V první práci jsme se zaměřili na vliv sleziny v procesu reakce organismu na intravenózně a intraperitoneálně podaný lipopolysacharid u laboratorních morčat po splenektomii a sledovali vychytávání LPS v Kupfferových buňkách v časech 15, 30 a 60 minut, kopírujících průběh vzestupu tělesné teploty laboratorních morčat po podání LPS. Pro jednoznačnou identifikaci LPS v makrofázích jsme používali fluoresceinem označený lipopolysacharid (FITC-LPS).

Zjistili jsme, že splenektomie vede k augmentaci odpovědi, tedy ke vzniku horečky u nízké dávky, která nezpůsobila horečku u kontrolní skupiny zvířat a k vyššímu vzestupu teploty po podání LPS proti příslušným kontrolám. Tento nárůst teploty u splenektomovaných zvířat proti pozitivním kontrolám byl nezávislý na cestě podání a době experimentu (7 vs 30 dní) po provedené splenektomii. Kinetika a způsob vychytávání LPS

se rovněž splenektomií výrazně ovlivnila. Zatímco splenektomie neovlivnila první výskyt LPS v Kupfferových buňkách, kde byl detekovatelný již v 15. minutě po intravenózním a 60. minutě po intraperitoneálním podání LPS, u splenektomovaných zvířat byl zjištěn intenzivnější signál LPS v Kupfferových buňkách a prodloužila se doba, po kterou bylo LPS detekovatelné v Kupfferových buňkách. Toto pozorování bylo nezávislé na cestě podání a době experimentu po splenektomii.

Zvýšené vychytávání LPS Kupfferovými buňkami po splenektomii je v souladu s předcházejícími pozorováními u laboratorních potkanů a myší po splenektomii (Andersson and Bengmark, 1989; Shirai et al., 1988; Yamada et al., 1990).

Zajímavým faktem je, že zvýšené vychytávání FITC-LPS Kupfferovými buňkami se pojí s pozorovaným nárůstem teploty proti pozitivní kontrolní skupině zvířat. Otázkou zůstává, zda nárůst teploty po podání FITC-LPS u splenektomovaných morčat je způsoben čistě vyšší dávkou FITC-LPS v Kupfferových buňkách danou kompenzací funkce chybějících makrofágů sleziny nebo slezina a její makrofágy modulují systém vychytávání LPS a následně celou febrilní odpověď. Tuto otázku jsme se snažili zodpovědět v naší následující práci.

V naší druhé práci jsme reprodukovali pozorování zjištěná po splenektomii zvířat u experimentálního modelu podvazu splenické žíly, tj. augmentaci febrilní odpovědi na intraperitoneální podání 2 μg/kg LPS, subfebrilní dávky u kontrolní skupiny, a zvýšené vychytávání LPS Kupfferovými buňkami také po intraperitoneálním podání 75 μg/kg fluoresceinem značeného LPS. Rovněž tato dávka je subfebrilní a čas stanovený pro sledování vychytávání FITC-LPS (60 min po léčbě) byl vybrán s ohledem na minimum aktivity v tomto čase u kontrolní skupiny. Aktivní role sleziny pak byla ověřena podáním „splenického extraktu“ získaného

homogenizací a následnou centrifugací sleziny ze zvířat předléčených LPS v intervalech 5, 15 a 30 min po léčbě. Podání „splenického extraktu vedlo k oslabení zvýšené febrilní reakce na intraperitonálně podané LPS u splenektovaných zvířat, a to až na úroveň odpovědi pozitivní kontrolní skupiny. Toto oslabení febrilní odpovědi bylo doprovázeno sníženou reakcí Kupfferových buněk v podobě sníženého vychytávání značeného LPS u splenektovaných zvířat. Zmíněné výsledky potvrzují náš dříve postulovaný regulační vliv sleziny prostřednictvím faktoru sdíleným portální cirkulací a regulujícím reakci Kupfferových buněk.

Konfirmace regulačního vlivu sleziny na funkci různých typů makrofágů byla také prokázána v práci Shih-Ching et al, kde autoři zjistili zvýšenou produkci propyrogeních cytokinů TNF-alfa, Il-6 a IL-10 u Kupfferových buněk, peritoneálních a alveolárních makrofágů jako důsledek jejich stimulace LPS u myší po splenektomii. Na druhou stranu došlo k poklesu produkce těchto cytokinů u mononuklárů (Shih-Ching et al., 2004). Augmentace pyrogenní odpovědi prostřednictvím zvýšené produkce pyrogenních cytokinů po splenektomii prokázaná Shih-Ching je vzhledem k předcházejícím experimentům (Perlik et al., 2005; Li et al., 2006; Steiner et al., 2009; Nilsberth et al., 2009) spíše nepravděpodobná, vzhledem k jasně prokázané dominantní roli PGE_2 v periferní signalizaci LPS mediované horečky u laboratorních morčat.

V souladu s naším pozorováním o regulační funkci sleziny s augmentací febrilní odpovědi proti kontrolám je práce Greisman et al, který pozoroval vyšší febrilní odpověď u králíka a u člověka po splenektomii při opakovaném podání endotoxinu 5. den po zahájení experimentu. Na druhou stranu, iniciální dávka endotoxinu v den 0 neukázala signifikantní rozdíl ve febrilní odpovědi, kontrolní a splenektomované skupiny 3-4 týdny po vlastním zákroku, což může být do značné míry způsobeno metodologickými rozdíly, například volbou dávky, časem zvoleným pro sledování horečky, případně rozdíly v regulační roli sleziny mezi

živočišnými druhy (Romanovsky and Petersen, 2003). Tyto rozdíly jsou zřejmé i ze zmiňované práce Greisman et al., kde autoři pozorovali, že při následné imunizaci denně podávaným endotoxinem došlo k oslabenému vývoji tolerance vůči endotoxinu spojenému s vyšší febrilní odpovědí 3. - 4. den po splenektomii. Rozdíl ve febrilní odpovědi mezi kontrolní skupinou a skupinou po splenektomii však nebyl pozorovaný u imunizovaných zvířat 6. den po splenektomii (Greisman et al., 1975), což naznačuje vývoj kompenzačního mechanismu po imunizaci u skupiny králíků po splenektomii. Tento kompenzační mechanismus je však u člověka pomalejší a i po 10 dnech imunizace vykazuje vyšší febrilní odpověď skupina splenektomovaných pacientů proti příslušným kontrolám. Uvedená práce však jednoznačně podtrhuje regulační vliv sleziny na vývoj febrilní odpovědi po stimulaci endotoxinem.

Součástí zájmu našich prací zabývajících se regulační funkcí sleziny byla také bližší identifikace možného mediátoru. V důsledku přípravy splenického extraktu v naší práci a jeho filtraci se zdá jako pravděpodobné, že se jedná o malý peptid pod 10kD nebo lipid. Následným zpracováním extraktu s odstraněním bílkovinné části jejich vysrážením, jsme se snažili identifikovat, zdali termoregulaci ovlivňuje extrakt obsahující spíše látku bílkovinné či lipidické struktury. Naše výsledky naznačují, že k větší atenuaci febrilní odpovědi u morčat po podvazu splenické žíly dochází po zpětném podání splenického extraktu obsahujícím lipidickou složku.

Zdroj, stejně jako vlastní látka, a signalizace musí být však ještě detailněji ozřejmeny. Nabízí se dříve prokázaná produkce PGE_2 splenickými makrofágy po kontaktu s LPS (Ge et al., 1997; Marshall et al., 1993; Roland et al., 1999; Tanaka et al., 1996), který by teoreticky, jako zpětnovazebný mechanisms, mohl tlumit reakci Kupfferových buněk.

Alernativně Steiner et al., (2006) prokázali u laboratorních potkanů, že COX-1 je důležitý enzym pro regulaci hypotermie asociované s podáním vysoké dávky LPS, což prokázali selektivní inhibicí COX-1 a COX-2. V souladu s naším zjištěním, že slezina aktivně moduluje LPS navozenou horečku, postulovali, že tento vliv může být mediován regulační funkcí sleziny prostřednictvím COX-1 spojeného s prostaglandin D synthasou a takto generovaného PGD_2 (Steiner et al., 2009). Aplikace PGD_2 byla totiž v minulosti asociována s hypotermií (Ueno et al., 1982) a navíc výšená hladina této syntázy byla právě dříve pozorována ve slezině (Jowsey et al., 2001). Mezi další kandidáty fungující jako endogenní antipyretické látky můžeme zařadit natriuretický peptid, NO a další látky odvozené od arachidonové kyseliny (Spencer 2008). Detailní popis mechanismu inhibice LPS navozené horečky však doposud nebyl dostatečně prostudován a nabízí se zde proto prostor pro další výzkum.

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The spleen modulates the febrile response of guinea pigs to LPS

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Feleder, Carlos, Zhonghua Li, Vit Perlik, Allison Evans, and Clark M. Blatteis. The spleen modulates the febrile response of guinea pigs to LPS. *Am J Physiol Regul Integr Comp Physiol* 284: R1466–R1476, 2003. First published March 6, 2003; 10.1152/ajpregu.00378.2002.—The febrile responses of splenectomized (Splex) or sham-operated (Sham) guinea pigs challenged intravenously or intraperitoneally with lipopolysaccharide (LPS) 7 and 30 days after surgery were evaluated. FITC-LPS uptake by Kupffer cells (KC) was additionally assessed 15, 30, and 60 min after injection. LPS at 0.05 μg/kg iv did not evoke fever in Sham animals but caused a 1.2°C core temperature (T_c) rise in the Splex animals. LPS at 2 μg/kg iv induced a 1.8°C greater T_c rise of the Splex animals than of their controls. LPS at 2 and 8 μg/kg ip 7 days postsurgery induced 1.4 and 1.8°C higher fevers, respectively, in the Splex than Sham animals. LPS at 2 and 8 μg/kg ip 30 days postsurgery also increased the febrile responses of the asplenic animals by 1.6 and 1.8°C, respectively. FITC-LPS at 7 days was detected in the controls within KC 15 min after its administration; the label density was reduced at 30 min and almost 0 at 60 min. In the Splex group, in contrast, the labeling was significantly denser and remained unchanged through all three time points; this effect was still present 30 days after surgery. Similar results were obtained at 60 min after FITC-LPS intraperitoneal injection. Gadolinium chloride pretreatment (−3 days) of the Splex group significantly reduced both their febrile responses to LPS (8 μg/kg ip) and their KC uptake of FITC-LPS 7 days postsurgery. Thus splenectomy increases the magnitude of the febrile response of guinea pigs and the uptake of systemically administered LPS.

fever; splenectomy; Kupffer cells; fluorescein isothiocyanate-lipopolysaccharide

FEVER IS A COMMON SIGN of various diseases, and its proper management is still an important issue in modern medicine. Over the past few years, many clinical observations of overwhelming postsplenectomy bacterial infections have been reported (4). *Streptococcus pneumoniae* is the etiologic agent in ~80% of these cases (9, 10, 27), but gram-negative bacteremia is also implicated in many instances (27). Infected asplenic patients generally exhibit higher fevers than infected eusplenic patients (25). Elevations in postoperative body core temperature (T_c) have also been described after splenectomy (Splex) in patients (25, 41). The basis of these observations has not yet been studied systematically. Indeed, to our best knowledge, the role of the spleen in the febrile response has not yet been investigated, notwithstanding its own fundamental participation and that of fever in the host defenses against infections.

Although pulmonary intravascular macrophages constitute the first filter encountered by intravenously or intraperitoneally injected lipopolysaccharide (LPS), the rate of LPS clearance and detoxification by these cells is slow so that LPS spills over into the general circulation (37). Consequently, neutrophils, monocytes, and other macrophages within the vasculature, including hepatic [Kupffer cells (KC)] and splenic macrophages (SMO), also contribute to the intravascular clearance of LPS (2, 14, 24). Of these, the KC are quantitatively the most important. They constitute 80% of all resident mononuclear phagocytes in the body (24), and the liver is thus considered to be the principal organ responsible for clearing LPS from the blood (24, 27). Hence, the liver is also considered to be the primary source of production of LPS-induced pyrogenic cytokines, the endogenous mediators of fever (11). Conscious guinea pigs challenged with LPS, but pretreated with the KC inhibitor gadolinium chloride, exhibit falls rather than rises in T_c (33), thus supporting the critical intermediary role of KC for LPS fever induction. Hepatic branch vagotomy also inhibits the development of LPS fever (19, 35), further implicating the liver in this response.

The spleen also has a role in bacterial clearance. Thus intravenously injected immunoreactive LPS (2.5 μg/g body wt) was detected as intact or fragmented LPS in the SMO of rats; staining gradually decreased from 24 h to 1 wk (13). LPS was almost exclusively localized in the red pulp of the spleen, especially in the marginal zone, where this macrophage population is predominant (13, 14). Significant amounts of LPS were also found bound to splenic tissue (100 ng LPS/g tissue) after intravenous LPS (13 μg/kg) administration to rabbits (32, 40). It has been suggested that the spleen may thus also be a critical clearance organ of injected LPS (13, 40). Indeed, in Splex LPS-treated (1 μg/kg) dogs, plasma endotoxin concentration is higher than that in control dogs, further suggesting that the spleen

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may normally participate in its clearance (26). In support, in the presence of impaired liver function, endotoxin clearance by SMO is increased (16). There is also evidence of a specific 80-kDa LPS-binding protein on murine splenocytes (20).

SMO also affect the ability of macrophages elsewhere to handle pathogens. In particular, it has been shown that Splex alters KC function (1, 36), increasing both their number (7) and affinity (8) for LPS. Splex in mice impairs alveolar macrophage function (18), indicating that the spleen also modulates the activity of these macrophages (17). Moreover, the activities of interleukin (IL)-1β and granulocyte colony-stimulating factor are different depending on whether the mice are splenectomized or eusplenic (4, 15).

It is increasingly recognized that the spleen liberates factors into the portal circulation that modulate KC function (8, 23). For example, SMO produce abundant IL-1β (13). Considerable evidence indicates that the action of IL-1β, the production of which is induced by LPS, is a key event in the generation of fever (11). It has been speculated that it would be advantageous to the host to minimize the production of IL-1β by KC in response to LPS to moderate the host response to inflammation (13). Hence, it would appear that KC and SMO may interact to modulate LPS uptake, and this communication may be mediated by cytokines. Because both KC and SMO produce IL-1β and other cytokines involved in the febrile response, the present study was undertaken to evaluate the possible contribution of the spleen to the febrile response to LPS and its interaction with liver macrophages as regards, particularly, LPS uptake by these cells.

MATERIALS AND METHODS

Animals

Male Hartley guinea pigs (300–350 g; Charles River Laboratories, Wilmington, MA) were used in these experiments. The animals were quarantined for 1 wk, three to a cage, before any experimental use. Tap water and food (Agway Prolab guinea pig diet) were available ad libitum. The ambient temperature in the animal room was 23 ± 1°C; lightness and darkness were alternated, with light on from 0600 to 1800. After quarantine, to moderate the psychological stress associated with the experiments, the animals were trained to the experimental procedure for 1 wk (daily for 4 h) by handling and placement in individual, locally fabricated, wire mesh confiners designed to prevent their turning around and to minimize their forward and backward movements, but without causing restraint stress. All animal protocols were approved by the University of Tennessee Health Science Center Animal Care and Use Committee and fully conform with the standards established by the United States Animal Welfare Act and by the documents entitled "Guiding Principles for Research Involving Animals and Human Beings" (5).

Drugs

FITC-LPS was from *Escherichia coli*, serotype O111:B4 (lot no. 75H4036; 4.4 μg FITC/mg LPS; Sigma-Aldrich, St. Louis, MO); fluorescein sodium salt (lot no. 26H3407; Sigma-Aldrich) was the control label. LPS was *Salmonella enteriditis* (batch no. 651628; Difco Laboratories, Detroit, MI). $GdCl_3$ hexahydrate (lot no. 121K3655) was purchased from Sigma-Aldrich. The vehicle for all the solutions was pyrogen-free saline (PFS, 0.9% NaCl, USP; Abbott Laboratories, Chicago, IL). Heparin was purchased from Elkins-Sinn (Cherry Hill, NJ).

Surgical Procedures

All animals received the antibiotic gentamicin sulfate (6 mg/kg im) prophylactically before any surgical procedure. Sterile techniques were used. For intravenous injections, the animals were prepared by inserting under ketamine-xylazine (35–5 mg/kg im) anesthesia, a siliconized catheter (ID 0.020 in., OD 0.037 in.; Baxter Healthcare, McGraw Park, IL), prefilled with heparinized (10 IU/ml) PFS, through the left jugular vein into the superior vena cava of each guinea pig. The distal end of the catheter was passed subcutaneously and exteriorized on the top of the head, knotted, rolled into a coil, and placed inside a protective polypropylene shield that was fixed to the skull with dental acrylic cement and four self-tapping, miniature stainless steel screws. Gentamicin sulfate was administered during the following 2 days. The catheters were flushed with heparinized (3 IU/ml) PFS daily; 48 h before an experiment, heparinized PFS was replaced with PFS only (38).

To remove the spleen, a lateral, 3-cm subcostal incision was made on the left side after the jugular cannulation. This approach facilitated the removal of the greater omentum from the left upper quadrant and allowed displacing the stomach away from the spleen. The spleen was lifted gently by placing its body over a blunt grasper across its inferior pole. The gastrosplenic ligament was depressed, the splenocolic and phrenocolic ligaments were dissected, and all local vessels were ligated using 4–0 silk suture. As the inferior pole was freed, the propping grasper was moved gradually more anteriorly, thus allowing the spleen to be dissected in segments, and elevated, starting at the lower pole and working toward the hilar vessels. The splenic hilar vessels and short gastric vessels were isolated, divided, and ligated. Next, the proximal splenic artery was also ligated. The spleen was freed completely and removed from the abdominal cavity. The abdomen was then reexplored, with attention to the pedicle vessels, short gastrics, and diaphragmatic bed. The operative field was cleaned, and all port sites were closed.

The sham operation was performed similarly except that, after the displacement of the stomach and spleen, these organs were returned to the abdominal cavity, and the wound was closed. The animals were allowed to recover for 48 h. They were then trained to the experimental procedure described above until the experiments were performed.

Temperature Recording

Seven and 30 days after this surgery, the guinea pigs, fully conscious in the individual confiners to which they had been trained, were placed under a plastic hood (free air circulation through open ports) to prevent undue disturbances from noise and fluctuations in ambient temperature (23 ± 1°C). T_c of the guinea pigs were monitored constantly and recorded at 2-min intervals for the duration of the experiments (6 h) on a Macintosh Plus lMb microcomputer through an analog-to-digital converter, using precalibrated copper-constantan thermocouples inserted 5 cm in the colon. The data were displayed digitally on a monitor, printed on an ImageWriter printer, and stored on a diskette. A 90-min stabilization period, to achieve thermal equilibrium, preceded all the measurements. To obviate possible effects of circadian variations,

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all the experiments were begun at the same time of day (0830).

Fluorescent Microscopy

PFS (0.3 ml), fluorescein sodium salt (0.0007 μg/μl PFS; this dose is equivalent to the amount of fluorescein in the FITC-LPS conjugate), or 75 μg FITC-LPS/kg was injected intravenously or intraperitoneally. At 15, 30, and 60 min after administration, the animals, under ketamine-xylazine anesthesia, were attached to a perfusion tray, the jugular veins were exposed, and, using a 28-g ⅝ needle and a tuberculin syringe, 0.1 ml heparin (10 IU/ml) and 1 ml of 1% sodium nitrite were injected. Venous blood (5 ml) was collected; leukocytes-rich plasma was prepared by centrifuging (1,500 rpm, 15 min) the whole blood. The abdomen was incised (1 cm) on the midline, and 10 ml Hanks' balanced salt solution was pipetted into the abdominal cavity, swirled and mixed, and then collected. This peritoneal cavity lavage solution and the leukocyte-rich plasma were dropped to slides, incubated at 37°C for 40 min, and then fixed with 2% paraformaldehyde at −40°C for 15 min. The slides were mounted with anti-fade mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) and covered with coverslips for later analysis. For the collection of tissue samples, the animal's thorax was opened, and normal saline was perfused through the left ventricle until the fluid exiting the right atrium was clear of blood. Paraformaldehyde (250 ml of 4%) was then infused for 20 min, after which the guinea pigs were laparotomized and a 0.5 × 0.5 × 0.5 cm cube was excised from the liver (middle left lobe) of each animal. Tissue samples were similarly collected from the brain (hypothalamic region), lungs (middle left lobe), left kidney (upper one-third), and mesenteric lymph nodes. All the tissues were stored in 20% sucrose-4% paraformaldehyde solution for later cryostat sectioning. Slices (10 μm thick) were cut and mounted on glass slides, using Vectashield anti-fade mounting medium, and covered with coverslips. The slides were viewed, and images were collected using a fluorescent microscopy system consisting of a Nikon Diaphot microscope with a fluorescein filter (488 λ) coupled to a MacQuadra 950 computer system with a Power Mac processor 601, Vaytek software for deconvolution, and IPLab Spectrum software for image collection in conjunction with a cooled charge-coupled device camera (Photometric model 250 CH). Digital processing of the images was done using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA). For quantitative analysis, five areas of 25 × 25 mm^2 each were selected randomly, and the fluorescent patches in these areas were counted.

Erythrocyte Sedimentation Rate

Erythrocyte sedimentation rate (ESR) was measured in all the animals using the Westergren method, a rapid, accepted, and simple indicator of latent infection (6a). It was assessed 60 min before an experiment.

Experimental Design

Experiment 1: effects of intravenous LPS on T_c. Sham and Splex guinea pigs were challenged with a low (0.05 μg/kg) or a high (2 μg/kg) dose of LPS in 0.9 ml PFS or PFS (0.9 ml/kg) administered intravenously 7 days after the surgery. T_c was monitored continuously from 90 min before to 360 min after drug treatment.

Experiment 2: effects of intraperitoneal LPS on T_c. A low (2 μg/kg) or a high (8 μg/kg in 0.9 ml PFS) dose of LPS or PFS (0.9 ml/kg) was administered intraperitoneally 7 and 30 days after the surgery to other Sham and Splex guinea pigs. The latter treatment day was chosen postfacto to determine whether the responses observed at 7 days were transitory or permanent. T_c was measured continuously as in *experiment 1*.

Experiment 3: effects of Splex on FITC-LPS uptake. To visualize the presumptive differential distribution of LPS in Sham and Splex guinea pigs, the uptake of its fluorescent analog, FITC-LPS (75 μg/kg iv or ip), by the liver, lungs, brain, kidneys, peritoneal macrophages, lymph nodes, and leukocytes of Sham and Splex guinea pigs was evaluated 7 and 30 days after the surgery at 15, 30, and 60 min after its intravenous or intraperitoneal injection. This dose was selected on the basis of its intravenous and intraperitoneal pyrogenic potencies, determined in preliminary studies to suitably approximate those of 2 μg unlabeled LPS/kg delivered intravenously and intraperitoneally (our standard pyrogenic iv dose is 2 μg/kg; see, e.g., Ref. 21) in intact, conscious animals (Fig. 1, *A* and *B*). Although 37.5 μg FITC-LPS, in fact, approximated more closely the febrile course induced by 2 μg of unlabeled LPS when both were delivered intravenously (Fig. 1*A*), it was not pyrogenic, and its fluorescence was not detectable in tissues after its intraperitoneal injection (22); consequently, this dose was not used for this experiment.

Experiment 4: effects of $GdCl_3$ on FITC-LPS uptake and T_c. To verify a posteriori whether KC in particular were the cell types that bound FITC-LPS and accounted for the associated

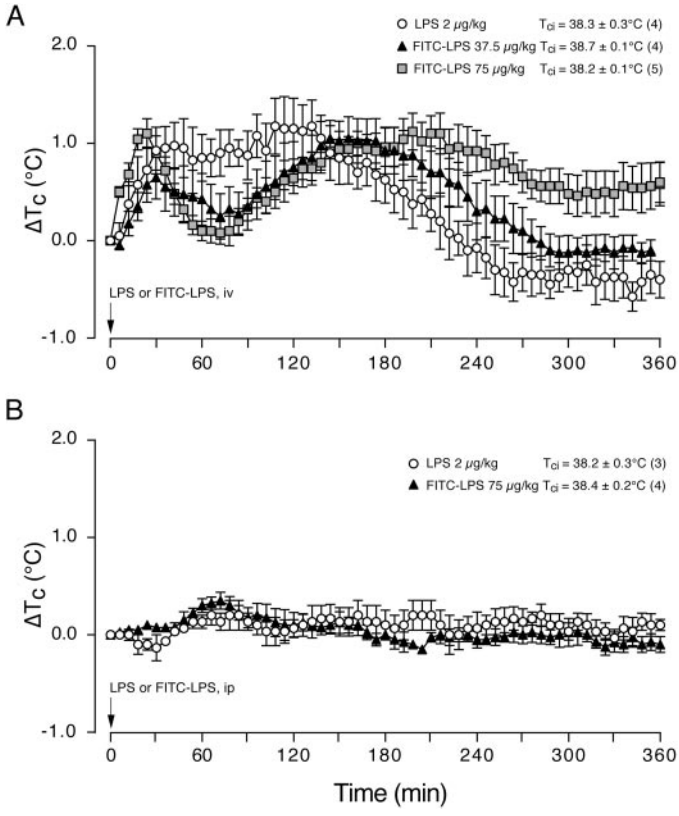

Fig. 1. Effects of unlabeled lipopolysaccharide (LPS; 2 μg/kg) and FITC-LPS (37.5 and 75 μg/kg) injected iv (*A*) or ip (*B*) on the core temperatures (T_c) of conscious, unoperated guinea pigs. T_c values are expressed as differences (ΔT_c) relative to their initial level [T_{ci}: average of the T_c over the last 10 min before the injection of pyrogen-free saline (PFS) or LPS]. Values are means ± SE; n = no. of animals.

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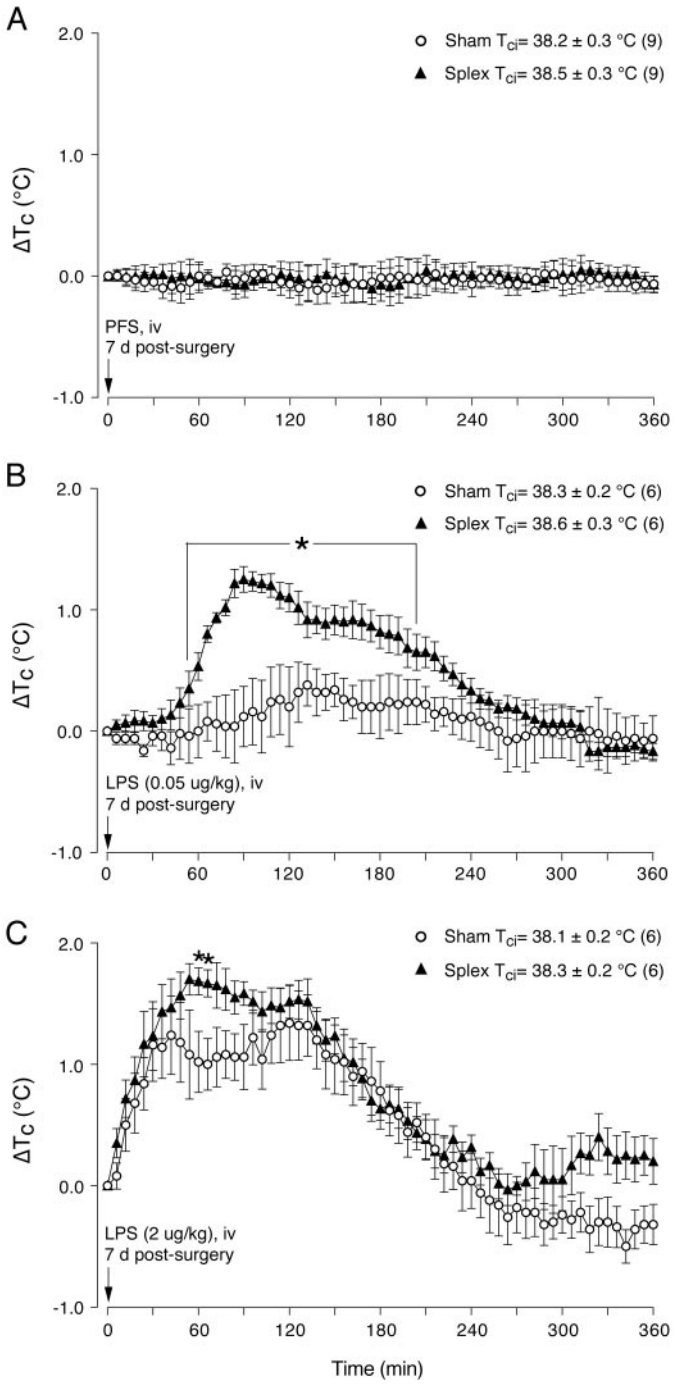

Fig. 2. Effects of PFS (*A*; 0.9 ml/kg) and LPS [0.05 (*B*) and 2 (*C*) μg/kg] injected iv on T_c of conscious Sham and splenectomized (Splex) guinea pigs 7 days after surgery. Conventions as in Fig. 1. *$P < 0.05$ relative to Sham.

T_c changes observed in this study, Sham and Splex guinea pigs were injected 7 days postoperatively with 75 μg FITC-LPS/kg ip, 3 days after the intravenous injection (in the penile vein, under light metofane anesthesia) of 7.5 mg $GdCl_3$/kg (21). The animals were killed 60 min after the FITC-LPS injection and perfused transcardially, their livers were excised and processed, and the fluorescent patches were counted as before. The intraperitoneal route and 60-min end point were chosen because the enhancement of the febrile response of the Splex animals to unlabeled LPS at 8 μg/kg ip was more manifest than that to LPS at 2 μg/kg iv (Figs. 2*C* and 3*B*) and because the density of FITC-LPS staining in these animals remained unchanged at 60 min after its intraperitoneal injection, whereas it was abating at this time after its intravenous injection (Fig. 4*A*). Other, similarly $GdCl_3$-pretreated Sham and Splex guinea pigs received 8 μg/kg unlabeled LPS intraperitoneally; their T_c were measured for 6 h, as before.

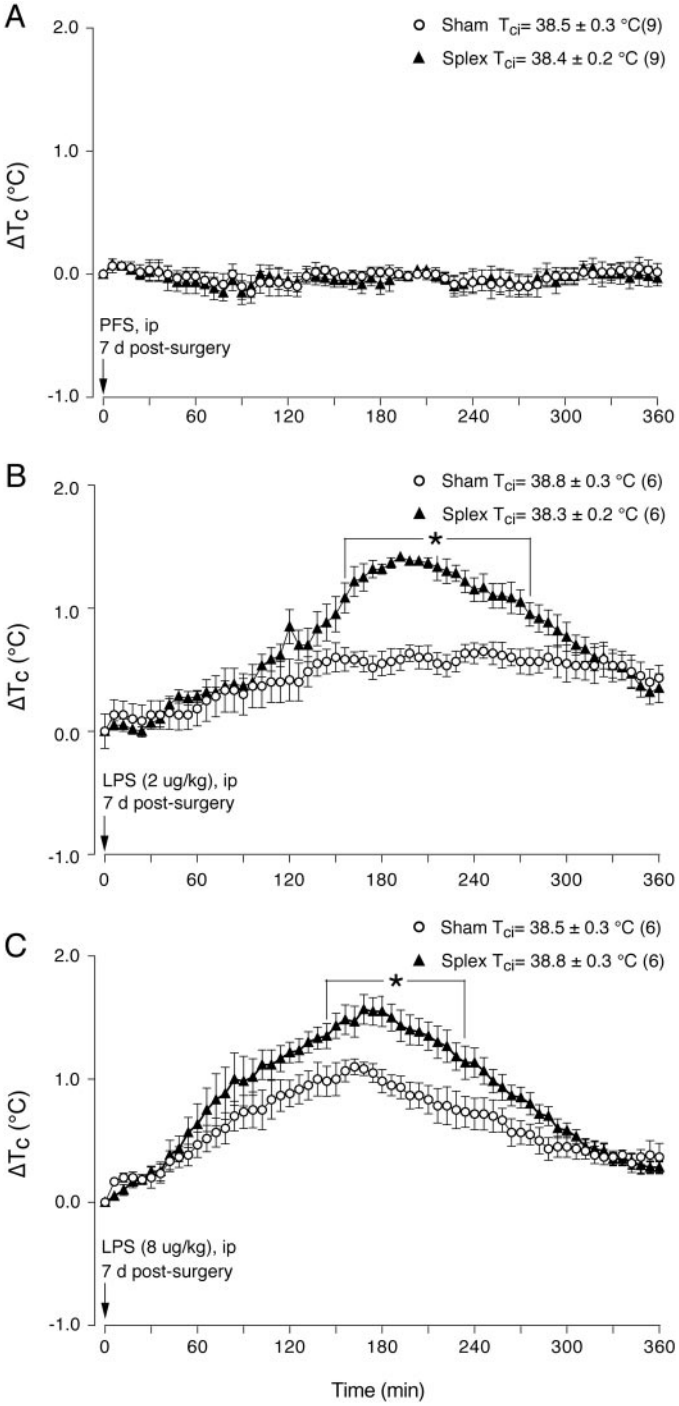

Fig. 3. Effects of PFS (0.9 ml/kg; *A*) and LPS [2 (*B*) and 8 (*C*) μg/kg] injected ip on the T_c of conscious Sham and Splex guinea pigs 7 days after the surgery. Conventions as in Fig. 1. *$P < 0.05$.

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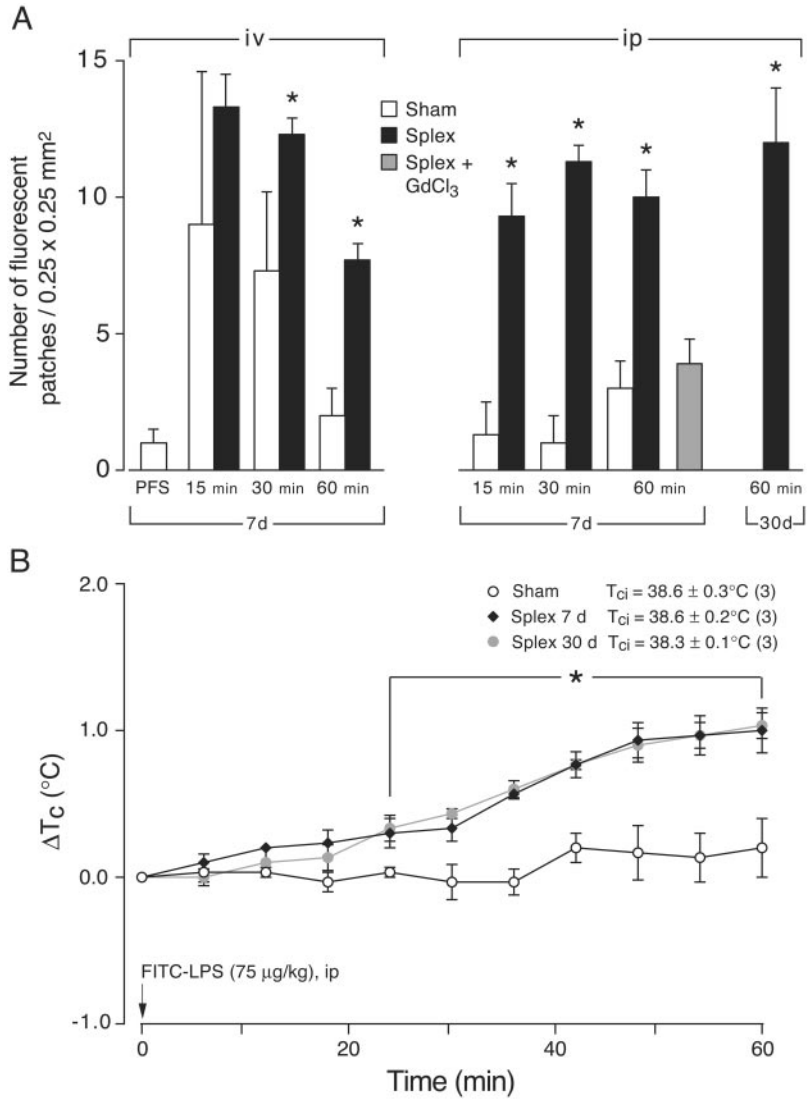

Fig. 4. *A*: densities of fluorescent labeling of Kupffer cells (KC) in livers of Sham, Splex-, and Splex + $GdCl_3$-pretreated guinea pigs 7 and 30 days postsurgery, measured 15, 30, and 60 min after FITC-LPS (75 μg/kg iv and ip). Bars are means ± SD; $N = 3$/group; *significantly different from corresponding Sham. *B*: effects of FITC-LPS (75 μg/kg ip) on the T_c of conscious Sham and Splex guinea pigs 7 and 30 days after surgery. Conventions as in Fig. 1. *$P < 0.05$.

Statistical Analyses

The results are reported as means ± SE. The values of T_c are changes from basal values [T_{ci} (initial), the T_c at 2-min intervals averaged over the last 10 min of the preceding 90-min stabilization period] plotted at 6-min intervals. Latencies of fever onset were defined as the intervals (in min) between the time of LPS injection (0 min) and that of the first T_c rise greater than 0.2°C (i.e., the SD of the T_c of PFS-treated guinea pigs) that continued uninterruptedly beyond 0.5°C. A two-way ANOVA followed by a Tukey-Kramer multiple comparisons test were used to compare the thermal courses between groups. The 5% level of probability was accepted as statistically significant.

RESULTS

ESR

No abnormality, i.e., no evidence of infection, was found in the Sham and the Splex groups (0.7 ± 0.3 and 0.8 ± 0.4 mm/h, respectively). No guinea pig died during the experiments.

Experiment 1: Effects of Intravenous LPS on T_c

The T_c values of both the Sham and Splex guinea pigs given PFS did not vary significantly over the 6-h duration of these experiments (Fig. 2*A*).

LPS at 0.05 μg/kg did not induce a statistically significant T_c rise in the Sham animals, but it did cause a significant biphasic T_c increase in the Splex guinea pigs (Fig. 2*B*). The first febrile peak was higher than the second, reaching ~1.2°C ~80 min after the LPS injection; the second peak occurred ~150 min after the injection. The return to basal T_c was gradual but essentially completed by ~270 min after LPS administration.

LPS at 2 μg/kg induced fever in the Sham guinea pigs with an onset latency of 10–12 min (Fig. 2*C*). The first febrile peak of ~1.3°C occurred at ~40 min after LPS administration and the second, also of ~1.3°C, at ~60 min after the first. The return to T_{ci} was gradual but essentially completed by ~250 min after LPS injection. The magnitude of the fever produced by this

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dose of LPS was significantly augmented in the Splex animals. Thus the first febrile rise was higher, to ~1.8°C, and the postfebrile recovery stabilized at a higher level than that of the Sham group (Fig. 2*C*). However, the onset latencies and the febrile courses were not significantly different in the Sham and Splex groups.

Experiment 2: Effects of Intraperitoneal LPS on T_c

PFS (7 and 30 days postsurgery) did not affect the T_c values of either the Sham or Splex animals (Figs. 3*A* and 5*A*).

As shown in Fig. 3*B*, LPS at 2 μg/kg 7 days postsurgery induced a significant T_c rise of ~1.4°C in the Splex animals. The rising phase began at ~30 min after LPS injection and culminated at 200 min. Thereafter, the return to T_{ci} was gradual and nearly complete by the end of the experimental period, 6 h later. In contrast, this dose of LPS caused only a small ~0.5°C increase in the T_c of the Sham animals; its onset latency was ~60 min. T_c reached its single maximum at ~150 min and gradually decreased throughout the remainder of the experimental period.

LPS at 8 μg/kg 7 days after the surgery induced a significant T_c rise of ~1.8°C in the Splex animals (Fig. 3*C*). The rise began quickly after the injection (45 min), and the single peak (1.5°C) was reached at ~170 min. T_c then decreased gradually and returned to control in ~300 min. In contrast, LPS produced a significantly smaller T_c increase, ~1.0°C, in the Sham guinea pigs; the onset latency was ~45 min, and the peak was reached at ~160 min. T_c then decreased gradually almost to its basal level 6 h after LPS injection.

LPS at 2 μg/kg 30 days postsurgery induced a significant T_c rise of ~1.6°C in the Splex guinea pigs (Fig. 5*B*); the latency of onset was ~45 min, and the T_c peak was reached at ~210 min. T_c decreased gradually toward its basal level over the next 150 min. By contrast, the Sham group exhibited a maximum T_c rise of only ~0.7°C. The onset latency was ~45 min, and the T_c peak was reached at ~210 min. T_c decreased thereafter slowly until the end of the experimental period.

LPS injected at 8 μg/kg 30 days postsurgery induced in the Splex animals a significant T_c rise of ~1.8°C, beginning promptly after the injection (Fig. 5*C*). The time to peak was ~170 min. The T_c then gradually fell until the end of the experiment. Again, the Sham group exhibited a lower T_c increase (~0.9°C); the onset latency was ~35 min, and the T_c peak was reached at ~170 min. T_c then declined gradually until the end of the experimental period.

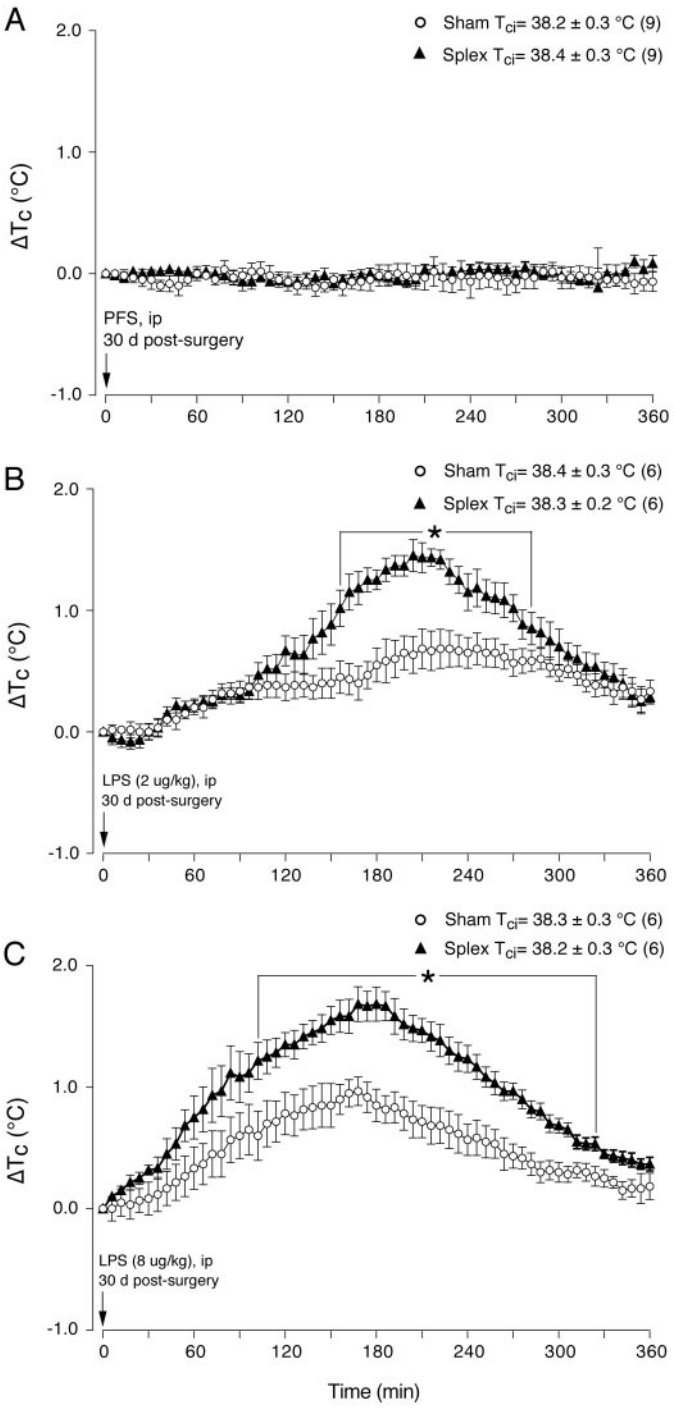

Fig. 5. Effects of PFS (0.9 ml/kg; *A*) and LPS [2 (*B*) and 8 (*C*) μg/kg] injected ip on the T_c of conscious Sham and Splex guinea pigs 30 days after surgery. Conventions as in Fig. 1. *$P < 0.05$.

Experiment 3: Effects of Splex on FITC-LPS Uptake

Only normal autofluorescence was observed after the intravenous and intraperitoneal injections of fluorescein sodium salt at a dose equivalent to its amount in the FITC-LPS conjugate (data not shown). The distribution and density of FITC-LPS fluorescence in the lungs, brain, kidneys, leukocytes (delivered intravenously or intraperitoneally), and peritoneal macrophages and mesenteric lymph nodes (intraperitoneally only) of Sham and Splex guinea pigs 7 and 30 days postsurgery were not different in the two groups (data not shown). On the other hand, differences were apparent between the livers of the two groups. Thus intravenous FITC-LPS appeared in the livers of Sham and Splex guinea pigs 7 days postoperatively at 15, 30, and 60 min as patches of granular fluorescence within,

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presumptively, KC in the liver sinusoids (Fig. 6). At 15 min, there was no demonstrable difference in the density of fluorescence between the two groups. In the Sham animals, the density of fluorescence decreased at 30 min and was virtually zero at 60 min, whereas in the Splex animals the labeling remained unchanged over the first 30 min and then decreased by ~40% at 60 min. These differences are expressed quantitatively in Fig. 4*A*.

FITC-LPS administered intraperitoneally 7 days postsurgery similarly appeared in the livers of the Splex guinea pigs 15, 30, and 60 min later as patches of granular fluorescence in the sinusoids but persisted at the same density throughout all three time points (Figs. 4*A* and 7). This enhanced uptake of FITC-LPS was associated with rising T_c values (Fig. 4*B*). In contrast, no such T_c elevations occurred in the Sham animals, although a little, but statistically insignificant, fluorescent labeling was evident at 60 min. The livers of the Splex guinea pigs still contained dense labeling 30 days postoperatively 60 min after intraperitoneal FITC-LPS; the density of these fluorescent patches was not significantly different from that 7 days postoperatively (Fig. 8). These results are illustrated quantitatively in Fig. 4*A*. The livers of Sham and Splex animals 7 days postoperatively, 15, 30, and 60 min after intravenous or intraperitoneal PFS showed only small amounts of diffuse autofluorescence; examples are shown in Figs. 8 and 9.

After intravenous (60 min) but not after intraperitoneal FITC-LPS, fluorescent patches also appeared in the hepatocytes of both the Sham and Splex animals (Figs. 6–8).

Experiment 4: Effects of $GdCl_3$ on FITC-LPS Uptake and T_c

$GdCl_3$ pretreatment 7 days postsurgery reduced the febrile responses of both the Sham and Splex guinea pigs to 8 μg LPS/kg, injected intraperitoneally 3 days after the $GdCl_3$, to the same levels (Fig. 10), but those of the Splex animals were depressed quantitatively more than their counterparts not pretreated with $GdCl_3$ (Fig. 3*C*). Thus LPS-induced rises of the T_c values of both groups of animals were delayed significantly in onset (~60 vs. ~45 min) and virtually abolished (~0.4 vs. ~1.5°C), although they peaked at the same time (~170 min). The reductions of these responses were associated with parallel, significant decreases in the densities of FITC-LPS labeling in the livers of these $GdCl_3$-pretreated guinea pigs (Figs. 4*A* and 9) compared with their untreated Splex counterparts (Fig. 7).

DISCUSSION

The present results show that significantly higher fevers occurred in Splex guinea pigs than in their Sham controls 7 and 30 days after their surgery in

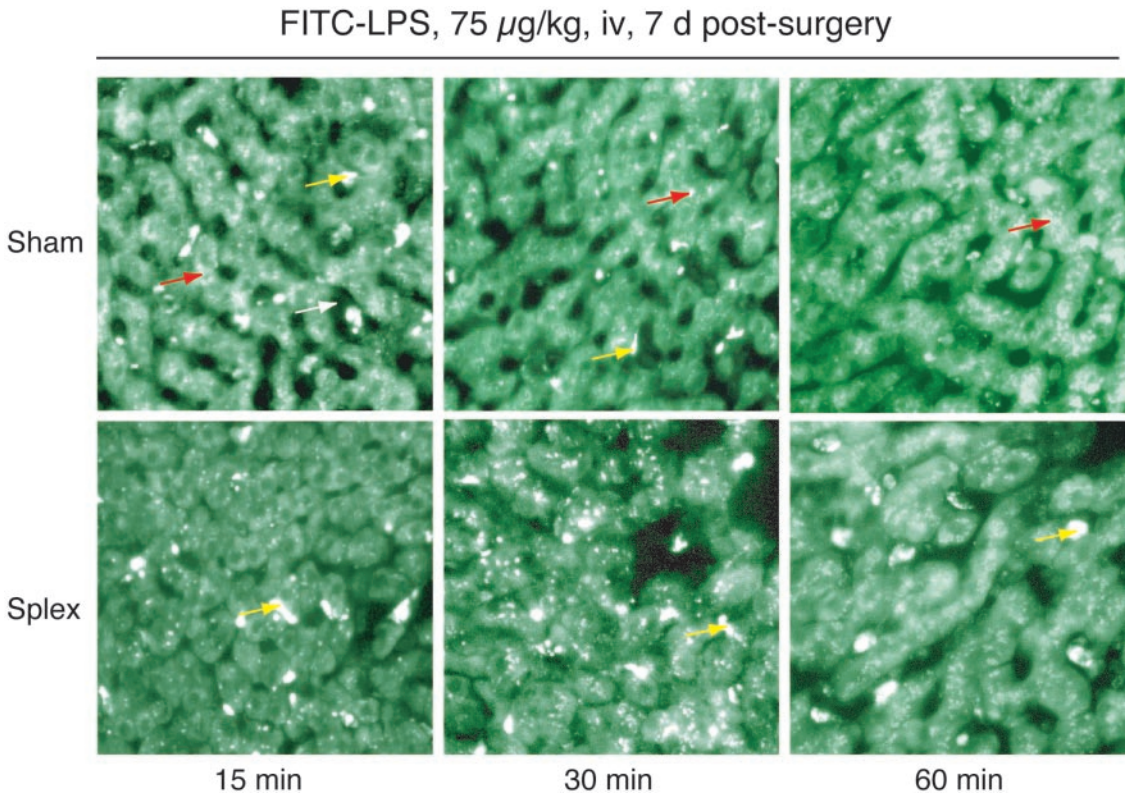

Fig. 6. Autofluorescence of hepatocytes (red arrows) and FITC-LPS labeling of KC (yellow arrows) in liver sinusoids (white arrows) of Sham and Splex guinea pigs 7 days postsurgery, 15, 30, and 60 min after FITC-LPS (75 μg/kg iv). Magnification: ×200.

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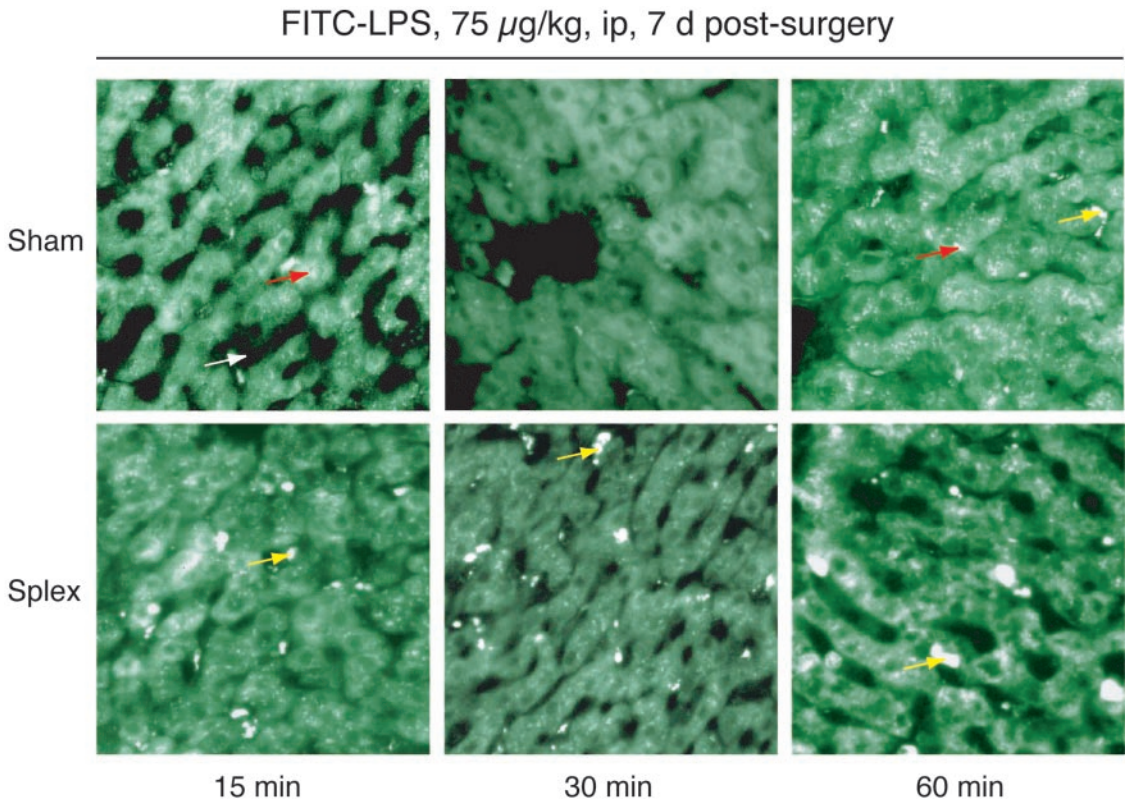

Fig. 7. Autofluorescence of hepatocytes and FITC-LPS labeling of KC in livers of Sham and Splex guinea pigs 7 days postsurgery, 15, 30, and 60 min after FITC-LPS (75 μg/kg ip). Magnification: ×200. Conventions as in Fig. 6.

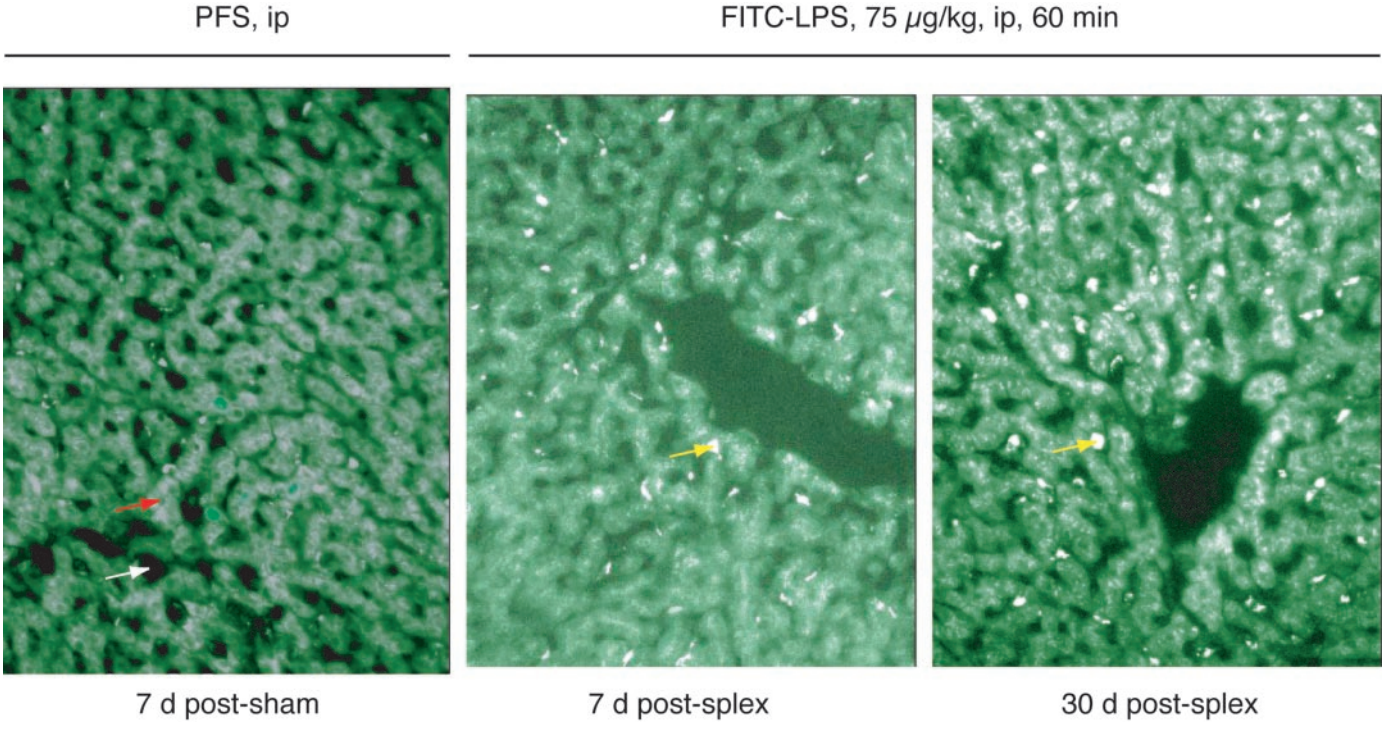

Fig. 8. Autofluorescence of hepatocytes and FITC-LPS labeling of KC in livers of Sham and Splex guinea pigs 7 and 30 days postsurgery, 60 min after FITC-LPS (75 μg/kg ip). Magnification: ×100. Conventions as in Fig. 6.

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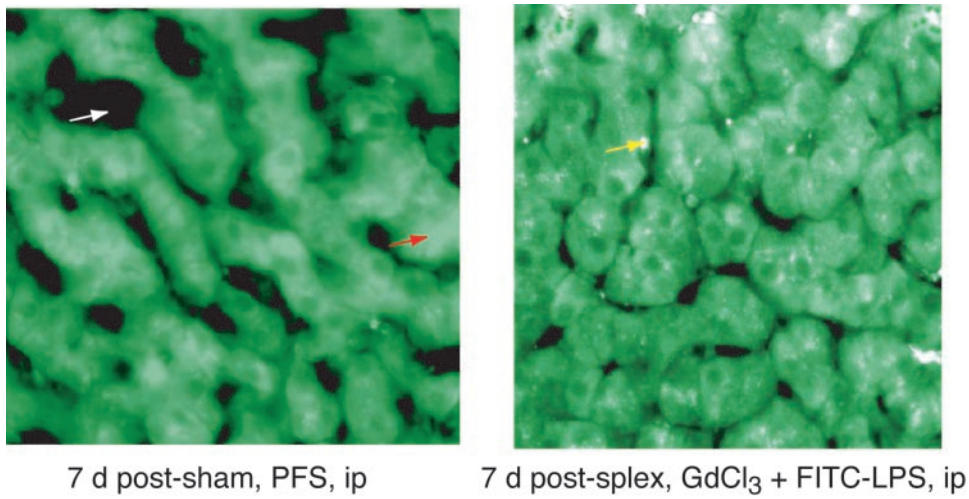

Fig. 9. FITC-LPS labeling of KC in livers of $GdCl_3$-pretreated Splex guinea pigs 7 days postsurgery, 60 min after FITC-LPS (75 μg/kg ip). The autofluorescence of the liver of PFS-treated Sham guinea pigs is shown for comparison. Magnification: ×100. Conventions as in Fig. 6.

response to both intravenous and intraperitoneal LPS. Indeed, doses minimally pyrogenic in Sham-operated animals caused greatly enhanced fevers in Splex guinea pigs. Furthermore, this effect of Splex was still robustly present 30 days postsurgery, indicating that it was not a transitory consequence of the procedure itself. These results further show that the observed, intensified fevers were associated with a significantly larger uptake of LPS by KC. Thus FITC-LPS injected intravenously labeled more KC and remained in these cells for a longer time in Splex than in Sham guinea pigs. Similarly, FITC-LPS injected intraperitoneally was taken up by these phagocytes more rapidly in Splex than in Sham guinea pigs. Again, this effect was still present 30 days post-Splex. In contrast, the uptake of LPS by other mononuclear phagocytes was not differentially affected by Splex. Elimination of KC by pretreatment with $GdCl_3$ attenuated both the febrile responses and the intensity of the labeling in the livers of both Sham and Splex animals, further implying a close link between LPS-induced fever and the liver.

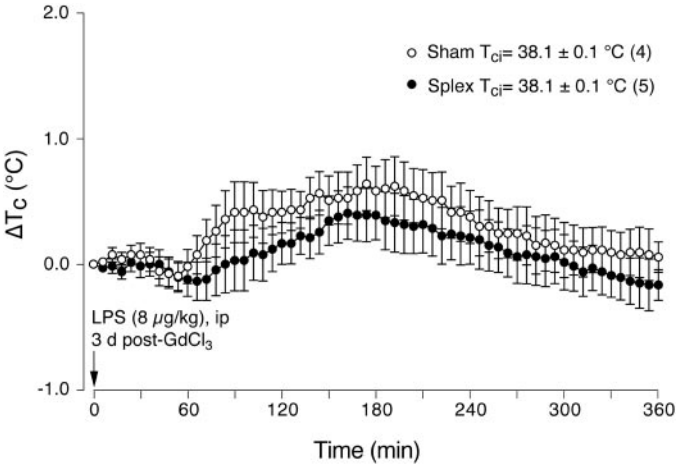

Fig. 10. Effects of LPS (8 μg/kg) injected ip on the T_c of conscious Sham and Splex guinea pigs 7 days after surgery, pretreated 3 days before the experiment with $GdCl_3$. Conventions as in Fig. 1.

To our best knowledge, this is the first experimental study demonstrating that Splex augments the febrile response to LPS. These results are consonant with clinical findings in which infected asplenic patients manifest higher fevers than infected eusplenic patients (25). Elevations in postsurgery T_c without any prior apparent infection have also been reported after Splex (25, 40), analogous to the data shown in Figs. 2, 3, and 5, *B* and *C*. A similar increase in the hepatic uptake of radiolabeled heat-killed *Escherichia coli* after their systemic administration was reported in Splex rats (6, 34). Also, in Splex mice with bone marrow irradiation-induced monocytopenia, both the number of KC and their autoradiographic uptake of tritiated thymidine were increased significantly (39). It has also been reported that the loss of splenic phagocytosis after removal of the spleen may be compensated by increased phagocytic activity of KC in rats (1, 9). However, Splex did not cause any change in the number of KC in rats (8), although, in other studies using mice, Splex did increase this number (7, 28). Hence, it is possible that the increased uptake of LPS by KC observed in the present study simply represents an effort by the KC to counterbalance the absence of the SMO. However, were this the case, one might expect that the magnitude of the febrile response would be unaffected or perhaps reduced a little but not enhanced as was observed. These data suggest, therefore, that the spleen may exert a modulating influence, presumably inhibitory, on the uptake of LPS by the liver. The present data alone do not allow determination of whether the increased density of fluorescence indicates an increase in the total number of activated KC, in the number of LPS receptors/KC, or in the functional activity of individual KC. Indeed, despite its high resolution, a difficulty inherent in the present imaging technique is that it causes blurs and halos from fluorescence emitted above or below the focal plane, thereby not allowing discrimination between fluorescence in or on the KC. This difficulty is compounded when the

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labeled cells are aggregated, as probably was the case here.

The mechanism by which the spleen may modulate, i.e., inhibit, the uptake of LPS by KC remains speculative. Several alternatives are possible. For example, SMO in the marginal zone of the spleen reportedly secrete quantitatively more IL-1α and IL-1β in response to LPS and to gram-negative bacteria (13). Because the induction of fever is thought to be mediated by the release of IL-1β in response to exogenous pyrogens (11) and if SMO, like KC, were another large source of this IL-1β, it might be advantageous to the host to normally minimize IL-1β production by the liver to limit fever height. Hence, the absence in Splex guinea pigs of an inhibitory influence on KC by IL-1β from the spleen could result in an increased production of hepatic IL-1β, thereby augmenting the febrile response. In this regard, KC are, in fact, directly exposed to splenic products via the portal vein (23). Among the factors that influence hepatic macrophagic activation state are PGE_2, transforming growth factor-β, IL-10, IL-6, tumor necrosis factor (TNF)-α, and interferon-γ. These could thus participate in the cross-talk between the spleen and the liver (2, 23). Indeed, it has been shown that in vitro pretreatment of KC with PGE_2 significantly decreases subsequent KC responses to LPS (2, 8). Hepatic TNF-α production is increased in Splex dogs compared with in control dogs, inferring an inhibitory influence by the spleen on hepatic TNF-α production (26). Splex reportedly also decreases the LPS-induced plasma IL-6 levels of rats more than can be attributed to the absence of splenic IL-6 production alone, hence suggesting a decrease in total, nonsplenic IL-6 levels (3). Other data have also implicated the spleen as an important mediator in the activation of nonsplenic IL-6 secretion by LPS (26). Because IL-6 is a feedback inhibitor of TNF secretion (11, 12), the lack of splenic IL-6 could lead to the increased KC production of TNF-α. If TNF-α levels were higher in the Splex guinea pigs, this could further account for the higher fevers of these animals; IL-1β, IL-6, and TNF-α are all involved in the production of fever by exogenous pyrogens (11). Hence, the present results suggest that hepatic macrophages, primed in our model by their lack of continuous exposure to presumptive splenic inhibitory products in the portal blood, may produce more febrigenic cytokines and/or factors, and/or possibly could express more cells, more LPS receptors/KC, or a greater functional activity than unprimed KC.

In conclusion, the present data indicate that LPS induces higher fevers in Splex than in Sham guinea pigs. This is associated with, and possibly causally related to, an increased LPS uptake by individual KC and/or an increased number of LPS-reactive KC. A factor released by the spleen could modulate KC LPS uptake and, hence, cytokine production. Whether such a factor is secreted by the spleen in the splenic vein and whether it carries signals to the KC remains to be clarified. However, regardless of the particular splenic factor responsible or of its mechanism of action, these data show that Splex is associated with higher fevers after LPS administration, indicating a participation of the spleen in the course of LPS-induced fever. Taken together, these data suggest that the apparently enhanced avidity of KC for bacterial endotoxin could account for the higher fevers of the Splex guinea pigs to a given dose of LPS, whether administered intravenously or intraperitoneally, compared with the febrile responses of Sham guinea pigs. Moreover, the absence of differences in the density of fluorescence in the brain, lungs, kidneys, lymph nodes, peritoneal macrophages, and monocytes between the Splex and Sham animals would further support the pivotal role of the liver in LPS-induced fever production, as suggested by previous observations (22, 31).

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Putative antihyperpyretic factor induced by LPS in spleen of guinea pigs

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Feleder, Carlos, Vit Perlik, Ying Tang, and Clark M. Blatteis. Putative antihyperpyretic factor induced by LPS in spleen of guinea pigs. *Am J Physiol Regul Integr Comp Physiol* 289: R680–R687, 2005. First published May 26, 2005; doi:10.1152/ajpregu.00022.2005.—We reported previously that the onset of LPS-induced fever, irrespective of its route of administration, is temporally correlated with the appearance of LPS in the liver and that splenectomy significantly increases both the febrile response to LPS and the uptake of LPS by Kupffer cells (KC). To further evaluate the role of the spleen in LPS fever production, we ligated the splenic vein and, 7 and 30 days later, monitored the core temperature changes over 6 h after intraperitoneal (ip) injection of LPS (2 μg/kg). Both the febrile response and the uptake of LPS by KC were significantly augmented. Like splenectomy, splenic vein ligation (SVL) increased the febrile response and LPS uptake by KC until the collateral circulation developed, suggesting that the spleen may normally contribute an inhibitory factor that limits KC uptake of LPS and thus affects the febrile response. Subsequently, to verify the presence of this factor, we prepared splenic extracts from guinea pigs pretreated with LPS (8 μg/kg ip) or pyrogen-free saline, homogenized and ultrafiltered them, and injected them intravenously into splenectomized (Splex) guinea pigs pretreated with LPS (8 μg/kg ip). The results confirmed our presumption that the splenic extract from LPS-treated guinea pigs inhibits the exaggerated febrile response and the LPS uptake by the liver of Splex guinea pigs, indicating the presence of a putative splenic inhibitory factor, confirming the participation of the spleen in LPS-induced fever, and suggesting the existence of a novel antihyperpyretic mechanism. Preliminary data indicate that this factor is a lipid.

Kupffer cells; fluorescein isothiocyanate-labeled lipopolysaccharide; antipyresis; splenic vein ligation

MANY STUDIES utilizing different approaches have indicated that the resident macrophages of the liver, the Kupffer cells (KC), are central to the clearance of bacterial endotoxic LPS from the body and for fever production (7, 19, 21). Because pyrogenic cytokines are produced largely by macrophages (6), KC constitute the largest pool of macrophages in the body (2, 4), and the liver is perfused by 25% of the cardiac output (17), the KC are considered to be the principal source of pyrogenic cytokines evoked by circulating LPS. In support, we (15) recently showed that the onset of the febrile response to LPS, irrespective of its intravenous (iv) or intraperitoneal (ip) route of administration, is temporally correlated with the appearance of LPS in the liver and its uptake by KC and that this mutual relationship is abolished by the prior administration of gadolinium chloride, a lanthanide that temporarily inactivates KC. However, other macrophages in intimate contact with the blood also clear circulating LPS and produce pyrogenic cytokines, e.g., splenic macrophages (SMO), but their relative contribution to fever production and, indeed, whether it is essential, is not entirely clear (11, 27). In a previous study, we (9) found that removal of the spleen of guinea pigs results in significantly enhanced febrile responses to both iv and ip LPS, and that these hyperpyrexias are associated with an increased uptake of LPS by KC. These effects, moreover, are persistent because their robustness is undiminished 30 days after splenectomy. These results agree with clinical experience showing that infected asplenic patients generally exhibit higher fevers than infected eusplenic patients (20). Elevations in postoperative body core temperature (T_c) have also been described in splenectomized patients immediately after surgery (20, 28).

It is possible that this effect simply reflects an increased avidity by the KC for LPS to counterbalance the absence of the SMO. However, were this such a compensatory event, the magnitude of the febrile response to LPS should be unaltered rather than augmented, as is actually observed. On the other hand, if the spleen normally exerted an inhibitory influence on the uptake of LPS by the liver, its removal could abrogate this possible action. Indeed, the spleen liberates factors, e.g., PGE_2 and transforming growth factor, into the portal circulation that downregulate KC function (4, 18). The removal of such KC-regulatory factor(s) could thus account for the increased febrile response and LPS uptake we observed in splenectomized (Splex) guinea pigs.

The results reported here are consonant with such a possibility. They indicate that the enhanced febrile response to LPS and its associated increased uptake of LPS by KC exhibited by Splex guinea pigs are reproduced by splenic vein ligation (SVL). However, these effects persist only until collateral vessels develop. An extract prepared from the spleens of LPS-treated guinea pigs attenuates both the exaggerated fever and the KC LPS uptake of Splex LPS-treated recipients. Preliminary, crude characterization of this putative splenic inhibitory factor suggests that it is a lipid.

MATERIALS AND METHODS

Animals

Male Hartley guinea pigs (300–350 g on arrival; Charles River Laboratories, Wilmington, MA) were used in these experiments. The animals were quarantined for 1 wk, three to a cage, before any experimental use. Tap water and food (Agway Prolab guinea pig diet) were available ad libitum. The ambient temperature (T_a) in the animal room was 23 ± 1°C, the housing T_a recommended by the Institute for

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Laboratory Animal Research, Commission on Life Sciences, National Research Council (12); light and darkness were alternated, with lights on from 0600 to 1800. After quarantine, to moderate the psychological stress associated with the experiments, the animals were trained to the experimental procedures for 1 wk (daily for 4 h) by handling and placement in individual, locally fabricated semicircular wire-mesh confiners designed to prevent their turning around and to minimize their forward and backward movement, without causing restraint stress. All the protocols were approved by the University of Tennessee Health Science Center Animal Care and Use Committee and fully conformed to the standards established by the U.S. Animal Welfare Act and by the documents entitled "Guiding Principles for Research Involving Animals and Human Beings" (1).

General

All glassware, plasticware, instruments, and cannulas used in these studies were sterilized by autoclaving. Electrochemical grade, high-purity water (Baxter Healthcare, Muskegon, MI) was used exclusively in the preparation of all solutions. Before use, the stock solutions were passed through a sterile 0.22-μm Miller-GS filter unit (Millipore, Bedford, MA), as an added precaution against bacterial contamination. The absence of endotoxic contamination in all fluids not containing LPS by design was verified by the *Limulus* amebocyte lysate test (Pyrochrome; Associates of Cape Cod, Falmouth, MA).

Drugs

FITC-labeled LPS was purchased from Sigma-Aldrich (cat. no. F-3665, lot no. 75H4036; St. Louis, MO). It was from *Escherichia coli* 0111:B4, the only kind currently available commercially and also the same as that used in our previous studies (9); fluorescein sodium salt (FSS; cat. no. F-6377, lot no. 26H3407; Sigma-Aldrich) was the control label. Unlabeled LPS was *Salmonella enteritidis* LPS B (batch no. 651628; Difco Laboratories, Detroit, MI), the same LPS batch we used in our previous studies (9, 15). The vehicle for all solutions was pyrogen-free saline (PFS, 0.9% NaCl, USP; Abbott Laboratories, Chicago, IL). Heparin was purchased from Elkins-Sinn (Cherry Hill, NJ).

Injection Procedures

For iv injections, the guinea pigs received a prophylactic subcutaneous (sc) injection of the antibiotic chloramphenicol (20 mg/kg) before the surgery and, 1 h later, were anesthetized with ketamine-xylazine (35 and 5 mg/kg, respectively) intramuscularly (im). Under aseptic conditions, usually the right jugular vein was exposed and freed from adhering tissue. A sterile siliconized catheter (inner diameter 0.020 in., outer diameter 0.037 in.; Baxter Healthcare, Deerfield, IL) containing heparinized (10 IU/ml) PFS was inserted and gently guided into the inferior vena cava. The free end of the catheter was pulled under the skin to the head, exteriorized onto the top of the head, knotted, and rolled into a coil. This coil was placed inside a polypropylene shield (a centrifuge microtube with a screw cap, with its cone cut off) that was fixed to the skull with dental acrylic cement and covered with its screwed-on cap. The neck wound was sutured and cleansed with 10% povidone-iodine solution and treated with nitrofurazone powder. After this surgery, the animals received a bolus 10-ml PFS sc injection and pain medication (butorphanol, 0.05 mg/kg sc), and, for two more days, sc chloramphenicol. The catheters were flushed with heparinized (3 IU/ml) PFS daily, except on the last day before an experiment, when PFS alone was used (26). During flushing, the performance of the catheters was verified by *1*) unimpeded withdrawal of blood into the catheter, *2*) low resistance to injection, and *3*) absence of locomotor and vocal responses from the animals to the injection. One week was allowed for recovery from this surgery before beginning an experiment.

For ip injections, the drugs were injected directly with a sterile 23-gauge 5/8 needle and tuberculin syringe into the peritoneum of the conscious animals.

Splenic Vein Ligation

All the animals received the antibiotic chloramphenicol (20 mg/kg sc) prophylactically before a surgical procedure. To ligate the splenic vein, a lateral 3-cm incision was made on the left subcostal side under ketamine-xylazine (35 and 5 mg/kg im, respectively) anesthesia and aseptic conditions. This approach facilitated the removal of the greater omentum from the left upper quadrant and allowed displacement of the stomach away from the spleen. The spleen was lifted gently by placing its body over a blunt grasper across its inferior pole. The splenic vein was ligated with 4-0 silk suture. After ligation, the stomach and spleen were returned to the abdominal cavity. The wound was closed in two layers, with a running 4-0 silk suture. Sham operations were performed in the same manner without ligating the blood vessel. The postoperative measures were the same as described for the iv injections above.

Splenic and Muscle Extract Preparation

To verify the presence of a splenic inhibitory factor, we prepared under aseptic conditions splenic extracts from guinea pigs. Using a method previously demonstrated to successfully yield a functional, hypotensive splenic extract (14), we removed the spleen and a psoas muscle (the control tissue) 5, 15, and 30 min after the administration of LPS or PFS from deeply anesthetized (ketamine-xylazine) guinea pigs, cleared the tissues of blood by perfusing with PFS, and rinsed them in ice-cold PBS; the same weight of tissue was used for each extraction. The tissues were then homogenized and centrifuged at 4,500 *g*, 4°C for 20 min. The supernatant was centrifuged at 14,000 *g*, 4°C for a further 10 min. Because our initial working hypothesis was that the splenic factor could be either a small peptide or a lipid, the supernatant was ultrafiltered with a Centripep-10 centrifugal concentrator (mol mass cutoff 10 kDa; Amicon, Beverly, MA), and the rest was discarded; 1.5 ml of spleen extract was normally obtained per donor animal.

To determine the chemical identity of the splenic factor, i.e., whether it is a peptide or a lipid, 15-min splenic extracts were prepared as above and incubated at 90°C for 80 min to denature peptides or passed through a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) to remove nonpolar compounds, mainly lipids. The collections were stored at −70°C.

Temperature Recording

A 90-min stabilization period to achieve thermal equilibrium preceded all the measurements. The T_c of the conscious guinea pigs, loosely restrained in the individual confiners to which they had been trained, were monitored continuously from 90 min before to 300–360 min after an injection. The data were recorded at 2-min intervals for the duration of the experiments on a Macintosh Plus 1 Mb microcomputer through an analog-to-digital converter, using precalibrated copper-constantan thermocouples inserted 5 cm into the colon. They were displayed digitally on a monitor, printed on an ImageWriter printer, and stored on a diskette. Fever indexes (FI) were derived from the area under the 360-min fever curve by imaging with a cooled charge-coupled device (CCD) camera (Photometric model 250 CH) and then scanning the images with NIH Image version 1.61. These values were converted to degrees Celsius by interpolating from a calibrated standard area that was 1°C high and 1-h long.

Fluorescent Microscopy

As described previously (15), an animal, under deep ketamine-xylazine anesthesia, was attached to a perfusion tray 60 min after FITC-LPS administration. The left jugular vein was exposed in the neck, and 0.1 ml of heparinized PFS and 1 ml of 1% sodium nitrite were injected with a 25-gauge 5/8 needle and a tuberculin syringe. Subsequently, for collection of liver samples, the animal's thorax was opened and normal saline was perfused through the left ventricle until the fluid exiting the right atrium was clear of blood; 250 ml of 4% paraformaldehyde was then infused for 20 min. Next, the guinea pig was

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laparotomized and a 0.5-cm × 0.5-cm × 0.5-cm cube was excised from the middle part of the left liver lobe of each animal. The tissue was immediately stored in 20% sucrose-4% paraformaldehyde solution, for later cryostat sectioning. Ten-micrometer-thick slices were cut, mounted on glass slides with antifade mounting medium (Vectashield; Vector Laboratories, Burlingame, CA), and covered with coverslips.

Slides were viewed and images were collected with a fluorescent microscopy system consisting of a Nikon Diaphot microscope with a fluorescein filter (488λ) coupled to a MacQuadra 950 computer system with a Power Mac processor 601, Vaytek software for deconvolution, and IPLab Spectrum software for image collection in conjunction with a cooled CCD camera. Digital processing of the images was done with Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA). The fluorescent patches were counted over five randomly selected 0.25 mm × 0.25 mm areas of tissue; no adjustment was made for the size or intensity of the individual fluorescent patches.

Experimental Protocols

Experiment 1: effect of SVL on T_c. Seven and thirty days after surgery, sham-operated and SVL guinea pigs were challenged ip with LPS at a dose of 2 μg/kg or with its vehicle (PFS, 0.5 ml/animal). This dose and route of LPS administration were used to replicate the conditions under which the augmenting febrigenic and KC LPS uptake effects of splenectomy were the most manifest in our previous study (9). T_c was monitored continuously for 360 min.

Experiment 2: effect of SVL on FITC-LPS uptake by KC. To visualize the expected, differential distribution of LPS in sham-operated and SVL guinea pigs, the uptake of its fluorescent analog, FITC-LPS (75 μg/kg ip), by the liver was evaluated in separate experiments 7 and 30 days after surgery, 60 min after its ip injection. This protocol was chosen because the density of fluorescent labeling normally present in KC 60 min after iv or ip FITC-LPS injection at this dose is minimal but is significantly increased after splenectomy (9, 15); 75 μg of FITC-LPS/kg ip is approximately pyrogenetically equivalent to 2 μg of native LPS/kg ip (15). FSS was the control label.

After each experiment, the guinea pigs' spleen and surrounding tissues were examined to verify that the splenic vein had successfully been completely ligated and to determine whether new collateral veins had meanwhile developed around the ligated splenic vein.

Experiment 3: effects of splenic and muscle extracts on T_c and FITC-LPS uptake by KC. To verify the presence of a splenic inhibitory factor, splenic and psoas muscle extracts prepared as described above were injected iv (one donor extract per recipient) into sham-operated and Splex guinea pigs pretreated ip with 8 μg of LPS/kg. This dose is our standard ip dose for intact guinea pigs; it induces a fever not different from that caused in Splex animals by 2 μg of LPS/kg ip (9). T_c was monitored continuously from 90 min before to 360 min after drug treatment. FITC-LPS (75 μg/kg ip) uptake by the liver was evaluated in separate experiments 60 min after its ip injection 7 days after surgery.

Experiment 4: effects of protein- and lipid-free extracts on T_c. The extracts obtained after the injection of LPS as described above were injected into recipient guinea pigs treated with PFS or LPS, as before. Again, T_c was monitored continuously from 90 min before to 300 min after LPS treatment.

Statistical Analysis

Results are reported as means ± SE. The values of T_c are changes from basal values [T_{ci} (initial T_c): the T_c at 2-min intervals averaged over the last 10 min of the preceding 90-min stabilization period] plotted at 6-min intervals. The data were evaluated by a repeated-measures analysis of variance model, where *factor 1* was the between-group factor (the experimental treatment) and *factor 2* the within-subject factor (the different sampling periods). The analyses were performed with Instat 3 (GraphPad software; Instant Biostatistics, San Diego, CA); each variable was considered to be independent. Latencies of fever onset were defined as the intervals (in min) between the time of LPS injection (0 min) and that of the first T_c rise >0.2°C (i.e., the SD of the T_c of PFS-treated sham-operated guinea pigs) that continued uninterruptedly beyond 0.5°C. The unpaired Student's *t*-test was used to evaluate the differences in fluorescent density of livers of the various groups. The 5% level of probability was accepted as statistically significant in all experiments.

RESULTS

Experiment 1: Effect of SVL on T_c

Seven days after surgery, LPS at 2 μg/kg ip induced low-magnitude T_c rises in the sham-operated guinea pigs, as pre-

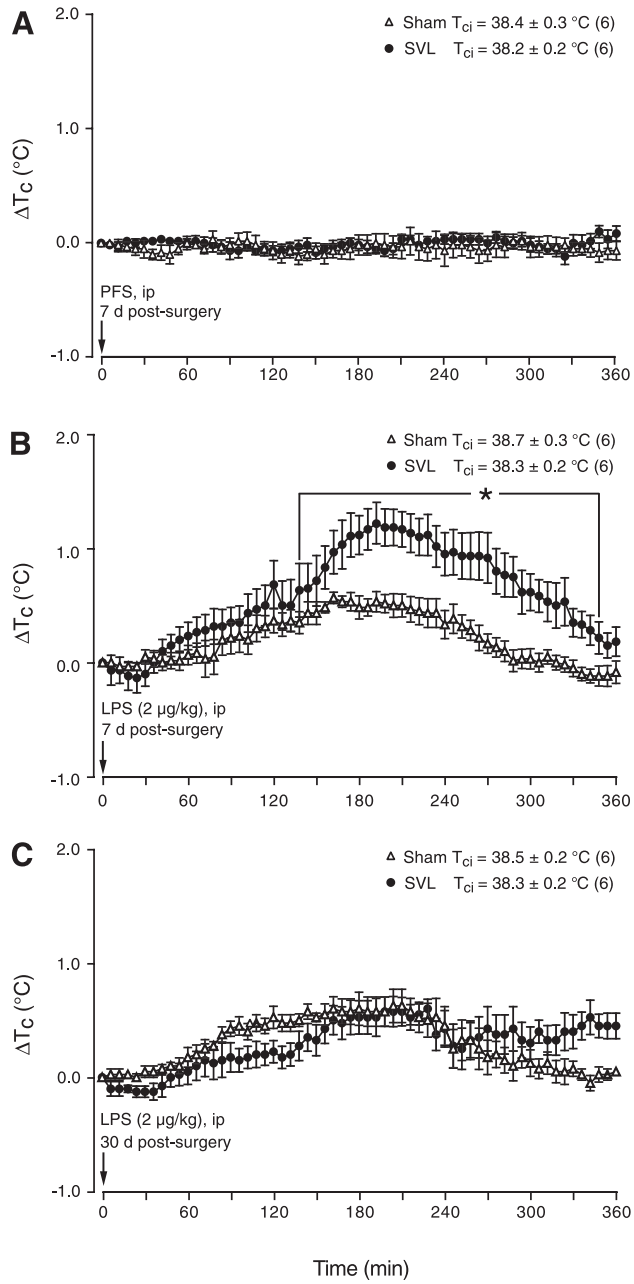

Fig. 1. Effects of pyrogen-free saline (PFS, 0.5 ml/animal ip; *A*) or LPS (2 μg/kg ip) on the core temperature (T_c) of conscious, sham-operated (Sham) and splenic vein-ligated (SVL) guinea pigs 7 (*B*) and 30 (*C*) days after the surgery. Values are means ± SE for no. of animals in parentheses. ΔT_c, change in T_c; T_{ci}, initial T_c. *$P < 0.05$, relative to Sham.

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viously described (9). These fevers had onset latencies of ~80 min and peak magnitudes of ~0.5°C occurring at ~160 min (Fig. 1*B*). In contrast, the same dose of LPS caused significant and long-lasting T_c increases in the SVL guinea pigs ($P < 0.05$). T_c began to rise at 30 min, reached a ~1.2°C peak at ~190 min, and then gradually returned to basal T_c; recovery was essentially complete by the end of the experiment. Thirty days after surgery, the fevers of the LPS-treated sham-operated and SVL guinea pigs were not significantly different from each other over the 6-h duration of these experiments and were similar to those of the sham-operated animals 7 days after surgery (Fig. 1*C*). PFS did not affect the T_c of either the sham-operated or SVL animals 7 days after surgery (Fig. 1*A*); this test was not repeated 30 days after surgery.

Experiment 2: Effect of SVL on FITC-LPS Uptake by KC

Only normal autofluorescence was observed 7 days after surgery in the livers of the sham-operated and SVL guinea pigs after ip injection of PFS (not illustrated; see Ref. 9) or FSS, the latter at a dose equivalent to its amount in the FITC-LPS conjugate (15). On the other hand, abundant labeling was observed as granular fluorescent patches within presumptive KC in the hepatic sinusoids and hepatocytes of SVL guinea pigs 60 min after FITC-LPS injection. However, in contrast, the fluorescent labeling was virtually absent 30 days after surgery (Fig. 2*A*). Figure 2*B* quantifies this difference ($P < 0.05$).

Complete ligation of the splenic vein was verified postmortem on *days 7* and *30* after surgery. No vessels were visible 7 days after surgery around the ligated portal-splenic vein, but new collateral veins were evident 30 days after surgery (not illustrated). The gross morphology of the SVL spleen appeared normal on both test days. There was also no evidence of splenomegaly or hematemesis.

Experiment 3: Effects of Splenic and Muscle Extracts on T_c and FITC-LPS Uptake by KC

As shown in Fig. 3A, LPS at 8 μg/kg ip 7 days after surgery induced T_c rises of ~0.9°C in the sham-operated guinea pigs, as previously described (9); their onset latencies were ~45 min. T_c reached its single maximum at ~170 min and, thereafter, gradually decreased throughout the remainder of the experimental period, returning to nearly its basal levels by ~5 h. In contrast, LPS at the same dose caused significantly greater T_c increases in the Splex guinea pigs (~1.6°C, $P < 0.05$; Fig. 3*B*), which, however, peaked at the same time as those in the sham-operated controls. Thereafter, T_c gradually returned toward basal levels, but recovery was not complete by the end of the experiment.

Psoas muscle extracts from intact guinea pigs treated with LPS injected iv into Splex guinea pigs similarly treated with LPS 7 days after their surgery did not modify the exaggerated febrile response of the recipient animals (Fig. 3*C*). The iv

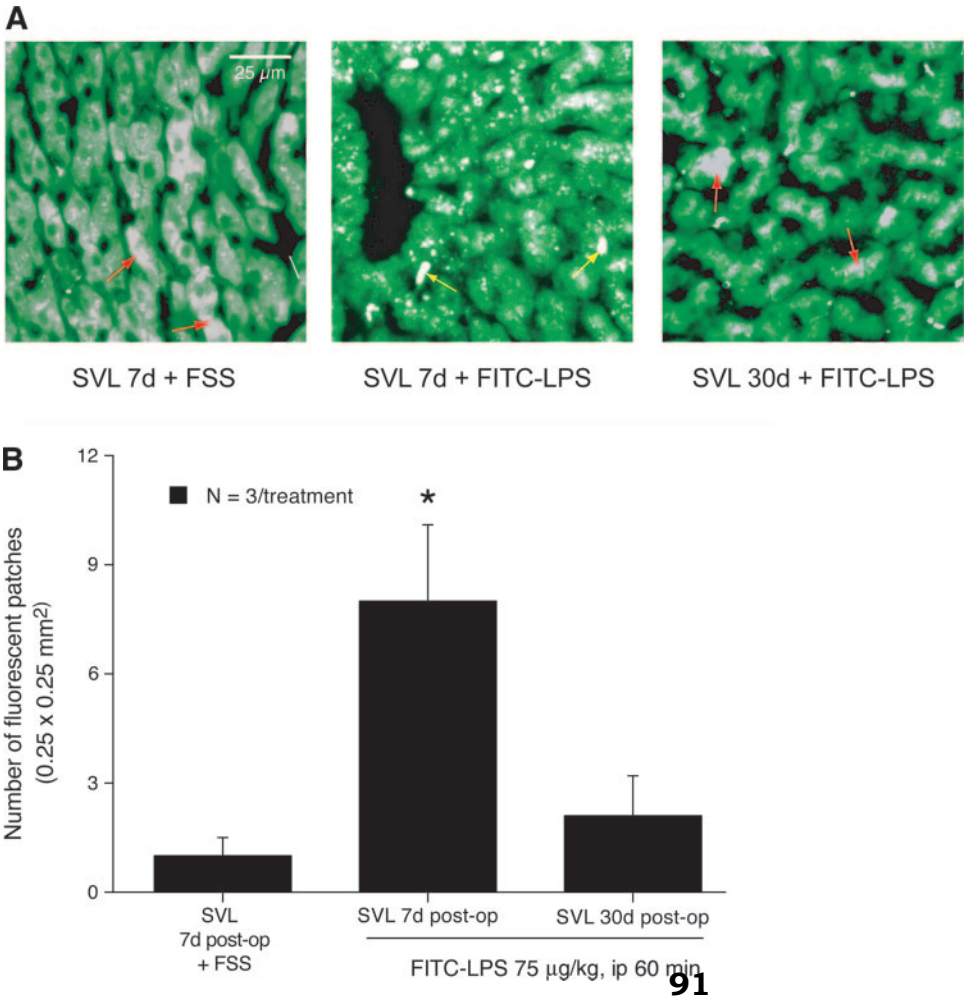

Fig. 2. *A*: fluorescent micrographs of 10-μm liver sections of SVL (7 and 30 days after surgery) guinea pigs excised 60 min after ip injection of fluorescein sodium salt (FSS) or FITC-labeled LPS (FITC-LPS) (75 μg/kg). White arrow, liver sinusoids, yellow arrow, FITC-LPS-labeled Kupffer cells (KC); red arrows, autofluorescent hepatocytes. Magnification ×200. *B*: densities of fluorescent labeling of KC in livers of SVL (7 and 30 days after surgery) guinea pigs, measured 60 min after ip injection of FSS or FITC-LPS (75 μg/kg). Bars are means ± SE; n = 3/treatment group. *$P < 0.05$, relative to Sham.

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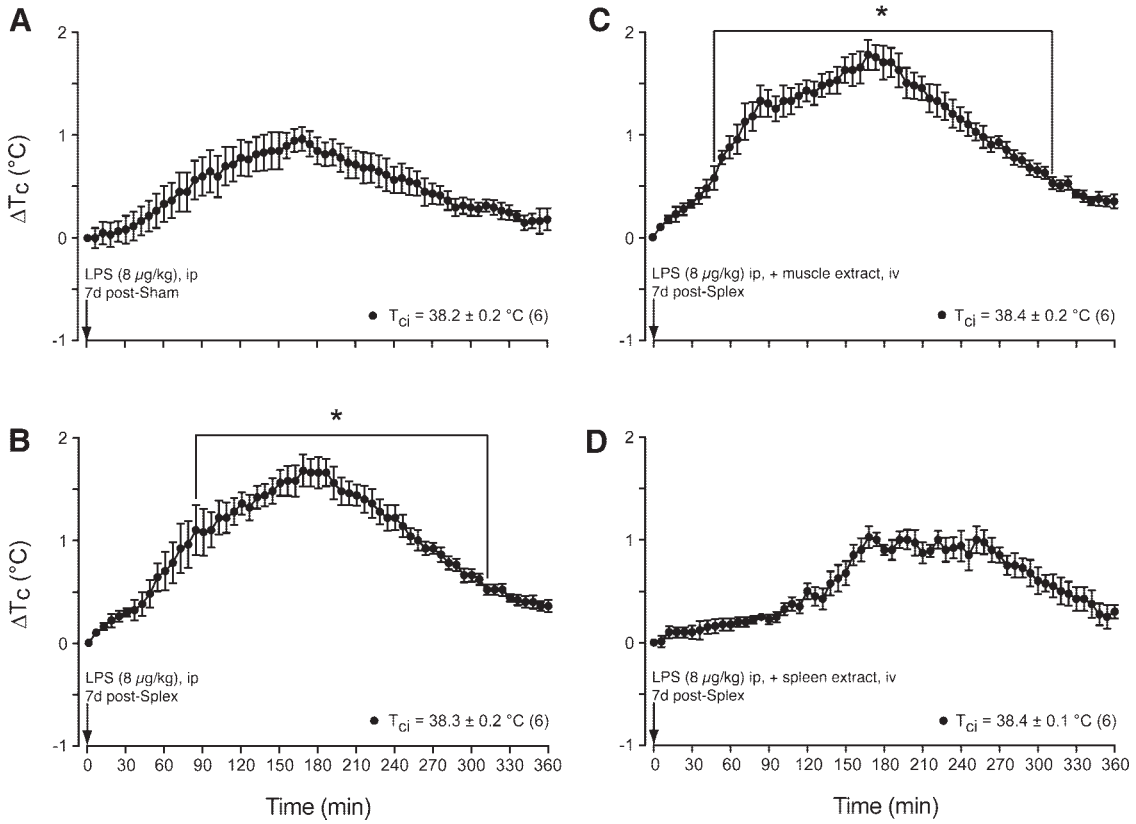

Fig. 3. Effects of LPS (8 μg/kg ip) on the T_c of conscious Sham (*A*) and Splex (*B*) guinea pigs and Splex guinea pigs that additionally received 1.5 ml iv of the 15-min psoas muscle (*C*) and splenic (*D*) extracts 7 days after surgery. Abbreviations and conventions as in Fig. 1. *$P < 0.05$, relative to Sham.

injection of 5-min splenic extracts from the donor guinea pigs also did not affect the febrile response to LPS of the recipient Splex animals, but the 15- and 30-min splenic extracts significantly reduced the magnitudes of the exaggerated fevers of these Splex guinea pigs (Fig. 3*D*), returning them to the levels of the sham-operated animals. T_c in the latter did not begin to rise until ~60 min after the injection, reached a ~1.0°C peak at ~160 min, stabilized for ~1.5 h, and then gradually returned to basal T_c; recovery was not fully complete by the end of the experiment. These febrile curves, however, were not significantly different from those observed in the sham-operated guinea pigs treated with ip LPS (Fig. 3, *A* and *D*). These results are summarized as FI in Fig. 4.

Seven days after surgery, FITC-LPS fluorescent patches were abundant within the sinusoids and hepatocytes of the recipient Splex guinea pigs 60 min after iv injection of the 15-min muscle extracts [Fig. 5*A*, *left*; the same results were obtained 7 days after splenectomy + FITC-LPS alone (9)], but this fluorescent labeling was significantly reduced in the livers of the Splex guinea pigs treated with the 15-min splenic extracts (Fig. 5*A*, *right*).

Experiment 4: Effects of Protein- and Lipid-Free Extracts on T_c

The iv coadministration of PFS and both the lipid- and protein-free splenic extracts had no effect on the animals' T_c (Fig. 6, *A* and *B*). In contrast, the coadministration of LPS and both extracts caused an initial, transient ~0.5°C fall in the T_c of the Splex recipients (Fig. 6, *C* and *D*); its nadir was

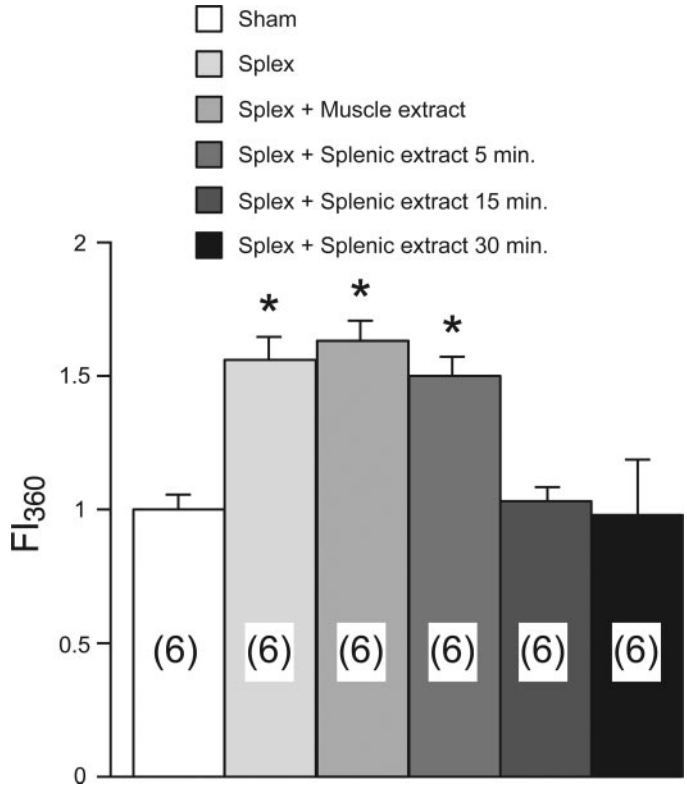

Fig. 4. Three hundred sixty-minute fever index (FI_{360}) of conscious Sham and Splex guinea pigs that received 1.5 ml iv of splenic extract obtained from donor guinea pigs killed 5, 15, and 30 min after LPS challenge (8 μg/kg ip). Abbreviations and conventions as in Fig. 1. *$P < 0.05$, relative to Sham.

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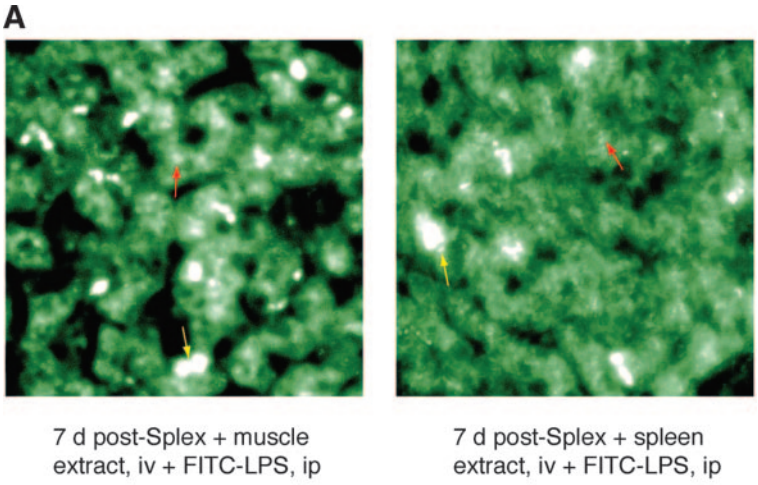

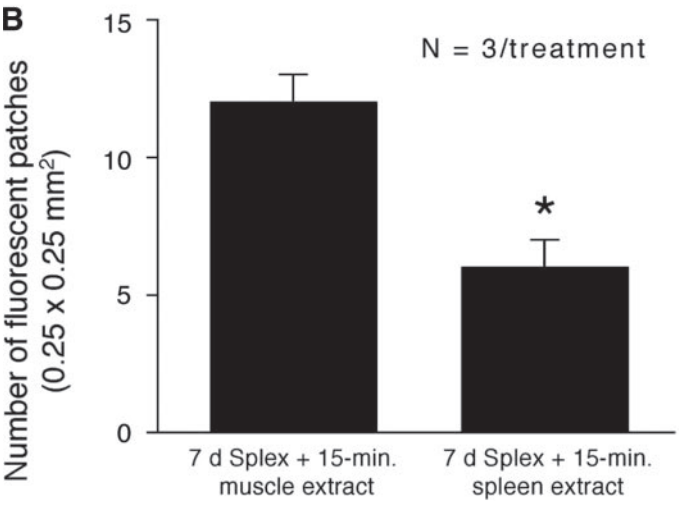

Fig. 5. *A*: fluorescent micrographs of 10-μm liver sections of Splex (7 days after surgery) guinea pigs excised 60 min after the injection of FITC-LPS (75 μg/kg ip) and of muscle (1.5 ml iv) or splenic (1.5 ml iv) extract. Yellow arrows, FITC-LPS-labeled KC; red arrows, autofluorescent hepatocytes. Magnification ×200. *B*: quantified representation of the same data. Bars are means ± SE; $n = 3$/treatment group. *$P < 0.05$, relative to muscle extract.

reached 15 min after injection. This hypothermic effect was presumably due to an interaction of LPS (but not PFS) and these preparations, the nature of which is undetermined at this time. The T_c of both the lipid- and protein-free-treated guinea pigs increased immediately thereafter. The T_c of the lipid-free extract-treated animals reached a peak of ~1.0°C at ~210 min. It then gradually decreased throughout the remainder of the experimental period; recovery, however, was not complete by the end of the experiment (Fig. 6*C*). In contrast, the T_c rise exhibited by the Splex guinea pigs treated with the protein-free extract reached only 0.5°C. This rise was significantly smaller than that caused by the lipid-free extract ($P < 0.05$; Fig. 6*D*). Both rises, however, peaked at the same time and subsequently also decreased at the same rate.

DISCUSSION

The present results show that significantly higher fevers occurred in response to ip LPS in SVL guinea pigs than in their sham-operated controls 7 days after surgery. However, 30 days after surgery, when new collateral vessels apparently draining from the spleen into the portal vein became evident around the ligated splenic vein, these effects were considerably moderated. These results also show that the observed intensified febrile response 7 days after SVL was associated with a significantly increased uptake of LPS by KC, but that this uptake was again normal 30 days later. These findings suggest, therefore, that a factor released by the spleen and flowing to the liver via the splenic-hepatic portal vein modulates KC uptake of LPS and hence the febrile response. Because the splenic extracts from ip LPS-treated donor guinea pigs injected iv into Splex guinea pigs also challenged with ip LPS significantly attenuated their exaggerated febrile response and their elevated uptake of LPS, both to levels essentially identical to those of sham-operated controls similarly treated, the production of a putative splenic counterregulatory factor may be inferred. Preliminary, crude characterization of the factor indicates that it is a lipid. Thus these results add further support to our earlier suggestion (9) that the spleen may normally exert a limiting effect on the uptake of LPS by KC, so that, in its absence, the magnitude of LPS-induced fever is greatly augmented. The present results also reinforce the notion that KC are central to the febrile response to LPS (15).

In validation of the hypothesis that the spleen may release a factor that limits LPS uptake by KC and the febrile response, extracts prepared from the spleens of intact donor guinea pigs

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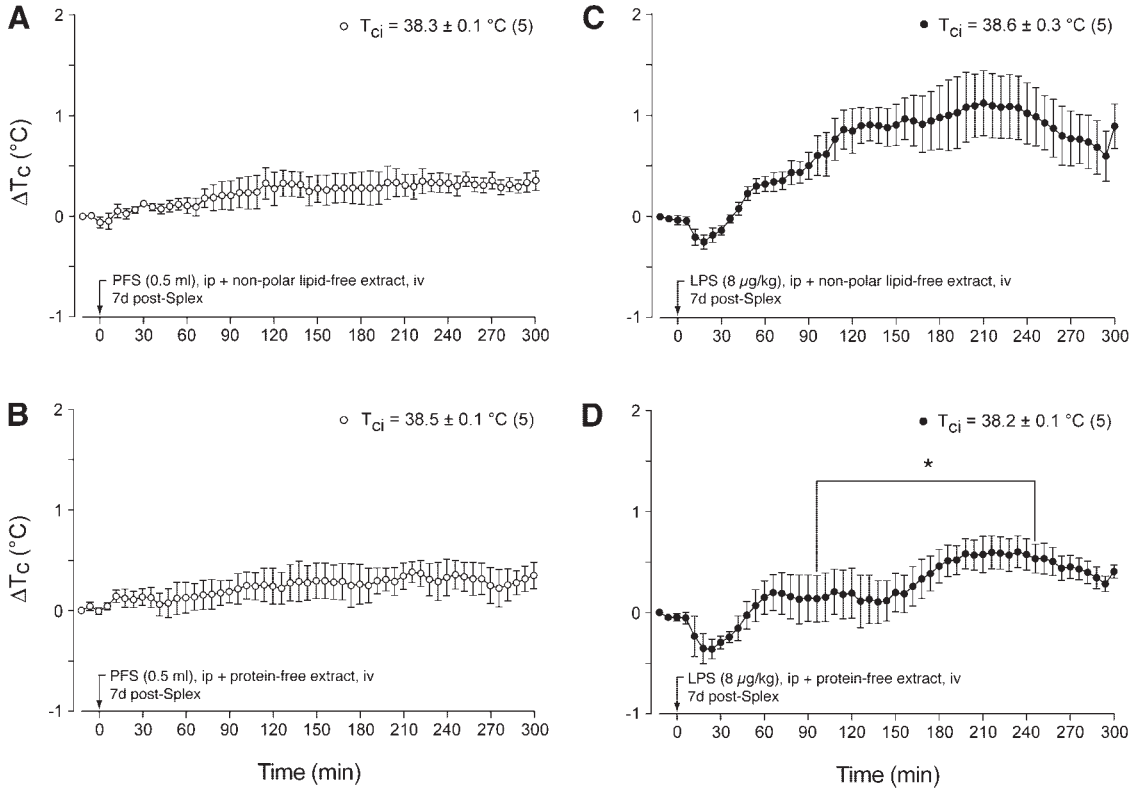

Fig. 6. Effects of iv injection of 1.5 ml of the splenic lipid-free (*A* and *C*) and protein-free (*B* and *D*) extracts on the T_c of Splex guinea pigs coinjected with PFS (0.5 ml, *A* and *B*) or LPS (8 μg/kg ip, *C* and *D*) 7 days after surgery. Abbreviations and conventions as in Fig. 1. *$P < 0.05$, relative to *A*.

15 and 30 min after ip injection of LPS, injected iv into recipient Splex guinea pigs also treated with ip LPS, attenuated the exaggerated febrile response of the latter to LPS, as well as the augmented LPS uptake by KC. On the other hand, the extracts collected from donors 5 min after the injection of LPS did not affect these enhanced responses of the recipient Splex guinea pigs. Because the inhibitory activity of the extracts was not detectable in samples collected earlier than 15 min, it may be conjectured that the effective splenic factor probably does not exist in a preformed state but rather is produced and released after a relatively short delay in response to the presence of LPS in the blood. The shortness of this interval would suggest that it could be a lipid mediator rather than a protein mediator, because the transcription and translation of the latter would be expected to require more time. Indeed, our preliminary and very crude analysis of the chemical nature of this compound suggests that it is a lipid. It is unlikely that this factor is an artifact of the extraction procedure because the 5-min extract was not effective but the subsequent extracts, obtained by the same procedure, were effective.

Although the specific identity of this factor is still uncertain, the fact that the active principle passed through a microporous filter having a nominal molecular mass cutoff of 10 kDa suggests that it is small, perhaps a prostanoid. SMO in the marginal zone of the organ secrete PGE_2 in response to LPS and gram-negative bacteria (11), consequently directly exposing KC to splenic PGE_2 via the portal vein (18). PGE_2 is also released by the spleen into the splenic vein during hemorrhagic and septic shock (22, 24). It has been shown in vitro that PGE_2 inhibits LPS-induced TNF-α and nitric oxide formation via prostaglandin receptors EP_2 and EP_4 on KC in time- and concentration-dependent manners (3, 10). Because fever is thought to involve the release of cytokines by KC in response to exogenous pyrogens (7), the delivery of splenic PGE_2 to the liver via the splenic-portal vein could be a mechanism, whereby cytokine production by the liver could be modulated; i.e., it could be a counterregulatory mechanism that limits fever height. The specific effect of PGE_2 on KC LPS uptake, however, is unknown.

The modulatory effect of the factor putatively released by the spleen reappeared after collateral vessels connecting the spleen and the liver had developed between 7 and 30 days after SVL. Hence, a direct vascular communication between the spleen and the liver would appear to be necessary for this factor to exert its effect on KC, inferring that the amount released by the spleen under normal conditions could be very small. It has been reported in dogs that, although the volume of the spleen and its venous pressure rise immediately after SVL, the splenic parenchymal pressure gradually returns to control levels over the following hours because of the dilation of short, collateral vessels that drain the venous blood into the left gastric and gastroepiploic veins, preventing hematemesis and splenic enlargement (13); the spleens of the present SVL guinea pigs also appeared normal. Because these vessels, in turn, empty into the splanchnic venous circulation, which then drains into the systemic circulation, its dilution in the general circulation could reduce its efficacy, thus rendering it ineffective under the present experimental conditions. The fact that the bolus iv injection of the splenic extract (presumably representing a large dose of the splenic factor) was effective in inhibiting the LPS-induced hyper-

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pyrexia of the present Splex guinea pigs would support this interpretation. However, the restoration of the full blood supply from the spleen to the liver 30 days after SVL still needs to be demonstrated specifically. Indeed, the possibility that no factor was released at 7 days after SVL because of functional impairment of the spleen consequent to the original, acute insult provoked by the SVL cannot yet be ruled out.

It is noteworthy that this putative inhibitory factor did not abrogate the entire fever response to LPS and restore the basal T_c, but rather returned the exaggerated fever to its usual febrile level. This antihyperpyretic action is therefore different from the antipyretic actions of, e.g., arginine vasopressin or α-melanocyte-stimulating hormone, which attenuate fever by acting centrally rather than in the periphery (16). On the other hand, this factor is similar to, e.g., glucocorticoids, which act in the periphery by inhibiting the production of pyrogenic cytokines, but different from them by limiting LPS uptake by KC. It is interesting to speculate that this mechanism could thus be another among those that prevent T_c from rising to extremely high levels during gram-negative bacterial infections, i.e., that set fevers' upper limits (16).

It remains to be determined which cells release this factor and what activates them. One possibility is that circulating LPS itself could be the trigger that directly stimulates the release of the splenic factor, presumably from SMO. Although LPS at 8 μg/kg is cleared rather slowly from the peritoneum, it nevertheless appears in the blood, its level gradually increasing during the first 60–90 min after ip injection (5, 21). Another possibility is that this factor could be induced by the autonomic nervous system reflexively activated by LPS (25). It is now generally recognized that, in the spleen, this system promotes the release of anti-inflammatory mediators (8, 23). Norepinephrine or ACh released by autonomic efferents to the spleen could therefore potentially stimulate the rapid release of this factor.

In conclusion, these data confirm our previous findings in Splex guinea pigs that the spleen may modulate the febrile response to LPS. This effect appears to be mediated by a humoral factor flowing from the spleen to the liver that limits the uptake of LPS by KC and, hence, the febrile response. It is prevented by SVL and is restored concomitantly with vascular collateralization, supporting its splenic source. The attenuation of the exaggerated febrile response of Splex guinea pigs to LPS by the administration of the extracts prepared from the spleens of LPS-treated donors further indicates the existence of this putative inhibitory factor. A novel, previously unrecognized, endogenous antihyperpyretic mechanism is therefore inferred.

ACKNOWLEDGMENTS

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GRANTS

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4.3. Centrální mediace signálu v preoptické oblasti hypothalamu

Centrální mediací febrilních reakcí v preoptické oblasti hypothalamu se zabývá práce autorského kolektivu: Feleder, C., Perlik, V., Blatteis, C.M.: *Preoptic α1- and α2-noradrenergic agonists induce, respectively, PGE2-independent and PGE 2-dependent hyperthermic responses in guinea pigs.* Práce byla presentována v American Journal of Physiology - Regulatory Integrative and Comparative Physiology v roce 2004 a práce autorského kolektivu: Feleder C., Perlik V. and Blatteis C.M.: *Preoptic norepinephrine mediates the febrile response of guinea pigs to lipopolysaccharide,* prezentovaná ve stejném periodiku v roce 2007.

V současné době není shody v tom, jaké jsou interakce mezi norepinephrinem (NE) a prostaglandinem E_2 (PGE_2) v preoptické oblasti (POA) během horečky. Dobře je dokumentována skutečnost, že periferní pyrogeny aktivují noradrenergní cesty v mozku (Dun and Wang, 1995; Kabiersch et al., 1988), a že NE je uvolňován v POA v souvislosti s febrilní odpovědí (Linthorst et al., 1995; Mefford and Heyes, 1990). Zapojení PGE_2 ve febrilní reakci je zřejmé, ale dynamika a zdroje pyrogenem zvýšených hladin PGE_2 v POA je zatím kontroverzní. Pro podporu níže uvedené hypotézy, stejně tak pro pochopení existujících interakcí, by bylo nutné prokázat, že tvorba PGE_2 NE-dependentní je mediována v preoptické oblasti COX-2, s ohledem na to, že panuje shoda, že COX-2 je izoforma enzymu, který katalyzuje PGE_2 jako odpověď na pyrogenní stimuly (Quan et al., 1998; Steiner et al., 2001; Zhang et al., 2003).

Pro posouzení výše uvedených skutečností jsme následně provedli studii zaměřenou na testování následujících hypotéz: 1) COX-2 je izoenzym, který prostřednictvím působení norepinephrinu (NE) zprostředkovává expresi PGE_2 v preoptické oblasti (POA); 2) Součástí této regulace jsou adrenoceptory (AR), respektive jejich sub-typy (α_1- AR; α_2- AR).

Pro experiment dle etických standardů byla použita morčata (Hartley guinea pigs) o hmotnosti 301 – 350 g. Po antibiotické profylaxi byla v celkové anestezii dle metody popsané dříve (Quan and Blatteis, 1989a; 1989b) stereotakticky zavedena kanyla pro mikrodialýzu do levé preoptické oblasti a fixována na lebku. Vlastní experimentální procedury probíhaly ve stejnou denní dobu. Teplota (T_c) byla monitorována kontinuálně a zaznamenávána v intervalech 2 minut po celou dobu trvání pokusu, vzorky perfuzátu byly odebírány v intervalech 30 minut a testovány imunoassay metodou. Výsledky jsou prezentovány jako průměry ± odchylka, na 5% hladině významnosti.

Pro identifikaci teplotního efektu v preoptické oblasti a identifikaci adrenoceptorů α_1- AR a α_2- AR, které tento efekt mohou zprostředkovat, jsme zvolili metodu farmakologické stimulace, respektive blokády a perfundovali preoptickou oblast NE, cirazolinem (α_1- AR agonista), clonidinem (α_2- AR agonista), prazosinem (α_1- AR antagonista) nebo johimbinem (α_2- AR antagonista) po dobu 6 hodin, dále prazosinem – 1 hodinu a následně citrazolinem – 5 hodin, nebo johimbinem - 1 hodinu a clonidinem – 5 hodin. Šestihodinový interval pro jednotlivé fáze experimentu byl zvolen s ohledem na teplotní průběh endotoxinové horečky (Steiner et al., 2001; Sehic et al., 1996).

Vzhledem k tomu, že α_2-AR agonista clonidin, na rozdíl od α_1-AR agonisty cirazolinu, vyvolal změny hladin PGE_2 a teploty, pro identifikaci předpokládaného COX-izoenzymu v tomto ději jsme perfundovali preoptickou oblast COX-1 inhibitorem (SC-560), a poté COX-2 inhibitory - nimesulidem, nebo MK-0663 po dobu 1 hodiny a následně clonidinem po dobu 5 hodin. Teplota a PGE_2 hladiny byly měřeny stejným způsobem. Tímto byl prakticky testován efekt - inhibice na teplotu a PGE_2.

Na základě získaných poznatků z prvních dvou experimentálních částí jsme pro validaci preoptického efektu NE provedli mikrodialýzu POA po dobu jedné hodiny prazosinem, johimbinem, a nebo MK-0663, následovanou pětihodinovou perfuzí NE. Pro vyloučení možného efektu 0,01% askorbátu, jako antioxidačního vehikula s nízkou hodnotou pH jsme provedli perfuzi POA cirazolinem a clonidinem v kombinaci i jednotlivě.
Selektivní α_1-AR agonista cirazolin indukoval v této studii promptní T_c vzestup bez ovlivnění bazálních preoptických hladin PGE_2, zatímco selektivní α_2-AR agonista clonidin vyvolal časný propad T_c a zpozdil teplotní vzestup, obě reakce byly spojené s paralelními změnami v hladinách PGE_2 v POA oblasti. Termální efekt obou těchto agonistů byl validován jejich blokádou antagonisty – prazosinem a johimbinem. Kromě toho, obojí vzestup teploty a preoptických hladin PGE_2, působené

clonidinem byly potlačeny intra-POA mikrodialýzou COX-2 inhibitorů, nimesulidu a MK-0663, ale jen počáteční pokles těchto proměnných byl potlačen COX-1 inhibitorem SC-560.

Inhibice COX-1 oslabovala clonidinem indukovanou T_c a PGE_2 propad, nikoliv však NE vyvolanou hypertermii, zatímco COX-2 inhibice potlačovala obojí, clonidinem a NE-indukovanou T_c a PGE_2 vzestup. Souběžná infuze cirazolinu a clonidinu reprodukovala pozdní vzestup T_c působený clonidinem in-solo, ale ne časný propad a také ne časný vzestup produkovaný cirazolinem; na druhou stranu odpověď PGE_2 byla podobná NE. Prazosin (α_1-AR antagonista) a yohimbin (α_2-AR antagonista) blokoval efekty jejich agonistů.

Tyto závěry ukazují, že α_1- a α_2-agonista AR aplikovaný do preoptické oblasti CNS morčete evokuje zřetelnou teplotní odpověď. Podobnou reakci (lékově vyvolanou horečku) u pacientů popsali autoři Kelesidis and Kelesidis (2009) u pacientů, léčených vysokou dávkou clonidinu. $Alfa_1$-agonista produkuje rychlou, PGE_2 nezávislou teplotní odpověď zatím co α_2-AR stimulace působí časný propad teploty a pozdní COX-2/PGE_2 dependentní teplotní vzestup. Tyto reakce jsou v nervovém systému indukovány presynaptickým působením norepinephrinu prostřednictvím α-receptorů s následnou modulací izoenzymu COX-2. Zjištěné skutečnosti poskytují nová fakta o teplotních regulacích a jsou v souladu s později prezentovanou prací autorů Corley and Rawls (2009). Přestože přítomnost α_1-ARs na termosenzitivních neuronech POA byla demonstrována již dříve různými autory (Mallick and Islam, 2002; Medgett and Ruffolo, 1988), termální odpověď různých látek na intra-POA microinjekce α_1-AR agonistů byly všeobecně inkonzistentní (Clark, 1987; Clark and Lipton, 1986). Cirazolinem indukovaná hypertermie vzniká v tomto případě bez dalších přídatných změn v hladinách PGE_2 v preoptické oblasti, tedy je vyvolána rychle a bez zprostředkování PGE_2. Tato zjištění jsou nová a napovídají, že mechanismus působení na neurony je přímý. Předpokládáme tedy, že

působení α_1-ARs na postsynaptické teplotně senzitivní nebo teplotně nesenzitivní neurony přímo redukuje, respektive zesiluje aktivitu těchto neuronů, obojí podporuje teplotní konzervaci dle již dříve prezentovaných modelů Hammela (1965) a Boulanta (1996). Specifické α_1-receptory účastny v tomto hypertermickém efektu byly již dříve identifikovány. Mallick et al. (2000) popsal, že stimulace norepinephrinem zvyšuje aktivitu Na-pumpy v mozku laboratorního potkana a je zprostředkována přes α_{1A}-receptory. Na základě výše uvedeného, by představované závěry mohly naznačit, že NE uvolňovaný endogenně v POA by mohl působit promptní hypertermickou akci, zprostředkovanou rychlou aktivací postsynaptických α_1-ARs. Tento děj zapojuje mechanismus druhého posla. NE indukuje produkci cAMP v neuronech hypothalamu (Etgen and Petitti, 1987). Je však otázkou, zda se tak děje prostřednictvím α_1- nebo α_2-receptorů (Quesada and Etgen, 2000; Schaad et al., 1987). Kromě toho bylo zjištěno, že cAMP zvyšuje aktivitu krysích POA termosenzitivních neuronů (Boulant, 1996), tj. účinek, který by podporoval tepelné ztráty a následně pokles T_c (Hammel, 1965). Lze dovodit, že clonidinem vyvolaný pokles T_c zjištěný v této studii může být zprostředkován přes hypotermické působení cAMP uvolňovaný přes α_2-ARs (neboť efekt byl blokován před-léčením johimbinem). Efekt působení α_2-agonistů popisují autoři Kendall et al. (**2010**) s odkazem na práci autorů Tranquilli et al. (2007). α_2-agonisté inhibují uvolňování NE ze synaptických štěrbin cestou G-proteinu, který snižuje tvorbu cAMP, a kromě toho α_2-agonisté uplatňují svůj vliv i prostřednictvím regulace aktivity iontových kanálů (Tranquilli et al., 2007). Prezentované výsledky poskytují další informaci o tom, že termální odpověď je zprostředkována α_2-receptory a katalyzována COX-2 izoenzymem. COX-2 je exprimována konstitučně v mozkových buňkách, provedené studie demonstrují, že ačkoliv oba COX-izoenzymy jsou regulovány různými zánětlivými podněty, například periferním podáním LPS je COX-2 exprimována ve větší míře (Vane et al., 1998) v astrocytech, mikroglii, perivaskulárních a endoteliálních buňkách

(Elmquist et al., 1997; Li et al., 1999; Matsumura et al., 1998; Quan et al., 1998; Van Dam et al., 1993). Tyto skutečnosti jsou relevantní pro předpokládaný mechanismus febrilních reakcí jako odpovědi na periferní působení LPS, neboť febrilní reakce morčat na intravenozní podání PLS vykazuje charakteristickou, dvoufázovou odpověď, ve které COX-2 hraje větší roli v pozdní fázi horečky (Steiner et al., 2001). Pyrogenní informace může být předávána do mozku vagovými aferentními drahami (Yilmaz et al., 2008) a projikována do preoptických oblastí prostřednictvím noradrenergní aferentace (Blatteis et al., 2000; Simon set al., 1998; Watkins teal., 1995), norepinephrin je tak možné považovat za stěžejní regulační hormon ve febrilní odpovědi POA na LPS (Osaka, 2009). Tuto skutečnost potvrzují i závěry práce autorů Saito et al., (2008), kteří studovali termoregulační odpověď v mediální preoptické oblasti u chladového stresu.

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Preoptic α_1- and α_2-noradrenergic agonists induce, respectively, PGE_2-independent and PGE_2-dependent hyperthermic responses in guinea pigs

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Feleder, Carlos, Vit Perlik, and Clark M. Blatteis. Preoptic α_1- and α_2-noradrenergic agonists induce, respectively, PGE_2-independent and PGE_2-dependent hyperthermic responses in guinea pigs. *Am J Physiol Regul Integr Comp Physiol* 286: R1156–R1166, 2004. First published February 12, 2004; 10.1152/ajpregu.00486.2003.—We have shown previously that norepinephrine (NE) microdialyzed into the preoptic area (POA) of conscious guinea pigs stimulates local PGE_2 release. To identify the cyclooxygenase (COX) isozyme that catalyzes the production of this PGE_2 and the adrenoceptor (AR) subtype that mediates this effect, we microdialyzed for 6 h NE, cirazoline (α_1-AR agonist), and clonidine (α_2-AR agonist) into the POA of conscious guinea pigs pretreated intrapreoptically (intra-POA) with SC-560 (COX-1 inhibitor) or nimesulide or MK-0663 (COX-2 inhibitors) and measured the animals' core temperature (T_c) and intra-POA PGE_2 responses. Cirazoline induced T_c rises promptly after the onset of its dialysis without altering PGE_2 levels. NE and clonidine caused early falls followed by late rises of T_c; intra-POA PGE_2 levels were closely correlated with this thermal course. COX-1 inhibition attenuated the clonidine-induced T_c and PGE_2 falls but not the NE-elicited hyperthermia, but COX-2 inhibition suppressed both the clonidine- and NE-induced T_c and PGE_2 rises. Coinfused cirazoline and clonidine reproduced the late T_c rise of clonidine but not its early fall and also not the early rise produced by cirazoline; on the other hand, the PGE_2 responses were similar to those to NE. Prazosin (α_1-AR antagonist) and yohimbine (α_2-AR antagonist) blocked the effects of their respective agonists. These results indicate that α_1- and α_2-AR agonists microdialyzed into the POA of conscious guinea pigs evoke distinct T_c responses: α_1-AR activation produces quick, PGE_2-independent T_c rises, and α_2-AR stimulation causes an early T_c fall and a late, COX-2/PGE_2-dependent T_c rise.

thermoregulation; cyclooxygenase inhibitors; prostaglandin E_2; noradrenergic agonists and antagonists; care temperature; norepinephrine

THERE IS MUCH EVIDENCE that norepinephrine (NE) and prostaglandin (PG) E_2 interact in many tissues, including nervous tissue (33). For example, an intimate association between NE and PGE_2 is well documented in the peripheral nervous system (21); to wit, the stimulation of sympathetic neurons induces the postsynaptic release of PGE_2, which then limits the further presynaptic release of NE, thereby modulating the activity of noradrenergic neurons. It is also well documented that NE induces PGE_2 synthesis in brain tissue (23, 60) where its feedback inhibition of the presynaptic release of NE has been similarly documented (3, 13, 54).

In conscious guinea pigs, NE microinjected into the preoptic-anterior hypothalamic area [POA, the locus of the thermal controller (7)] evokes body (core) temperature (T_c) rises (75, 47). Electrical stimulation of the ascending noradrenergic system in the brain stem yields the same result (67), whereas chemical sympathectomy abrogates this response (66). PGE_2 microinjected into the POA also is thermogenic (5). NE is quickly degraded and removed from the brain whereas PGE_2 is removed much more slowly (74), so that the action of the latter may outlast the effect of the former. Because the hyperthermia induced in cats by intracerebroventricularly injected NE was inhibited by pretreatment with the antipyretic aspirin, a cyclooxygenase (COX) inhibitor, the notion was advanced that the hyperthermic action of NE may be exerted through PGE_2 (30). Others (27), however, have suggested that PGE_2 may be thermogenic through NE because the destruction by 6-hydroxydopamine of the noradrenergic nerve terminals in the POA of rabbits attenuated the rise in T_c produced by intravenously injected LPS, while still others (40) found no mutual interaction between NE and PGE_2.

There is thus no consensus as yet as to how NE and PGE_2 may interact in the POA during fever. The issue has relevance to current hypotheses of the mechanism of fever induction. Thus it is well documented that peripheral pyrogens activate noradrenergic pathways in the brain (14, 26) and that NE is released in the POA in conjunction with the febrile response (31, 38). The involvement of PGE_2 in fever production is now all but axiomatic, but the dynamics and source of the pyrogen-induced elevation of preoptic PGE_2 levels are still controversial (5). If NE were the direct stimulus for its increased production, it might be expected from the preceding that it would begin promptly and that, in turn, the elevated PGE_2 levels would inhibit the further release of NE. Furthermore, to support the hypothesis of such an interaction in fever production, it would be necessary to show that the induction of PGE_2 by NE is mediated in the POA by COX-2, since it is now generally agreed that COX-2 is the isoform of the enzyme that catalyzes the production of PGE_2 in response to pyrogenic stimuli (29, 48, 64, 77).

The present study was undertaken, therefore, to test the hypothesis that COX-2 is the isozyme that mediates the NE-enhanced expression of PGE_2 in the POA and to identify the adrenoceptor (AR) subtype(s) that may be involved in this effect. Because physiological stimuli cause NE to be released from its neuronal terminals not in a single burst but in repetitive pulses over a varying time until its stores become exhausted (2, 65) and because the repetitive pattern of delivery is better approximated by microdialysis, when NE is presented to the tissue over a longer duration, than by microinjection, when it is presented acutely in a microdroplet (47), we microdialyzed rather than microinjected NE and specific α_1- and α_2-AR

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agonists and antagonists into the POA of conscious guinea pigs pretreated intrapreoptically (intra-POA) with selective COX-1 and COX-2 inhibitors and measured the animals' T_c and POA PGE_2 levels. Our results validate the hypothesis and reveal additionally that the α-AR agonists of NE exert different effects. The activation of α_1-ARs evokes very promptly a T_c rise with no associated PGE_2 release, whereas the activation of α_2-ARs induces an initial T_c fall followed later by a T_c rise, the upregulation of COX-2 and the consequent production of PGE_2. However, when administered together (to mimic NE), the hyperthermic effect of α_1-AR stimulation is nullified by the initial hypothermic effect of the simultaneous activation of α_2-ARs. COX-1 does not appear to have a demonstrable role in the T_c rise caused by NE under these conditions but may be involved in the α_2-AR-mediated T_c fall.

MATERIALS AND METHODS

Animals

Male, pathogen-free, Hartley guinea pigs (301–350 g; Charles River Laboratories, Wilmington, MA) were used in these experiments. The animals were quarantined for 1 wk, three to a cage, before any experimental use. Tap water and food (Agway Prolab guinea pig diet) were available ad libitum. The ambient temperature (T_a) in the animal room was 23 ± 1°C; light and darkness alternated, with light on from 0600 to 1800. After quarantine, to moderate the psychological stress associated with the experiments, the animals were trained to the experimental procedure for 1 wk (daily for 4 h) by handling and placement in individual, locally fabricated, semicircular wire mesh confiners designed to prevent their turning around and to minimize their forward and backward movements, but without causing restraint stress; rodents readily adapt to such confinement and show no sign of discomfort or anxiety (56). All animal protocols were approved by the University of Tennessee Health Science Center Animal Care and Use Committee and fully conform with the standards established by the US Animal Welfare Act and by the documents entitled "Guiding Principles for Research Involving Animals and Human Beings" (1).

Surgical Procedure

All the animals received a prophylactic injection of the antibiotic gentamicin sulfate (6 mg/kg im) immediately before surgery and once a day for the following 2 days. Microdialysis guide cannulas were implanted by methods described previously (46, 47). Briefly, the guinea pigs were anesthetized with ketamine-xylazine (35–5 mg/kg im). Under aseptic conditions, a sterile, 17-mm-long, 17-gauge, thin-walled stainless steel guide cannula with a tightly fitting indwelling stylet was implanted stereotaxically into the left medial POA [anteroposterior = 11.6 mm, lateral = 1.0 mm, ventral = −9.0 mm, according to the atlas of Luparello (32)] and fixed to the skull with four self-tapping, miniature, stainless steel screws and dental acrylic cement. All the animals received fluid replacement therapy (5–10 ml of pyrogen-free saline sc) immediately after surgery and butorphanol (0.05 mg/kg twice a day) for postoperative pain control, as necessary. Training to the experimental procedure resumed 2 days postsurgery. Seven days were allowed for full recovery.

Drugs

The microdialysis perfusate and vehicle for all the drugs was sterile artificial cerebrospinal fluid (aCSF) for guinea pigs, prepared as previously described (60) with a final concentration (in mM) of 140.0 NaCl, 2.7 KCl, 1.0 $MgCl_2 \cdot 2H_2O$, 1.2 $CaCl_2 \cdot 2H_2O$, 2.0 Na_2HPO_4; osmolality of 290 mosmol/kgH_2O; and pH of 7.4, adjusted with 85% H_3PO_4. The following solutions were prepared just before use and stored in amber glass vials at room temperature: l-NE bitartrate (10 μg/μl), the NE antioxidant ascorbic acid (0.1% in aCSF), the α_1-AR agonist cirazoline (1 μg/μl), the α_1-AR antagonist prazosin (1 μg/μl), the α_2-AR agonist clonidine (2 μg/μl), the α_2-AR antagonist yohimbine (1 μg/μl), the selective COX-1 inhibitor SC-560 (5 μg/μl), the selective COX-2 inhibitors MK-0663 (0.5 μg/μl) and nimesulide (1 μg/μl), and their solvent DMSO (6% in aCSF). These concentrations were selected on the basis of our previous studies (47, 49–51, 55, 60). It should be noted in this regard that only 10% of the doses microdialyzed at 2 μl/min, as in this study, diffuses into the POA (47). MK-0663 was generously supplied by Merck Frosst Canada (Kirckland, Quebec, Canada); cirazoline was purchased from Tocris (Ellisville, MO). All the other drugs were purchased from Sigma-Aldrich (St. Louis, MO).

Microdialysis Probes

The microdialysis probes used in this study were adapted from the concentric design of Torto et al. (68) and constructed by us. Thus a 20-cm-long silica capillary (ID 50 μm, OD 125 μm; Polymicro Technologies, Phoenix, AZ) was inserted within a 15-cm-long piece of polyethylene tubing [PE 50 (ID 0.58 mm, OD 0.965 mm); Intramedic Clay Adams, Becton Dickinson, Sparks, MD] so that 2.1 cm of the capillary protruded from the distal end of the outer tubing. Next, a 2.5-cm-long, 26-gauge stainless steel tube (ID 75 μm, OD 154 μm; Polymicro Technologies, Phoenix, AZ) was slipped over the capillary a distance of 0.5 cm into the PE tubing so that 2 cm of the tube remained outside of the PE tubing and ~1 mm of the capillary extended beyond the distal tip of the tube. A nitrocellulose hollow fiber dialysis membrane (10-kDa nominal molecular mass cutoff, OD 150 μm, 2 mm fiber; Spectra/Por, Spectrum, Laguna Hills, CA.) was then fitted over the protruding capillary and its distal end sealed with a droplet of "5-min" epoxy (Devcon, Danvers, MA). The inner capillary thus served as the outlet and the PE tubing as the inlet of the perfusion medium.

Two hours before an experiment, the indwelling stylets of the guide cannulas were replaced by sterile microdialysis probes, so that their dialysis membrane tips protruded exactly 1 mm beyond the guide cannulas. They were fixed to the skull with tissue adhesive and immediately perfused with sterile aCSF via sterile 1-ml tuberculin syringes clamped to a syringe pump (model A-99; Razel Scientific Instruments, Stamford, CT) as the driver; to run six animals simultaneously, the pushers of two pumps were modified so that each could accommodate three syringes at a time. The flow rate of the perfusion was 2 μl/min (46). A 90-min stabilization period preceded all the experimental treatments.

After an experiment, the guinea pigs were euthanized by isoflurane overdose, and the brains were quickly removed and stored in 10% phosphate-buffered Formalin for later histological verification of the placement of the dialysis probe tips. Localization of the center of the dialysis probe within 0.5 mm of the medial POA was regarded as the correct placement (Fig. 1). Only the data from animals with confirmed preoptic placement of the probes are included in this report.

Physiological Measurements

Seven days after surgery, the guinea pigs, fully conscious, were loosely restrained in their individual wire mesh confiners at T_a 23 ± 1°C. The T_c of the guinea pigs were monitored constantly and recorded at 2-min intervals for the duration of an experiment on a Macintosh Plus lMb microcomputer through an analog-to-digital converter, using precalibrated copper-constantan thermocouples inserted 5 cm into the colon. The data were displayed digitally on a monitor, printed on an ImageWriter printer, and stored on a diskette. The effluents from the microdialysate probes were collected over 30-min intervals continuously throughout the experiments. The PGE_2 content of the samples was evaluated using a PGE_2 enzyme immunoassay kit (no. 931–001; Assay Designs, Ann Arbor, MI). To

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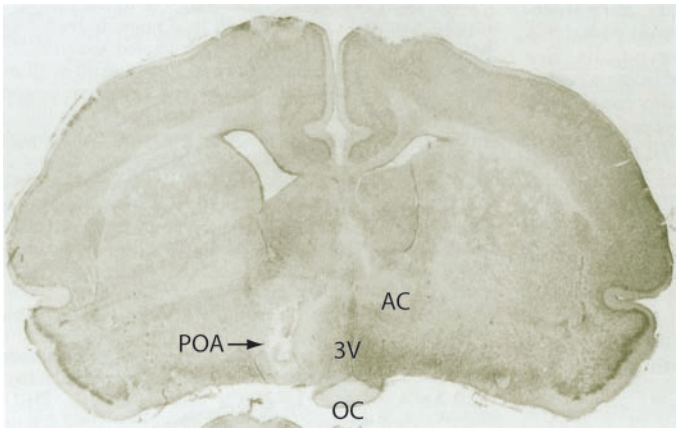

Fig. 1. Photomicrograph of a coronal section through the forebrain of a guinea pig, showing the track of a microdialysis probe guide cannula implanted into the preoptic area (POA). The guide cannulas were placed 7 days before an experiment (see materials and methods for details). AC, anterior commissure; 3V, third ventricle; OC, optic chiasma.

obviate possible effects of circadian variations, all the experiments were begun at the same time of day (0830).

Experimental Designs

Experiment 1: NE effects on T_c. To determine the thermal effect of intra-POA NE and to identify the α-AR subtype(s) that may mediate this effect, we microdialyzed NE, cirazoline, clonidine, prazosin, or yohimbine into the POA of conscious guinea pigs for 6 h, or prazosin for 1 h followed by cirazoline for 5 h, or yohimbine for 1 h followed by clonidine for 5 h. T_c and preoptic PGE_2 levels were measured as described earlier. The 6-h total duration of this procedure was chosen to align it with the temporal course of a prototypic endotoxic fever in conscious guinea pigs (60, 64).

Experiment 2: α_2-ARs and COX. Because clonidine, but not cirazoline, induced T_c-associated POA PGE_2 level changes (see results), to identify the COX isozyme presumptively involved in these effects, we microdialyzed SC-560, nimesulide, or MK-0663 for 1 h into the POA of conscious guinea pigs and clonidine for the following 5 h. T_c and preoptic PGE_2 levels were determined as before. The effects of these COX inhibitors on T_c and PGE_2 were also tested.

Experiment 3: validation of preoptic NE effects. To relate the results of *experiments 1* and *2* to the actions of intra-POA NE, we microdialyzed for 1 h prazosin, yohimbine, or MK-0663 into the POA of conscious guinea pigs, followed for 5 h by NE. T_c and preoptic PGE_2 levels were monitored as above.

Finally, to correlate the effects of NE to those evoked by the α_1-AR and α_2-AR agonists, but without the confounding effects of 0.1% ascorbate (low pH and antioxidant vehicle), we microdialyzed cirazoline and clonidine combined into a single solution intra-POA for 6 h. T_c and preoptic PGE_2 levels were monitored as above.

Statistical Analyses

The results are reported here as means ± SE. The values of T_c are changes from basal values [initial T_c (T_{ci}), the T_c at 2-min intervals averaged over the last 10 min of the preceding 90-min stabilization period] plotted at 6-min intervals. Latencies of T_c changes were defined as the intervals (in min) between the onset of a treatment (0 or 60 min) and the first T_c rise or fall greater than 0.2°C (i.e., the SD of the T_c of aCSF-treated guinea pigs) that continued uninterruptedly beyond ±0.5°C. The PGE_2 data are expressed as changes relative to their values before a treatment (P_0). A repeated-measures ANOVA was used to compare the T_c and PGE_2 changes between groups; *factor 1* was the between-groups factor (the treatment), and *factor 2* was the within-subjects factor (the different sampling periods), followed, if significant differences were found, by a point-by-point Tukey-Kramer multiple comparison test. The analyses were performed using Instat 3 (Graph Pad Software; Instant Biostatistics, San Diego, CA). Each variable was considered to be independent. The 5% level of probability was accepted as statistically significant.

RESULTS

Experiment 1: NE Effects on T_c

The intra-POA microdialysis of aCSF per se did not evoke any T_c and intra-POA PGE_2 changes (Fig. 2, *A* and *B*). In contrast, aCSF + 0.1% ascorbic acid, the NE antioxidant, caused a prompt, statistically significant approximately −0.6°C decrease in T_c (latency: 45 ± 12 min); it reached its nadir in 120 min and then returned to its basal level by ~210 min. The levels of PGE_2 in the microdialysate effluents, however, were not changed by this treatment (Fig. 2, *C* and *D*). NE + 0.1% ascorbic acid induced a biphasic thermal response. The first, a T_c fall, coincided in magnitude with that induced by ascorbic acid but lasted longer. It was followed by a second response, a T_c rise, that gradually began after ~120 min and peaked ~0.8°C above T_{ci}, a statistically significant increase: this rise was maintained until the end of the experiment. Preoptic PGE_2 levels increased significantly in these guinea pigs in approximate temporal correspondence with their T_c rises (Fig. 2, *E* and *F*).

The microdialysis of cirazoline evoked a statistically significant T_c rise very quickly after the onset of its administration (latency: 15 ± 6 min). The maximum (~1°C) was attained in ~150 min, continued unaltered for ~2 h, and then gradually declined even though the perfusion was continuing. PGE_2 levels, however, were not altered (Fig. 3, *A* and *B*). Prazosin perfused over the full 6 h of the experiment altered neither the T_c nor the PGE_2 levels of the guinea pigs (Fig. 3, *C* and *D*), but prazosin administered for 1 h before cirazoline completely abolished the effect of cirazoline on T_c; PGE_2 was not affected (Fig. 3, *E* and *F*).

Clonidine induced a gradual, significant T_c fall followed by a rapid, significant T_c rise compared with aCSF alone (Fig. 2*A*). The T_c decline began almost immediately after the onset of the dialysis (latency: 15 ± 6 min) and reached approximately −0.8°C in ~160 min. It then increased progressively until the end of the experiment to ~1°C above T_{ci}. Intra-POA PGE_2 levels changed significantly in close correlation with the T_c changes (Fig. 4, *A* and *B*). The perfusion of yohimbine over the 6-h duration of the experiment affected neither the T_c nor the PGE_2 levels of the guinea pigs (Fig. 4, *C* and *D*), but yohimbine administered for 1 h before clonidine completely abolished both the T_c and PGE_2 falls and rises caused by clonidine alone (Fig. 4, *E* and *F*).

Experiment 2: α_2-ARs and COX

The microdialysis of SC-560 for 1 h before that of clonidine did not alter the late effects of clonidine on T_c and intra-POA PGE_2 levels. However, it inhibited its early effects on these variables. PGE_2 levels began to increase significantly ~150 min after the onset of clonidine, rising continuously until the end of the experiment, in coincidence with T_c (Fig. 5, *A* and *B*). In contrast, the microdialysis of nimesulide or MK-0663 blocked the late rises of both T_c and PGE_2 evoked by the

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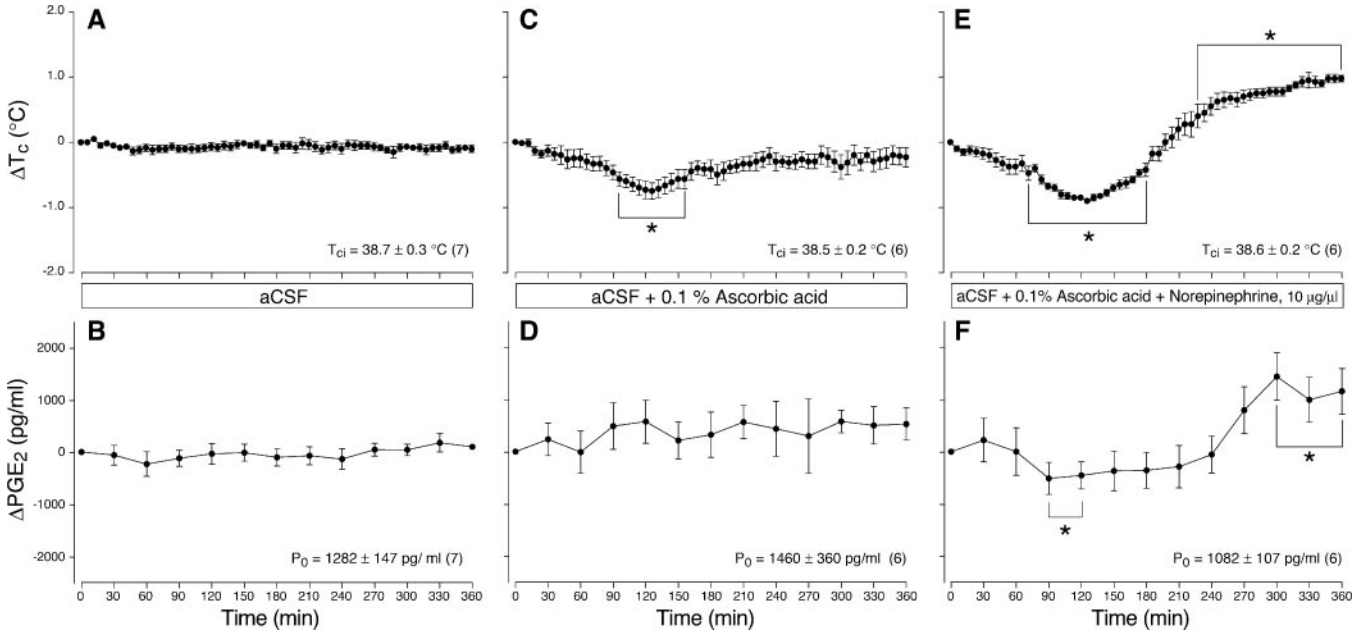

Fig. 2. Effects on the body (core) temperature (T_c) and preoptic PGE_2 levels, respectively, of artificial cerebrospinal fluid (aCSF; *A* and *B*), aCSF + ascorbic acid (*C* and *D*), and aCSF + ascorbic acid + norepinephrine (NE) (*E* and *F*) microdialyzed into the POA of conscious guinea pigs. T_c values are expressed as differences (ΔT_c) relative to their initial levels [T_{ci}: average of the T_c over the last 10 min before treatment (time 0)]. P_0, PGE_2 value before treatment. Values are means ± SE; (*n*), no. of animals. $^*P <$ 0.05.

microdialysis of clonidine alone; indeed, MK-0663 inverted the T_c rise into a significant fall, but neither had an effect on the early responses. PGE_2 levels changed significantly in close temporal correlation with these thermal courses (Fig. 5, *C–F*). None of the COX inhibitors or their vehicle, DMSO, microdialyzed for 6 h, significantly affected the T_c or the intra-POA PGE_2 levels of the guinea pigs (not illustrated).

Experiment 3: Validation of Preoptic NE Effects

The intra-POA microdialysis of prazosin for 1 h before that of NE attenuated the initial T_c fall caused by NE alone but did not alter the late rises of T_c and intra-POA PGE_2 levels induced by NE (Fig. 2, *E* and *F*). Thus the T_c increased gradually, beginning ~180 min after the start of the perfusion of NE and

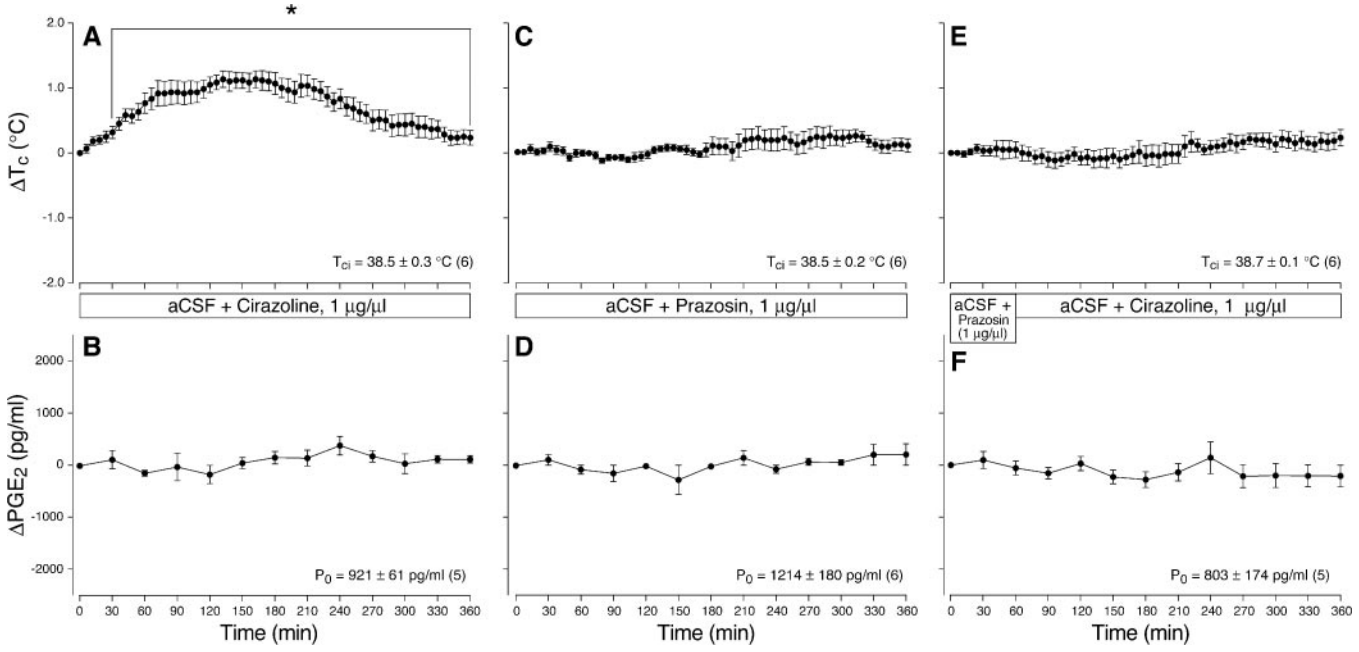

Fig. 3. Effects on T_c and preoptic PGE_2 levels, respectively, of the α_1-adrenoceptor (α_1-AR) agonist cirazoline (*A* and *B*), the selective α_1-AR antagonist prazosin (*C* and *D*), and prazosin + cirazoline (*E* and *F*) microdialyzed into the POA of conscious guinea pigs. Abbreviations and conventions as in Fig. 2. $^*P <$ 0.05.

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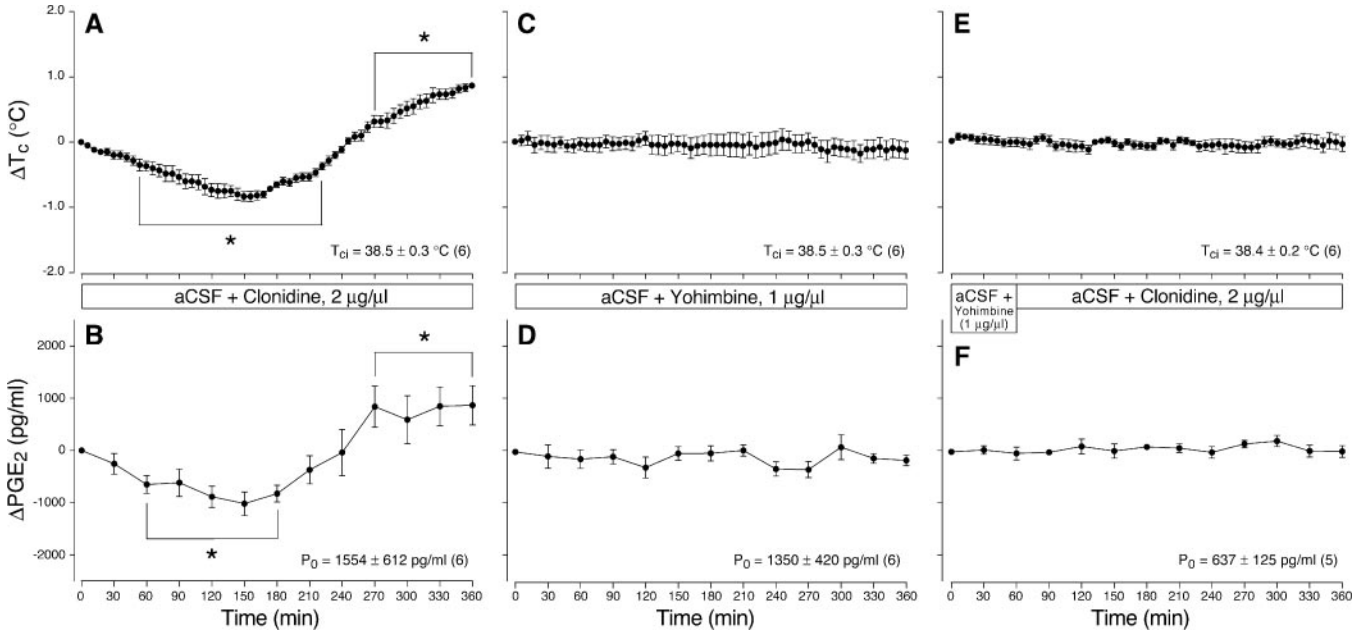

Fig. 4. Effects on T_c and preoptic PGE_2 levels, respectively, of the α_2-AR agonist clonidine (*A* and *B*), the α_2-AR antagonist yohimbine (*C* and *D*), and yohimbine + clonidine (*E* and *F*) microdialyzed into the POA of conscious guinea pigs. Abbreviations and conventions as in Fig. 2. *$P < 0.05$.

reaching a significant elevation of ~1°C by the end of the experiment. PGE_2 levels again changed in close correlation with the thermal courses (Fig. 6, *A* and *B*). Yohimbine microdialyzed intra-POA for 1 h before NE also prevented the early fall of T_c and the late rises of T_c and PGE_2 levels induced by NE. Similarly, MK-0663 completely abolished the T_c and PGE_2 level changes evoked by NE. Indeed, MK-0663 inverted both the T_c and PGE_2 late rises into falls, in close correlation with each other (Fig. 6, *C-F*).

The initial T_c falls produced by both NE (Fig. 2*E*) and clonidine (Fig. 4*A*) were abrogated when cirazoline and clonidine were microdialyzed together (Fig. 7*A*). However, the late T_c rises induced by both NE and clonidine were still evident. Thus T_c increased progressively from ~200 min after the onset of the perfusion until the end of the experiment (to ~1.2°C above T_{ci}). Preoptic PGE_2 levels, on the other hand, decreased significantly promptly after the onset of the experimental treatment and remained low for ~180 min and then

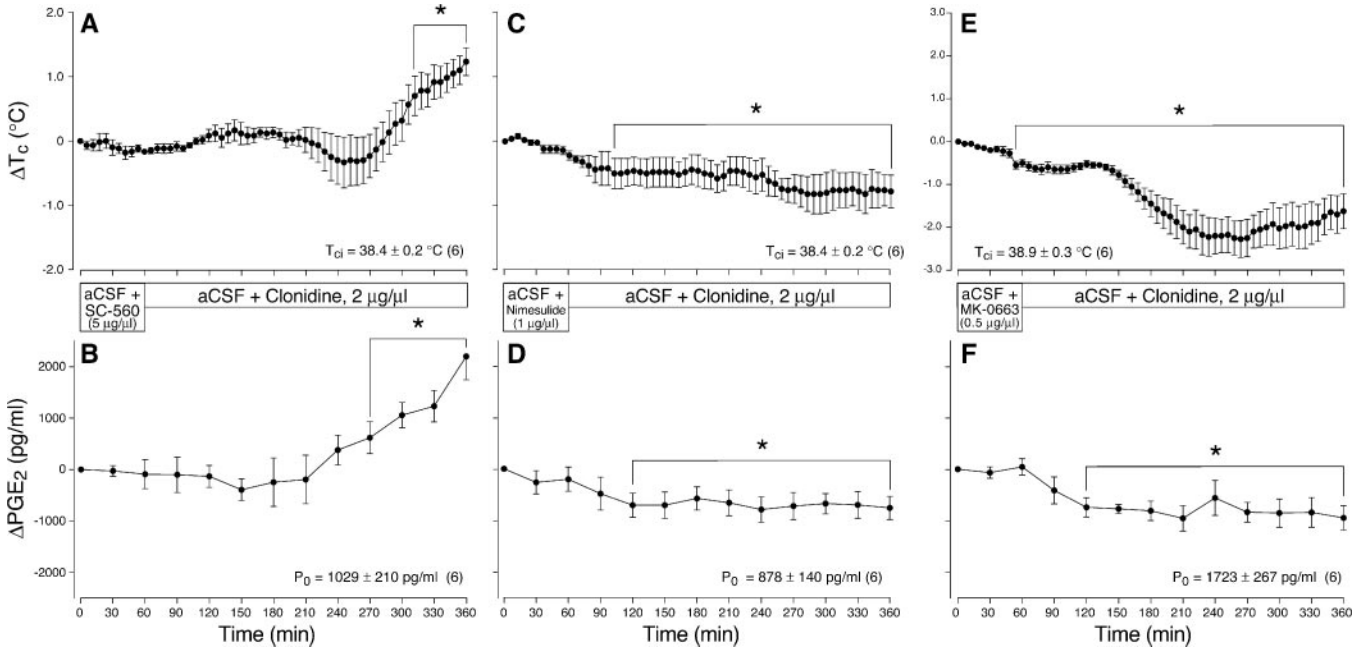

Fig. 5. Effects on T_c and preoptic PGE_2 levels, respectively, of the COX-1 inhibitor SC-560 + clonidine (*A* and *B*) and the COX-2 inhibitors nimesulide + clonidine (*C* and *D*) and MK-0663 (*E* and *F*) + clonidine microdialyzed into the POA of conscious guinea pigs. Abbreviations and conventions as in Fig. 2. *$P < 0.05$.

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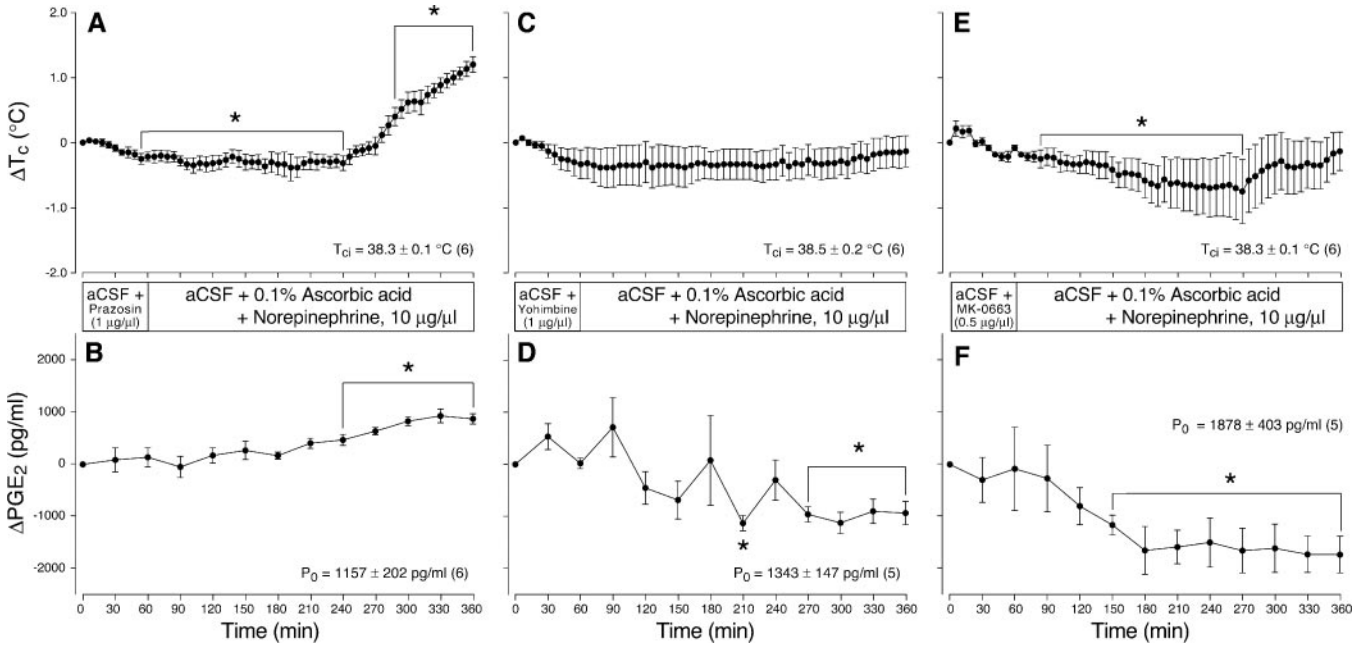

Fig. 6. Effects on T_c and preoptic PGE_2 levels, respectively, of the α_1-AR antagonist prazosin + NE (*A* and *B*), the α_2-AR antagonist yohimbine + NE (*C* and *D*), and the COX-2 inhibitor MK-0663 + NE (*E* and *F*) microdialyzed into the POA of conscious guinea pigs. Abbreviations and conventions as in Fig. 2. $^*P < 0.05$.

increased in correlation with the late T_c rise (Fig. 7*B*), analogously to the full response to clonidine (Fig. 4*B*) and the late response to NE (Fig. 2*F*).

DISCUSSION

The selective α_1-AR agonist cirazoline induced in this study prompt T_c rises without affecting basal preoptic PGE_2 levels, whereas the selective α_2-AR agonist clonidine caused early T_c falls and delayed T_c rises, both associated with parallel changes in the levels of POA PGE_2. The thermal effects of both these agonists were validated by their blockade by their respective antagonists, prazosin and yohimbine. Furthermore, both the increases in T_c and POA PGE_2 levels caused by clonidine were prevented by the intra-POA microdialysis of the COX-2 inhibitors nimesulide and MK-0663, but only the initial decreases of these variables were suppressed by that of the COX-1 inhibitor SC-560. (Because the hyperthermic effect of cirazoline was not accompanied by changes in the levels of POA PGE_2, the effects of the COX inhibitors were not tested in its regard.) The intra-POA microdialysis of NE reproduced the early and the late T_c and preoptic PGE_2 level changes induced by clonidine but not the early T_c rise evoked by cirazoline; both the clonidine-mediated effects were inhibited by yohimbine and MK-0663 but not by prazosin. Cirazoline and clonidine microdialyzed together replicated the late T_c rises but not the early T_c falls elicited by clonidine and NE and also not the early T_c rises caused by cirazoline; on the other hand, it induced both the POA PGE_2 falls and rises associated with clonidine and NE. The present results thus indicate that α-AR agonists microdialyzed into the POA of conscious guinea pigs evoke differentially mediated T_c responses. To the best of our knowledge, they are also the first demonstration in the nervous system that the induction of postsynaptic PGE_2 by presynaptic NE is mediated by α_2-ARs and modulated by COX-2.

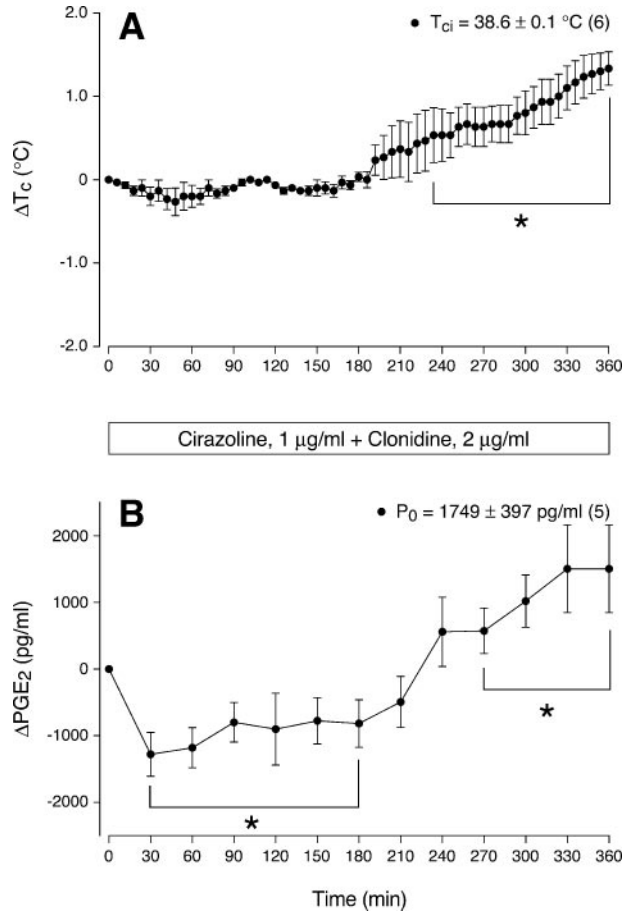

Fig. 7. Effects on T_c (*A*) and preoptic PGE_2 levels (*B*) of the α_1-AR agonist cirazoline + the α_2-AR agonist clonidine microdialyzed together into the POA of conscious guinea pigs. Abbreviations and conventions as in Fig. 2. $^*P < 0.05$.

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Although the presence of α_1-ARs on thermosensitive neurons in the POA has been demonstrated previously by various means (35, 37), the thermal responses of different species to intra-POA microinjected α_1-AR agonists have generally been inconsistent (9, 10). Indeed, we found in a previous study (50) that the α_1-AR agonist methoxamine microdialyzed into the POA of conscious guinea pigs for 3 h had no thermal effect. The very prompt hyperthermic response to the specific α_1-AR agonist cirazoline observed in this study was therefore unexpected. We have no explanation at this time for the discrepant responses to the two drugs other than to speculate that they may be accounted for by the lower potency of methoxamine compared with cirazoline (8, 63). The possibility that cirazoline may have leaked out of the central nervous system and acted on peripheral vascular α_1-ARs, causing cutaneous vasoconstriction, seems remote because the dose that was microdialyzed intra-POA in this study was effectively ~1,000 times lower than its minimally effective vasoconstrictive dose (18, 37). It is also possible that the observed T_c rises may have been caused by a stimulatory action of cirazoline not mediated by α_1-ARs, but this too is unlikely since its hyperthermic effect was inhibited by the specific α_1-AR antagonist prazosin (Fig. 3). Significantly, the cirazoline-induced hyperthermia occurred in this instance without any associated changes in the levels of intra-POA PGE_2; i.e., it was evoked quickly and without the intermediation of PGE_2. This finding is novel and suggests that its mode of action on neurons is direct. We propose therefore (Fig. 8) that the activation of α_1-ARs on postsynaptic warm-sensitive or thermoinsensitive neurons directly reduces or augments, respectively, the activities of these neurons, both responses promoting heat conservation according to the models of Hammel (19) and Boulant (7). Since these neurons, moreover, are thought to inhibit synaptically connected cold-sensitive neurons, these are concomitantly facilitated, stimulating heat production (not illustrated). The combination of these effector mechanisms raises T_c. The eventual decline of the hyperthermic action of cirazoline observed in the present study was presumably due to the desensitization of the relevant postsynaptic neurons, since prolonged exposure to a transmitter can gradually attenuate the responsiveness of neurons that originally were activated by it. The specific α_1-AR subclass involved in this hyperthermic effect remains to be identified; Mallick et al. (34) have reported that the NE-stimulated increase in sodium pump activity of rat brain homogenates is mediated by α_{1A}-ARs. Based on the foregoing, the present results would imply that NE released endogenously in the POA should exert a prompt hyperthermic action mediated by quickly activated postsynaptic α_1-ARs. A discrepancy, however, appears to exist: the presence of NE, as mimicked by its intra-POA microdialysis, induced a T_c fall rather than a T_c rise; it will be addressed later.

The intra-POA microdialysis of the α_2-AR agonist clonidine evoked in this study a biphasic thermal response, viz., an initial, early decrease in T_c followed by a late, extended T_c rise. Clonidine has consistently been reported to evoke T_c falls when microinjected intra-POA in various species (39, 55). In our own previous studies (50, 51), it also induced dose-dependent T_c falls in conscious guinea pigs when microdialyzed intra-POA for 3 h at doses from 0.5 to 10 $\mu g/\mu l$. It did so again in the present study, at 2 $\mu g/\mu l$ (Fig. 4). α_2-ARs occur on both presynaptic and postsynaptic neurons. The identity of their subclasses specifically involved in this effect was not investigated in the present study; in mice, both α_{2A}- and α_{2C}-ARs have been implicated in the hypothermic action of clonidine (24, 28, 58).

In the present study, this hypothermic response was accompanied, moreover, by a concurrent decrease in the animals' preoptic PGE_2 levels, a new finding. Like the T_c fall, it was blocked by pretreatment with the selective α_2-AR antagonist yohimbine. Unexpectedly, both the T_c and PGE_2 falls were also prevented by pretreatment with the COX-1 inhibitor SC-560 (Fig. 5). At least two possibilities could account for the latter surprising findings. Both tentatively involve the second messenger cAMP. Thus *1*) various lines of evidence have indicated that NE induces the production of cAMP in first-order neurons in hypothalamic tissue slices (17); it is controversial, however, whether it does so by stimulating α_1- or α_2-ARs (52, 59). It was also reported long ago (11, 12) that the intra-POA microinjection of its analog agonist, dibutyryl-cAMP, into rabbits and rats causes a rapid fall followed by a prolonged rise in T_c and that the latter rise is blocked by acetaminophen, then a nonspecific COX inhibitor, but lately a putatively specific inhibitor of the presumptive COX-1 variant, COX-3 (61). This late rise was attributed to PGE_2 generated in consequence of tissue damage associated with the microinjection procedure per se. Finally in this regard, it has also been

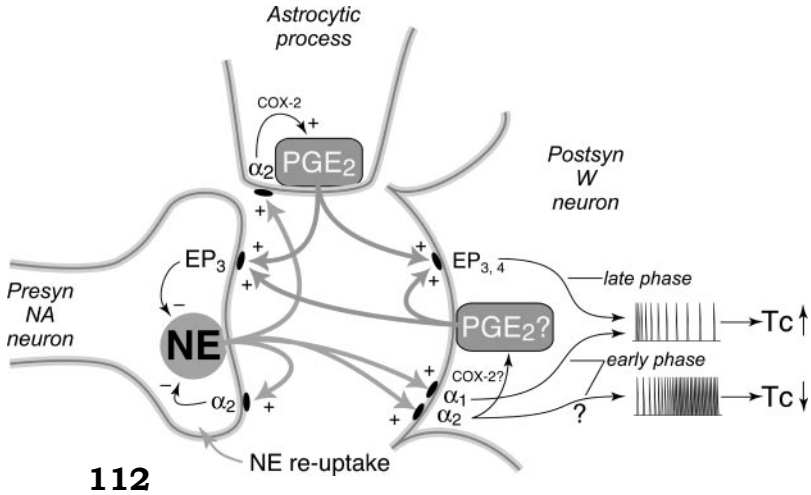

Fig. 8. Proposed mechanism of the thermogenic interaction of NE and PGE_2 in the POA of guinea pigs. See text for details. Modified from Ref. 5.

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shown that cAMP increases the activity of rat preoptic warm-sensitive neurons in slice preparations (7), an effect that would promote heat loss and, consequently, a T_c fall (7, 19). Taken together, these data infer that the clonidine-induced fall in T_c observed in the present study could have been due to the hypothermic action of cAMP released by α_2-ARs (since the effect was blocked by yohimbine pretreatment). This explication is insufficient, however, to also account for the decrease in preoptic PGE_2 that accompanied this T_c fall. Hence, *2*) it could be that the stimulation by clonidine of postsynaptic α_2-ARs also rapidly activates COX-1 (since the effect was blocked by SC-560) and induces the preferential production of PGD_2. cAMP is a well-known activator of COX expression (44), and, from a strictly stoichiometric point of view, if under these conditions PGD synthase were activated relative to (presumably cytosolic) PGE synthase, one would expect less PGE_2 and more PGD_2 to be produced; both synthases are downstream of COX-1 and would be competing for the same, initially limited substrate, arachidonic acid. PGD_2 has been shown to be hypothermic in various species (69), including guinea pigs (4), and it is also more abundant in brain than PGE_2 (20). COX-1 is expressed constitutively in most cells, including neurons, and its PG products mediate basic physiological functions in normal tissue; the involvement of COX-1-dependent PGD_2 in the observed effects is, therefore, quite plausible. Indeed, its blockade would prevent them, as was observed in this study. COX-1 blockade, however, does not affect the basal level of preoptic PGE_2 (not illustrated).

The continued microdialysis of clonidine converted the initial T_c and preoptic PGE_2 falls into concurrent, protracted increases (Fig. 4). Because both these rises were also inhibited by yohimbine pretreatment, they too were evidently α_2-AR mediated; and because, moreover, they were prevented by the prior microdialysis of the selective COX-2 inhibitors nimesulide and MK-0663, this late hyperthermic response was mediated by COX-2-dependent PGE_2. The production of PGE_2 by NE is well documented (23, 33, 60), but the AR subtype and COX isoform that mediate PGE_2 production were not identified in those earlier studies. The present data thus add the information that this response is α_2-AR mediated and specifically catalyzed by COX-2. COX-2 is expressed constitutively in brain cells, and various studies have demonstrated that, although both COX-1 and COX-2 enzymes are upregulated by various inflammatory stimuli, COX-2 is the more readily inducible isoform (72). Its induction in this study could have been initiated by cAMP simultaneously with its activation of COX-1. One to 4 h are required for new gene expression and translation of COX-2 in LPS-activated phagocytic, endothelial, and other relevant cells ex vivo and in vivo (25, 48, 77), a duration that coincides with the interval between the onset of the microdialysis of clonidine and NE and the beginning of the rises of T_c and the levels of POA PGE_2 induced by both these agonists in the present study (Figs. 2 and 4). The involvement of COX-2 under the present experimental conditions is therefore also concordant. In support, the late hyperthermic response to clonidine microinjected intracerebroventricularly into conscious mice is suppressed in COX-2 gene-ablated animals (unpublished observation). Although we have previously observed a biphasic hypo-/hyperthermic response to intra-POA microinjected (rather than microdialyzed) clonidine (55), in that study in contrast to the present one, the α_2-AR antagonist rauwolscine microinjected 10 min before clonidine abolished the hypothermia without affecting the subsequent hyperthermia; the latter was attenuated by the intramuscular injection of the nonspecific COX inhibitor indomethacin 20 min after the intra-POA microinjection of clonidine. This response was similar to that to dibutyryl-cAMP reported by Dascombe (11, 12). We interpreted those data as verifying the α_2-AR-mediated hypothermic action of clonidine and, like Dascombe, attributed the subsequent T_c rise to contamination of the thermal response to this agonist by PGE_2 released in the POA consequent to the acute inflammatory response to the microinjection procedure per se (57). However, in view of the present findings, we now suggest that the late reduced hyperthermic response to microinjected clonidine observed in that previous study (55) was also partly accomplished by α_2-AR-mediated, as contrasted only to injury-induced, upregulation of COX-2-dependent PGE_2.

The brain cell type(s) expressing COX-2 in the present instance was not investigated, but the increase of COX-2 after, for example, the peripheral administration of LPS is observed in astrocytes, microglia, perivascular cells, and cerebromicrovascular endothelial cells but only irregularly in neurons (16, 29, 36, 48, 71); neurons, in any case, secrete only small amounts of PGE_2 (22). Hence, we conjecture that the PGE_2 collected in the microdialysate effluents from the POA extracellular space in the present experiments was generated by astrocytic processes contacting noradrenergic synaptic regions rather than by postsynaptic neurons (41). We propose therefore (Fig. 8) that the evoked release of NE by discharging presynaptic noradrenergic neurons stimulates astrocytic α_2-ARs, generating arachidonic acid from membrane phospholipids; PGE_2 is then formed by the action of COX-2 on the arachidonic acid. Which phospholipase (PL) forms are activated and how COX-2 and microsomal PGE synthase are upregulated under these conditions remain to be determined. In rat vascular smooth muscle cells, NE-stimulated postjunctional α_2-ARs activate cytosolic PLA_2 [isoforms not identified (45)], but NE also binds to α_2-ARs coupled to phosphoinositide-specific phospholipases (PI-PL) C and D (45). In rat hypothalami, LPS upregulates secretory PLA_2-IIA [cell types not identified (25)]. The mode of action of PGE_2 on the activities of POA warm-sensitive and thermoinsensitive neurons is similar to that suggested earlier for α_1-AR-mediated responses, viz., a reduction and an increase in firing rates, respectively (53). The PGE_2-sensitive receptor involved in these neuronal effects may be the EP_3 and/or EP_4 subtypes; both have been linked to the development of fever and are present in the POA (15, 42, 43, 70, 76).

Although extensive, the data on the thermoregulatory actions of central NE are inconsistent, some indicating that NE reduces, others that it increases, and still others that it does not influence T_c (47, 49–51, 75). The discrepant results have been hard to reconcile because the methodologies, species, and doses employed by different investigators varied greatly. Indeed, we found, in common with other workers (66, 67, 75), that NE microinjected into the POA of conscious guinea pigs caused a rise in T_c but that, when microdialyzed, it evoked a hypothermic response (49–51). We attributed the T_c fall in the latter case to an artifact of the low pH (~3.5) of our perfusate and the neurotoxic effect of the potent antioxidant (sodium metabisulfite) present in that solution (Levophed). However, in

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that study, we discontinued the perfusion after 3 h and therefore missed its subsequent effects. The present finding that the intra-POA microdialysis of NE for 6 h induced first a fall (as before) and then a rise in both T_c and POA PGE_2 levels (analogous to the biphasic response to clonidine) and that both these effects were inhibited by yohimbine thus indicates that this biphasic response represents the authentic mode of action of NE microdialyzed for 6 h into the POA, i.e., that neither the observed falls nor the rises of these two variables were artifactitious and that both events were mediated by α_2-AR stimulation. On the other hand, the coadministration of cirazoline and clonidine, to mimic NE without its vehicle, 0.1% ascorbic acid, did not reproduce the initial NE-induced hypothermia although the fall in POA PGE_2 was still evident. We ascribe the absence of a fall in T_c under these conditions to the observed, opposite thermal actions of these agonists, i.e., the direct, α_1-AR-stimulated elevation of T_c by cirazoline was counteracted by the clonidine, α_2-AR-mediated induction of, presumptively, cAMP/PGD_2 and the consequent depression of T_c. It is not clear, however, why then the α_2-AR-mediated, hypothermic action of NE was not similarly antagonized by its α_1-AR-mediated, hyperthermic effect, and why also the blockade of the latter effect by prazosin did not enhance the T_c fall. However, because the NE and combined cirazoline-clonidine solutions differed in this study only by the presence of ascorbic acid in the former, it is possible that the acidity of that additive accounted for the different effects. Indeed, the hypothermic effect of NE was larger than that of ascorbic acid yet smaller than that of clonidine. Because, furthermore, the NE- and clonidine-induced decreases in POA PGE_2 levels were relatively similar, we speculate that pH could be more determinative of neuronal discharge activity (α_1-AR) than of astrocytic signal transduction (α_2-AR). Indeed, we found in an earlier study (60) that the intra-POA microdialysis of Levophed similarly abrogated the NE-induced changes in T_c but not those in PGE_2.

Discounting, then, the possible, confounding effect of the acidity of the NE solution and assuming that the observed responses to coadministered cirazoline and clonidine represent those of buffered NE, we conjecture that the hypothermic effect of endogenous NE released in the POA in response to a peripheral stimulus, in contrast to exogenous NE microdialyzed continuously into the POA, could be masked whereas its simultaneously activated hyperthermic effects could be manifested and occur in succession, i.e., the first (cirazoline) rapid in onset, α_1-AR mediated and PGE_2 independent, and the second (clonidine) delayed, α_2-AR mediated and COX-2/PGE_2 dependent. If existent, such a mechanism could be pertinent to a postulated mechanism of the febrile response to peripheral LPS that posits that the pyrogenic message may be transmitted to the brain by vagal afferents, ultimately arriving in the POA via ascending noradrenergic projections (6, 62, 73). To wit, the febrile response of guinea pigs to intravenous LPS is characteristically biphasic, and COX-2 plays a greater role in the late than in the early phase of the fever (64). If validated, this would implicate intra-POA NE pivotally in the modulation of the febrile response to LPS.

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V současné době existuje dostatek informací o tom, že zvýšená hladina PGE_2 v preoptické oblasti (POA) předního hypotalamu působí jako podstatný mediátor febrilní reakce (Blatteis, 1997; Ivanov and Romanovsky, 2004). Je obecně známé, že zvýšenou produkci tohoto PGE_2 katalyzuje inducibilní cyklooxygenáza COX-2. Diskutováno však je, jak dochází k jejímu zvýšení v POA. Předpokládají se různé mechanismy, včetně toho, že PGE_2 je tvořen a uvolňován z periferní cirkulace; (monocyty) nebo fixními buňkami (jaterní makrofágy) mononukleáry, nebo alternativně endoteliálními buňkami mozku, u nichž se předpokládá, že jsou aktivovány exogenními cirkulujícími pyrogeny - lipopolysacharidy (LPS) nebo pyrogenními cytokiny (interleukin IL-1β). PGE_2 prochází do mozku difuzí přes hematoencefalickou bariéru (BBB), nebo přes organum vasculosum laminae terminalis (OVLT) – tedy cirkumventrikulárním orgánem, jež nemá vyvinutou BBB (Banks et al., 1995; Blatteis et al., 2005; Blatteis and Sehic, 1997; Romanovsky et al., 2005). Tyto mechanismy jsou v podstatě založeny na humorálním transportu PGE_2 nebo jeho aktivačních faktorů (LPS, IL-1β) do mozku. Předpokládá se rovněž neurální mechanismus zodpovědný za vzestup hodnot PGE_2 v preoptické oblasti za febrilního stavu (Romanovsky et al., 1997; Sehic and Blatteis, 1996; Blatteis et al., 2000). Tato skutečnost je založena na představě, že periferní pyrogenní signál může být přenášen do medulla oblongata via nervi vagi a odtud přes ventrální adrenergní svazek do preoptické oblasti. Tato cesta je rychlejší než transport cirkulací a je velmi dobře zdokumentováno, že systémové podání exogenních a endogenních pyrogenů vyvolá promptní aktivaci noradrenergních zakončení v POA s následným uvolněním norepinephrinu (NE), (Lavicky and Dunn, 1995; Linthorst et al., 1995; Kendall et al., 2010). Je také dobře prokázáno, že NE indukuje tvorbu PGE_2 v mozkové tkáni in-vivo a in-vitro (Feleder et al., 2004; Hori et al., 1987; Blatteis, 1997; Malik and Sehic, 1990). Nově jsme popsali, že alfa-adrenergní agonista aplikovaný do POA morčete vyvolá

rozdílné zvýšení teploty (T_c). V jenom případě vzniká zvýšení teploty prostřednictvím aktivace α_1-receptoru, jež nesouvisí s uvolněním PGE_2, v druhém případě je zvýšení teploty spojeno s aktivací α_2-receptoru a COX-2 s následným zvýšením PGE_2 (Feleder et al., 2004). Intravenózní pyrogenní dávky LPS vyvolají u morčat rychle a charakteristicky dva následující vzestupy T_c, spojené se vzestupem PGE_2 (Quan and Blatteis, 1989).

Na základě těchto nálezů jsme zkoumali hypotézy: 1) zdali LPS febrilní reakce je zprostředkována preoptickým NE; 2) zda její dvě fáze jsou regulovány stejnou cestou.

Experimentální uspořádání pro potvrzení sledovaných hypotéz bylo analogické s předcházejícími pokusy. Za použití stejného animálního modelu byly provedeny celkem tři experimenty, stejným způsobem byly měřeny sledované hodnoty, stejné bylo i statistické zpracování dat a grafické vyjádření. Pro ověření definované hypotézy byly zvoleny tři experimentální modely: 1) Sledování efektu intravenózního (IV) podání LPS na hladiny norepinephrinu (NE) v preoptické oblasti pokusného zvířete; 2) Efekt α-antagonistů (prazosin, johimbin) v POA na hladiny PGE_2 po intravenózním podání LPS; 3) Efekt COX selektivních inhibitorů (selektivní COX-1 inhibitor SC-560, a selektivní COX-1 inhibitor acetaminophen, selektivní inhibitor COX-2 MK-0663) na hladiny PGE_2 v POA po intravenózním podání LPS.

Výsledky našeho experimentálního uspořádání potvrdily, že IV podání LPS trojnásobně zvýší bazální hodnoty NE v preoptické oblasti po 30 minutách po podání s návratem k normě během dvou následujících hodin. IV podaný LPS evokuje rychlou, dvoufázovu febrilní odpověď s nástupem první fáze 60 min po IV podání LPS a druhé fáze 90 min po první. Hladiny PGE_2 v preoptické oblasti korespondovaly s teplotními změnami. Návrat

k normě nastal cca po 300 minutách. Podaný prazosin výrazně zpozdil nástup a tempo nástupu teploty v první i druhé fázi febrilní odpovědi, a kromě toho značně oslabil nástup první i druhé febrilní odpovědi. Johimbin podaný do preoptické oblasti experimentálního zvířete neovlivnil průběh nástupu a výšku dosažené febrilní odpovědi, ale potlačil druhý vzestup horečnaté odpovědi tak, že teplota přetrvávala na původní hodnotě z první fáze beze změn až do konce experimentu. Ani prazosin ani johimbin nevykazoval tyto reakce u kontrolních zvířat, kterým byl podán apyrogenní roztok (PFS). Aplikace COX-1 selektivních inhibitorů (SC-560, acetaminophen) neovlivnila změny teplot v čase po podání LPS a hodnoty PGE_2 v preoptické oblasti, zatímco podání MK-0663 blokoval sekundární vzestup teploty (vyvolaný IV podáním LPS). Toto nemělo efekt na časnou odpověď této proměnné, nicméně došlo k depresi časného nástupu PGE_2 v preoptické oblasti a reverzi jeho pozdního vzestupu. Kontrolní podání PFS a DMSO ve stejném uspořádání a délce experimentu bylo bez výše popsaných efektů.

Výsledky výše popsaných selektivních blokád jednotlivých receptorů tak naznačují nové zjištění, že vzestup preoptických hladin PGE_2, který je spojen s druhou, nikoliv první fází LPS provokovanou febrilní odpovědí je COX-2 dependentní. Tuto skutečnost podporuje práce autorů Soriano et al., (2011), kteří LPS evokovanou febrilní odpověď redukovali exogenním ghrelinem, který snižoval hladiny PGE_2 v POA a práce autorů Soriano and Branco, (2010).

V souladu s předcházejícím zjištěním je i rychlý, s febrilní reakcí časově spojený, vzestup hladin NE v preoptické oblasti po IV. podání LPS, což odpovídá dosavadním představám o spojení termoregulace s centrálním noradrenergním systémem. Lze předpokládat, že α1-receptory aktivované NE jsou lokalizovány na post-synaptických teplotně senzitivních (nesenzitivních) neuronech v preoptické oblasti a jsou pod vlivem tohoto hormonu. Tento dřívější předpoklad je nyní již skutečností, což potvrzují práce dalších autorů (Imberi et al., 2008; Osaka, 2009; Rather et al.,

2008). Stimulace centrálních i periferních neuronů indukuje postsynaptické uvolnění PGE_2, které následně blokuje presynaptické uvolnění NE, což zpětně reguluje aktivitu noradrenergních neuronů (Hedqvist, 1977; Malik and Sehic, 1990). Norepinephrin, uvolňovaný v preoptické oblasti na základě vagem zprostředkované odpovědi na tvorbu PGE_2, vyvolává charakteristický bifázický vzestup teploty po IV podání LPS. Prezentovaná práce potvrdila že: význam NE v preoptické oblasti je v této reakci zásadní, nicméně mechanismus této regulace může být dvojí: 1) rychlý vzestup T_c aktivací α_1-AR bez účasti PGE_2, nebo 2) vývoj druhého teplotního vzestupu simultánní stimulací α_2-receptorů s tvorbou a uvolněním COX-2 dependentní PGE_2 v preoptické oblasti.

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Preoptic norepinephrine mediates the febrile response of guinea pigs to lipopolysaccharide

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Feleder C, Perlik V, Blatteis CM. Preoptic norepinephrine mediates the febrile response of guinea pigs to lipopolysaccharide. *Am J Physiol Regul Integr Comp Physiol* 293: R1135–R1143, 2007. First published June 20, 2007; doi:10.1152/ajpregu.00067.2007.—Norepinephrine (NE) microdialyzed in the preoptic area (POA) raises core temperature (T_c) via *1*) α_1-adrenoceptors (AR), quickly and independently of POA PGE_2, and *2*) α_2-AR, after a delay and PGE_2 dependently. Since systemic lipopolysaccharide (LPS) activates the central noradrenergic system, we investigated whether preoptic NE mediates LPS fever. We injected LPS (2 μg/kg iv) in guinea pigs prepared with intra-POA microdialysis probes and determined POA cerebrospinal (CSF) NE levels. We similarly microdialyzed prazosin (α_1 blocker, 1 μg/μl), yohimbine (α_2 blocker, 1 μg/μl), SC-560 [cyclooxygenase (COX)-1 blocker, 5 μg/μl], acetaminophen (presumptive COX-1v blocker, 5 μg/μl), or MK-0663 (COX-2 blocker, 0.5 μg/μl) in other animals before intravenous LPS and measured CSF PGE_2. All of the agents were perfused at 2 μg/min for 6 h. T_c was monitored constantly. POA NE peaked within 30 min after LPS and then returned to baseline over the next 90 min. T_c increased within 12 min to a first peak at ~60 min and to a second at ~150 min and then declined over the following 2.5 h. POA PGE_2 followed a concurrent course. Prazosin pretreatment eliminated the first T_c rise but not the second; PGE_2 rose normally. Yohimbine pretreatment did not affect the first T_c rise, which continued unchanged for 6 h; the second rise, however, was absent, and PGE_2 levels did not increase. SC-560 and acetaminophen did not alter the LPS-induced PGE_2 and T_c rises; MK-0663 prevented both the late PGE_2 and T_c rises. These results confirm that POA NE is pivotal in the development of LPS fever.

prostaglandin E_2; cyclooxygenase inhibitors; noradrenergic receptor antagonists; pyrogen signaling; body temperature

ABUNDANT EVIDENCE has implicated PGE_2, elevated by a systemic pyrogenic challenge in the preoptic area (POA) of the anterior hypothalamus (the fever-producing locus), as an essential mediator of the febrile response (for reviews, see Refs. 5 and 36). It is generally agreed that the increased production of this PGE_2 is catalyzed by inducible cyclooxygenase (COX)-2. How its level comes to rise in the POA is, however, a debated issue. Various mechanisms have been suggested, including that PGE_2 is produced and released peripherally by circulating (e.g., monocytes) or fixed (e.g., hepatic macrophages) mononuclear phagocytes, or, alternatively, by cerebral microvascular endothelial cells; these are presumed to be activated by the circulating exogenous pyrogen [e.g., lipopolysaccharide (LPS)] or by pyrogenic cytokines [e.g., interleukin (IL)-1β] induced by it. PGE_2 is then postulated to pass into the brain by diffusion, by transport through the blood-brain barrier (BBB), or through the organum vasculosum laminae terminalis (OVLT), a circumventricular organ in which the BBB is deficient (for reviews, see Refs. 2, 7, 9, 57). These mechanisms, thus, are predicated on the humoral transport of PGE_2 or its activating factor, i.e., LPS or IL-1β, to the brain.

A neural mechanism to account for the elevation of preoptic PGE_2 under febrile conditions has also been proposed (60, 63; for review, see Ref. 10). It is based on evidence suggesting that the peripheral pyrogenic signal could be transmitted centripetally via the vagus nerves to the medulla oblongata, thence via the ventral noradrenergic bundle to the POA. This pathway is quicker than circulatory transport, and it is well documented that the systemic administration of exogenous and endogenous pyrogens provokes the prompt activation of noradrenergic terminals in the POA and the consequent release of norepinephrine (NE; 39, 42; for reviews, see Refs. 20 and 43). It is also well established that NE induces the synthesis of PGE_2 in brain tissue in vitro and in vivo (23, 31; for reviews, see Refs. 5 and 45).

We reported recently that α-adrenoceptor (AR) agonists microdialyzed in the POA of conscious guinea pigs evoke two differentially modulated core temperature (T_c) rises, viz., one occurring very promptly and involving the activation of α_1-AR but with no associated PGE_2 release and the other occurring significantly later and involving the activation of α_2-AR, the participation of, specifically, COX-2, and the consequent production of PGE_2 (23). In guinea pigs, pyrogenic doses of intravenously injected LPS rapidly and characteristically evoke two successive T_c rises associated with concurrent PGE_2 rises (64). Based on these findings, we investigated whether LPS fever could be mediated by preoptic NE and its two phases regulated in the same way.

MATERIALS AND METHODS

Animals

Male, pathogen-free, Hartley guinea pigs (301–350 g body wt on arrival; Charles River Laboratories, Wilmington, MA) were used in these experiments. The animals were quarantined for 1 wk, three to a cage, before any experimental use. Tap water and food (Agway Prolab guinea pig diet) were available ad libitum except during the experiments. The ambient temperature (T_a) in the animal room was 23 ± 1°C, the housing T_a recommended by the Institute of Laboratory Animal Resources Commission on Life Sciences (32); light and darkness alternated, with light on from 0600 to 1800. After quarantine, to moderate the psychological stress associated with the experiments, the animals were trained for 1 wk daily for 4 h to the experimental procedures by handling and placement in individual, locally fabricated, semicircular wire mesh confiners designed to prevent their turning around and to minimize their forward and backward

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movements, but without causing excessive restraint stress; rodents readily adapt to such confinement and show no signs of discomfort or anxiety (53, 59). All animal protocols were approved by our institutional Animal Care and Use Committee and fully conform to the standards established by the United States Animal Welfare Act and by the American Physiological Society documents entitled *Guiding Principles for Research Involving Animals and Human Beings* (1).

General

All glassware, plasticware, instruments, and cannulas used in these studies were sterilized by autoclaving. Electrochemical-grade, high-purity water (Baxter Healthcare, Muskegan, MI) was used exclusively in the preparation of all the solutions. Before use, the stock solutions were passed through a sterile 0.22-μm Miller-GS filter unit (Millipore, Bedford, MA) as an added precaution against bacterial contamination. Absence of endotoxic contamination in all fluids not containing LPS by design were verified by the Limulus amebocyte lysate test (Pyrochrome; Associates of Cape Cod, Falmouth, MA).

Drugs. LPS was *Salmonella enteritidis* LPS B (batch no. 651628; Difco Laboratories, Detroit, MI), the same LPS batch we have used in all our previous studies; it was suspended in pyrogen-free saline (PFS, 0.9% NaCl, USP; Abbott Laboratories, Chicago, IL). Heparin was purchased from Elkins-Sinn (Cherry Hill, NJ) and dissolved in PFS. PFS was also the control drug for these solutions.

The microdialysis perfusate (and vehicle for all the drugs administered ic) was artificial cerebrospinal fluid for guinea pigs (aCSF; final concentration in mM: 140.0 NaCl, 2.7 KCl, 1.0 $MgCl_2 \cdot 2H_2O$, 1.2 $CaCl_2 \cdot 2H_2O$, 2.0 Na_2HPO_4; osmolality: 290 mosmol/kgH_2O; pH 7.4, adjusted with 85% H_3PO_4); it was prepared freshly each day and prewarmed to ~38°C for administration. Prazosin hydrochloride (catalog no. P7791, an α_1-AR antagonist; 1 μg/μl of aCSF) and yohimbine hydrochloride (catalog no. Y3125, an α_2-AR antagonist; 1 μg/μl of aCSF) were prepared just before use and stored in amber glass vials at room temperature. The selective COX-1 inhibitor SC-560 (catalog no. S2064; 5 μg/μl of aCSF), the selective COX-2 inhibitor MK-0663 (0.5 μg/μl of aCSF), the COX-1 splice variant COX-1v presumptively selective antagonist acetaminophen, USP (catalog no. A5000; 0.5 μg/μl of aCSF), and their solvent DMSO (6% in aCSF) were similarly prepared freshly for each use. These drug concentrations replicate those in our previous study (23). MK-0663 was generously donated by Merck (Rahway, NJ); DMSO (catalog no. 081-4) was purchased from Burdick and Jackson (Muskegon, MI). All the other drugs were purchased from Sigma-Aldrich (St. Louis, MO).

Surgical procedures. All animals received the antibiotic chloramphenicol (20 mg/kg body wt sc) 1 h before surgery and one time a day for the following 2 days. Immediately after surgery, the animals also received a subcutaneous bolus, 10 ml PFS injection and pain medication (butorphanol, 0.05 mg/kg body wt). All procedures were performed under ketamine-xylazine (35–5 mg/kg body wt im) anesthesia and aseptic conditions. Experiments were performed 7 days after the last of the following two surgical procedures, when the animals had recovered; 7 days also separated the two surgeries. Retraining was performed during the latter 4 days of this recovery period.

Jugular vein cannulation. In preparation for intravenous injections, a siliconized cannula (ID 0.020 in., OD 0.037 in.; Baxter Healthcare, McGraw Park, IL), prefilled with heparinized (10 IU/ml) PFS, was inserted in the left jugular vein and gently guided in the superior vena cava of each guinea pig. The free end of the cannula was passed subcutaneously toward the head, exteriorized on the top of the head, knotted, and rolled into a coil. This coil was then placed inside a protective polypropylene shield (a centrifuge microtube with a screw-cap, with its cone cut off) that was fixed to the skull with dental acrylic cement and four self-tapping, miniature stainless steel screws. The neck wound was sutured and cleansed with 10% povidone-iodine solution and treated with nitrofurazone powder. To maintain the patency of the inserted cannulas, these were flushed with 0.5 ml of heparinized (3 IU/ml) PFS every day after surgery until 3 days before an experiment, when PFS alone was used because of the inhibitory effect of heparin on complement activation (72); complement component 5a is a critical mediator of the febrile response of guinea pigs and mice to LPS (8).

Microdialysis. A sterile, 17-mm-long, 17-gauge, thin-walled stainless steel guide cannula with a tightly fitting indwelling stylet was implanted stereotaxically (Mechanical Developments, Trent H. Wells, Jr., South Gate, CA) in the left medial POA [anterior-posterior = 11.6 mm, lateral = 1.0 mm, ventral = −9.0 mm; according to the atlas of Luparello (44)] and fixed to the skull with four self-tapping, miniature, stainless steel screws and dental acrylic cement. Concentric microdialysis probes were constructed in our laboratory as previously described (23). Approximately 2 h before an experiment, the indwelling stylets of the guide cannulas were replaced by sterile microdialysis probes so that their dialysis membrane tips protruded exactly 1 mm beyond the guide cannulas. They were fixed to the skull with tissue adhesive and immediately perfused with sterile guinea pig aCSF via sterile 1-ml tuberculin syringes clamped to a syringe pump (model no. A-99; Razel Scientific Instruments, Stamford, CT) as the driver, for the duration of an experiment. To run six animals simultaneously, the pushers of two pumps were modified so that each could accommodate three syringes at a time. During the first 90 min after insertion of the probes [thermal stabilization period (see below)], the flow rate of the perfusion was adjusted to 4, 3, and 2 μl/min at 30-min intervals; it was maintained at the latter rate for a second 90-min period (the pretreatment control period) and also for the following experimental period. The effluents from the microdialysis probes were collected in vials chilled on ice over 30-min intervals continuously throughout the experiments from 90 min before to up to 360 min after a treatment (*time 0* = initiation of treatment) and analyzed later for their NE or PGE_2 content.

After an experiment, the guinea pigs were killed by isoflurane overdose, and the brains were quickly removed and stored in 10% phosphate-buffered Formalin for later histological verification of the placement of the dialysis probe tips. Localization of the center of the dialysis probe within 0.5 mm of the medial POA was regarded as the correct placement. Only the data from confirmed preoptic placements of the probes are included in this report.

Physiological Measurements

Temperature recording. To obviate possible effects of circadian variations, all of the experiments were begun at the same time of day (0830). A 90-min thermal stabilization period preceded all the experimental treatments. After their last surgery (7 days), the guinea pigs, fully conscious, were loosely restrained in the individual wire mesh confiners to which they had been trained at T_a 23 ± 1°C. The T_c values of the animals were monitored constantly from the beginning of the stabilization period and recorded at 2-min intervals for the duration of an experiment on a Macintosh Plus 1-Mb microcomputer through an analog-to-digital converter, using precalibrated copper-constantan thermocouples inserted 5 cm in the colon. The data were displayed digitally on a monitor, printed on an ImageWriter printer, and stored on a diskette for later analysis.

Biochemical analyses. NE. The NE content of the samples was evaluated using HPLC with EC detection. Microdialysate effluents were collected in 1 μl of 5% perchloric acid on ice and stored at −20°C until analysis. For analysis, samples (10 μl) were injected using a CMA 200 refrigerated automatic sampler (CMA Microdialysis, North Chelmsford, MA) on a 150 × 3-mm ODS C_{18} column (ESA, Bedford, MA) perfused by BAS 200A HPLC pumps (BAS, West Lafayette, IN) at 0.25 ml/min with a mobile phase containing 80 mM sodium dihydrogen phosphate monohydrate, 2.0 mM 1-octanesulfonic acid sodium salt, 100 μl/l triethylamine, 5 nM EDTA, and 10% acetonitrile, pH 3.0. NE levels were determined by using an ESA

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Coulochem II 5200A electrochemical detector with an ESA 5041 high-sensitivity microbore analytical cell and an ESA 5020 guard cell. Electrochemical detection was performed at 220 mV and 1.0 nA with the guard cell at 350 mV. All samples were analyzed in triplicates. The limit of detection for NE was 0.5 pg/10 μl. The chromatographic data were collected and analyzed with a PowerChrom system (AD Instruments, Castle Hill, NSW, Australia) and expressed as picogram per milliliter of sample. Basal values were defined as the average NE levels of the three samples before PFS or LPS administration (pretreatment control period).

PGE_2. The PGE_2 content of the microdialysate samples was analyzed using a commercial PGE_2 enzyme immunoassay kit (High Sensitivity PGE_2 EIA Kit no. 931-001; Assay Designs, Ann Arbor, MI). The PG synthetase inhibitor indomethacin (10 μg/ml) was added to all the blood samples immediately after collection. All samples were diluted before analysis in the assay buffer system provided by the manufacturer, according to the manufacturer's instructions. All samples were analyzed in duplicates. The detection limit of this assay was 8.26 pg/ml of sample. The data were expressed as changes (ΔPGE_2) relative to their values over the 90 min before a treatment (P_0).

Experimental Design

Experiment 1: Effect of LPS injected intravenously on the level of NE in the POA. To verify in our model that NE is released in the POA in virtually immediate response to the peripheral administration of LPS, collection of microdialysate effluents was begun 60 min before, immediately after the 90-min stabilization period, and continued, in this instance, for 300 min after (*time 0*) the intravenous injection of PFS (0.6 ml/kg) or LPS (2 μg/kg in 0.6 ml of PFS). The guinea pigs were conscious throughout this experiment. The samples were analyzed for their NE content as described above.

Experiment 2: Effects of α-AR antagonists microdialyzed in the POA on the febrile and preoptic PGE_2 responses to LPS intravenously. To determine whether NE presumptively released promptly in the POA after the intravenous administration of LPS mediates the ensuing febrile and PGE_2 responses, prazosin or yohimbine was microdialyzed (each at 1 μg/μl of aCSF) immediately following the 90-min stabilization period in the POA of conscious guinea pigs for 1 h, beginning 30 min before the intravenous injection of PFS or LPS. This design was chosen to replicate the conditions of our previous study (23). T_c values and preoptic PGE_2 levels were measured from 90 min before until 360 min after PFS or LPS injection, as described earlier.

Experiment 3: Effects of COX inhibitors microdialyzed in the POA on the febrile and preoptic PGE_2 responses to LPS intravenously. To identify the COX isozyme responsible for the rise in preoptic PGE_2 during the two characteristic rising phases of the febrile response of guinea pigs to intravenous LPS, the selective COX-1 inhibitor SC-560 (5 μg/μl), the selective COX-1v inhibitor acetaminophen [0.5 μg/μl; COX-1v (putative COX-3) has been implicated as a putative central fever mediator (11, 12, 16)], or the selective COX-2 inhibitor MK-0663 (0.5 μg/μl) was microdialyzed immediately following the 90-min stabilization period in the POA of conscious guinea pigs and continued for the duration of the experiment; the solvent of these inhibitors was 6% DMSO in aCSF. PFS or LPS was injected intravenously at *time 0*. T_c values and preoptic PGE_2 levels were determined as described previously.

Statistical Methods

The results are presented as means ± SE. The values of T_c are reported as changes (ΔT_c, in °C) from basal values [initial T_c (T_{ci}), the T_c at 2-min intervals averaged over the last 10 min of the preceding 90-min stabilization period] plotted at 6-min intervals. Latencies of fever onset were defined as the intervals (in minutes) between the time of LPS injection (0 min) and that of the first T_c rise >0.2°C (the SD of the mean T_{ci}) that continued uninterruptedly beyond 0.5°C. The NE data are expressed as their absolute values (in pg/ml) and the PGE_2 data as changes (ΔPGE_2, in pg/ml) relative to the mean ± SD of their three values before a treatment. A repeated-measures ANOVA was used to compare all changes between groups; factor 1 was the between-groups factor (the treatment) and factor 2 the within-subjects factor (the different sampling periods), followed, if significant differences were found, by a point-by-point Tukey-Kramer multiple-comparison test. The analyses were performed using Instat 3 (Graph Pad Software; Instant Biostatistics, San Diego, CA). Each variable was considered to be independent. The 5% level of probability was accepted as statistically significant.

RESULTS

Experiment 1

The intravenous injection of LPS produced a tripling of the basal level of NE in the POA interstitial fluid of the conscious guinea pigs, measured 30 min after this treatment (Fig. 1). NE then gradually returned toward its initial value over the following 2 h. The level of preoptic NE was not affected by the intravenous injection of PFS.

Experiment 2

Intravenous LPS produced its characteristic, prompt, biphasic fever; the onset latency was 11 ± 0.4 min (mean ± SD; Fig. 2*A*). The first febrile peak of ~1.4°C occurred at ca. 60 min after LPS administration, and the second of ~1.7°C ca. 90 min after the first. The return to T_{ci} was gradual, but essentially completed by ca. 300 min after LPS injection. Preoptic PGE_2 levels increased significantly in these conscious guinea pigs in

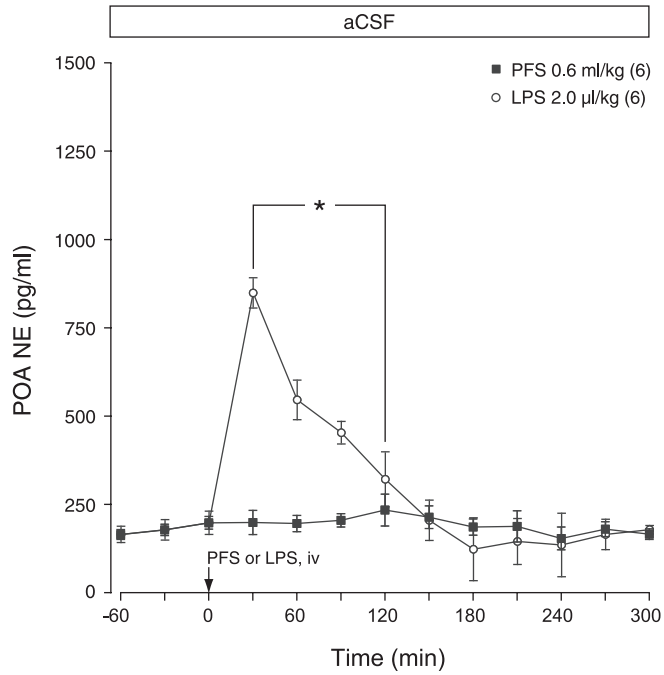

Fig. 1. Content of norepinephrine (NE) in microdialysate effluents collected over 6 h at 30-min intervals from the POA of conscious guinea pigs that received pyrogen-free saline (PFS, 0.6 ml/kg) or lipopolysaccharide (LPS, 2 μg/kg in PFS) iv at *time 0 min* (the end of a preceding 90-min stabilization period). aCSF, artificial cerebrospinal fluid. Values are means ± SE. *$P <$ 0.05 relative to PFS treatment.

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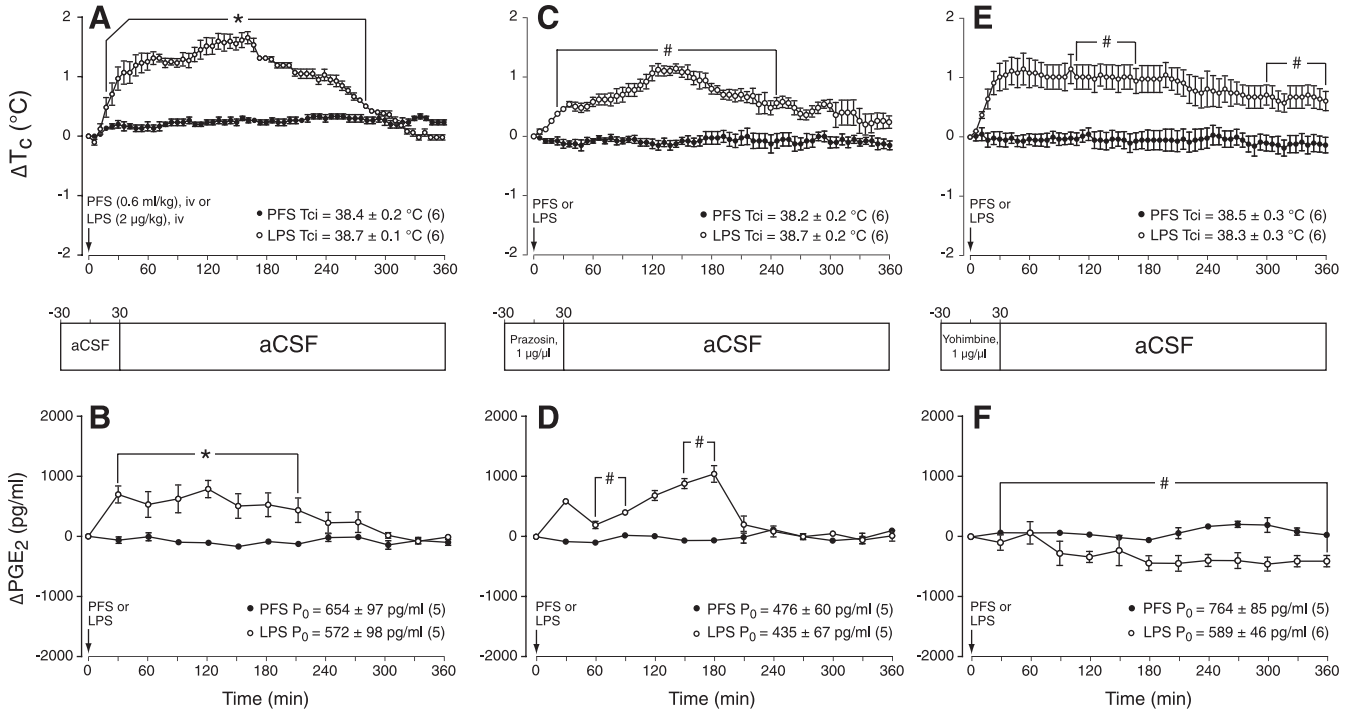

Fig. 2. Changes in the core temperature (T_c) and preoptic PGE_2 levels of conscious guinea pigs that received PFS or LPS iv at *time 0 min* (the end of a preceding 90-min stabilization period, *A* and *B*) and the α_1-AR antagonist prazosin (1 μg/μl of aCSF, *C* and *D*) or the α_2-AR antagonist yohimbine (1 μg/μl of aCSF, *E* and *F*) by continuous microdialysis (2 μl/min) for 6 h in the POA. The antagonist treatments began 30 min before the PFS or LPS injection and lasted 1 h. Values are differences (Δ) relative to their initial levels [T_{ci} or values over the 90 min before a treatment (P_0)] and are expressed as means ± SE. $*P < 0.05$ relative to the corresponding PFS treatments (*A–F*). $\#P < 0.05$ relative to LPS treatment (*C* and *E* or *D* and *F* vs. *A* and *B*, respectively).

close temporal correspondence with their T_c rise (Fig. 2*B*). Intravenous PFS had no effect on either of these variables.

The intra-POA microdialysis of prazosin significantly delayed the onset and slowed the rate of development of the first T_c rise; the onset latency was 19 ± 0.5 min ($P < 0.05$). It also greatly attenuated the height of the first and significantly reduced the maximum of the second febrile rises. Thus the first T_c rise increased only ~0.5°C, stabilized for ca. 30 min, and then resumed its rise to a second peak of ~1.1°C at ca. 150 min (Fig. 2*C*). T_c then gradually decreased. Prazosin microdialyzed in the POA, however, did not block the initial LPS-induced preoptic PGE_2 rise, which continued to its peak at 180 min; but it depressed its level at 60 min and augmented it at 180 min post-LPS (Fig. 2*D*) by comparison with its contour in the LPS only-treated counterparts (Fig. 2*B*).

Intra-POA-microdialyzed yohimbine did not affect the slope and height of the first febrile rise, but it suppressed the second rise so that the fever continued at its initial, lower, first-phase level uninterruptedly through the end of the experiment (Fig. 2*E*). However, strikingly, it totally suppressed the normally associated first and second POA PGE_2 rises (Fig. 2*F*).

Neither intra-POA prazosin nor yohimbine demonstrably affected the T_c and preoptic PGE_2 levels of the PFS intravenously treated controls (Fig. 2, *A–F*).

Experiment 3

Neither the microdialysis of SC-560 nor of acetaminophen altered the prototypical, temporally correlated effects of intravenous LPS on T_c and POA PGE_2 levels (Fig. 3, *A–F*). MK-0663, on the other hand, blocked the second rise of T_c normally evoked by intravenous LPS; it had, however, no effect on the early response of this variable (Fig. 3*G*). However, it depressed the early LPS-induced rise of preoptic PGE_2 and reversed its late rise (Fig. 3*H*). Neither PFS injected intravenously nor aCSF or DMSO, the vehicle of these COX inhibitors, microdialyzed intra-POA per se over the same duration, affected the T_c or the preoptic PGE_2 levels of the conscious guinea pigs (data not shown).

DISCUSSION

The present results verify that the biphasic fever characteristically evoked in conscious guinea pigs by the intravenous injection of a moderate, subseptic dose of LPS is attended by coincident increases in preoptic PGE_2 levels, as reported previously (64). They further show that this challenge rapidly provokes the release of NE in the POA of these animals and, hence, would support the notion that preoptic NE may play a pivotal role in febrigenesis. Thus α_1-AR antagonism by intra-POA prazosin greatly slowed the development and reduced the height of both the early and late phases of the febrile response to intravenous LPS, but without reducing the normally associated preoptic PGE_2 rises. α_2-AR antagonism by yohimbine, on the other hand, affected neither the development nor the height of the first febrile rise, but it prevented both the development of the second T_c rise and the preoptic PGE_2 rises associated with both intravenous LPS-induced febrile phases. Consequently, remarkably, fever was initiated and maintained under the latter conditions at its original height throughout the experiment in the total absence of a corresponding increase in the level of POA PGE_2. These findings would infer, therefore, that intra-

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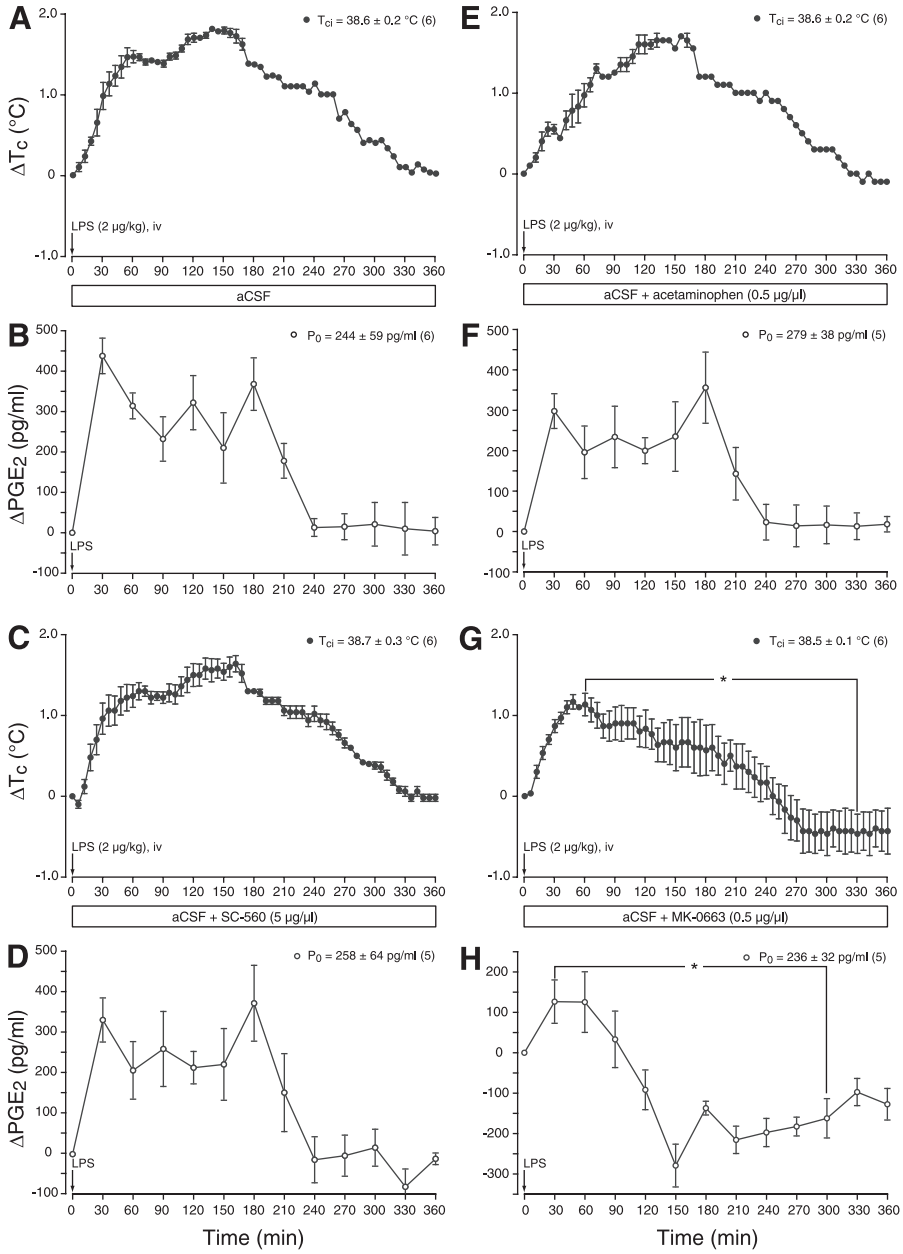

Fig. 3. Changes in the T_c and preoptic PGE_2 levels of conscious guinea pigs that received PFS or LPS (2 μg/kg in PFS) iv (*A* and *B*) and the cyclooxygenase (COX)-1 inhibitor SC-560 (5 μg/μl, *C* and *D*), the COX-1v inhibitor acetaminophen (0.5 μg/μl, *E* and *F*), or the COX-2 inhibitor MK-0663 (0.5 μg/μl, *G* and *H*) by continuous microdialysis for 6 h in the POA; the solvent of these inhibitors was 6% DMSO in aCSF. The COX inhibitor treatments began coincidentally with the PFS or LPS injection and continued for the duration of the experiments. Values are differences (Δ) relative to their initial levels and are expressed as means ± SE. PFS (in lieu of LPS), aCSF alone, or aCSF + 6% DMSO had no demonstrable effect on these variables (data not shown). $^*P < 0.05$ relative to no COX inhibitor treatment (*A* and *B*). Please note that the scale of the *y*-axis of *H* is different from those of *B*, *D*, and *F*.

POA PGE_2 is not essential for the manifestation of the second phase of intravenous LPS fever. The present results indicate, moreover, that, such as it exists, only the rise in preoptic PGE_2 that is associated with the second, but not with the first, phase of LPS fever is COX-2 dependent. This is also a novel finding, at variance with the conventional view (for reviews, see Refs. 5 and 36). Neither COX-1 nor COX-1v appeared to influence T_c or the production of POA PGE_2 during either phase.

The rapid elevation in the concentration of NE in the preoptic dialysate observed in this study following the intravenous injection of LPS and its temporal association with the early phase of fever are in agreement with previous findings in rats and mice treated with intraperitoneal LPS (18, 39, 42, 43, 73, 74). Indeed, it is well established that the peripheral administration of exogenous and endogenous pyrogens stimulates the increased metabolism of NE, especially in the hypothalamus (for reviews, see Refs. 20 and 43). It is also generally accepted that, notwithstanding certain species and other experimental variations regarding the up or down direction of its thermal effect, the central noradrenergic system is involved in thermoregulation (for review, see Ref. 75). In guinea pigs, electrical stimulation of the ascending noradrenergic system in the brain stem (67, 68) or NE microinjected in the POA (55) evokes T_c rises. Recently, we (23) reported that NE microdialyzed in the POA of conscious guinea pigs mediates, in fact, not one but two distinct T_c rises, each associated with the activation of a different α-AR class. Thus one rise is rapid in onset and α_1-AR-mediated, and the other is delayed, appearing significantly later than the former, and is α_2-AR mediated; the specific involvement of both AR classes was verified by the blockade of their thermal effect by their respective, selective antagonists. We also found in the same study that the early T_c

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rise provoked by intra-POA microdialyzed NE occurred without any demonstrable change in the level of PGE_2 in the POA, whereas the later rise was associated with a concurrent elevation of this level. Again, the noninvolvement of PGE_2 in the α_1-AR-induced T_c rise and, in contrast, the involvement of COX-2-dependent PGE_2 in that caused by α_2-AR activation were confirmed by their respective responses to selective COX-1 and COX-2 inhibitors (23). Also, in a parallel study (6), we found that the thermal responses of conscious wild-type *Cox-1* and *Cox-2* gene-deleted mice to the intracerebroventricular microinjection of α_1- and α_2-AR agonists corresponded exactly with those observed in these guinea pigs. In the present study, the biphasic febrile response of the conscious guinea pigs to intravenous LPS was associated with NE released in the POA and evidently developed in the same order as the two successive T_c rises induced by intra-POA microdialyzed NE per se, that is, the present first rise of T_c was induced by α_1-AR stimulation and was not associated with PGE_2 formation, whereas the second elevation of T_c was mediated by α_2-AR stimulation and associated with the delayed production of COX-2-dependent PGE_2. We suggest, therefore, that noradrenergic terminals in the POA, through their NE-induced, presumptively contemporaneous activation of both α-ARs, mediated the observed febrile response to LPS.

The present finding that prazosin attenuated the initial febrile rise, i.e., that α_1-AR stimulation mediated it without the intermediation of PGE_2, suggests that the α_1-ARs activated by NE are located on postsynaptic warm-sensitive or thermoinsensitive neurons in the POA and that NE directly reduces or augments, respectively, the activities of these neurons. According to the classical model of Hammel (29), both responses promote heat conservation. In support, the direct inhibition and excitation by cirazoline, an α_1-AR agonist, of the firing rates of, respectively, warm-sensitive and thermoinsensitive neurons have recently been reported in rat POA slice preparations (33). Because these neurons, moreover, are thought to inhibit synaptically connected cold-sensitive neurons, these are concomitantly facilitated, stimulating heat production, i.e., in combination, these effector mechanisms raise T_c. The specific α_1-AR subtype involved in this hyperthermic effect remains to be identified.

The findings that the second febrile rise and the associated increase of preoptic PGE_2 were both inhibited by yohimbine and the selective COX-2 inhibitor MK-0663 indicate that these subsequent, characteristic features of the febrile response to intravenous LPS were specifically mediated by α_2-AR-activated COX-2-dependent PGE_2, in agreement with the demonstrated actions of intra-POA microdialyzed clonidine, an α_2-AR agonist, and NE per se (23). It is significant in this regard that, under the present experimental conditions, the second peak of fever was ~0.5°C higher than the first and that this was also the extent of the fever attenuated by yohimbine (Figs. 2, *A* and *E*). The brain cell type expressing COX-2 in response to NE in these guinea pigs remains to be determined. Because in rat brain COX-2 mRNA becomes detectable in astrocytes, microglia, perivascular cells, and cerebromicrovascular endothelial cells, but only irregularly in neurons, ca. 1 h after the intravenous administration of LPS and pyrogenic cytokines (13, 15, 22, 70), hence after the onset of fever (latency ca. 10 min), we conjecture that the increased PGE_2 in the POA of the present guinea pigs was generated by astrocytic processes contacting noradrenergic synaptic regions. The delay imposed by its synthetic process probably accounts for the interval between α_2-AR stimulation and the appearance of PGE_2. The identity of the α_2-AR subtype involved in this effect also remains to be elucidated. The subsequent effect of the thus released PGE_2 on the activities of POA warm-sensitive and thermoinsensitive neurons is presumptively similar to that indicated earlier for α_1-AR-mediated responses. The PGE_2-sensitive receptor involved in these neuronal effects is probably the EP_3 subtype; it has been linked to the development of fever and is present in the POA (21, 69; for review, see Ref. 52). The receptor implicated in the inhibition of presynaptic NE release by PGE_2 has also been previously identified as the EP_3 subtype (62).

The notion that NE and PGE_2 may interact in the POA in fever production is not new, albeit that the mechanism of their cooperation in this function is in dispute. Thus it has been suggested that the hyperthermic action of NE may be exerted through PGE_2 because the T_c rise induced in cats by intracerebroventricularly microinjected NE is inhibited by pretreatment with aspirin (50). A similar involvement of NE was described originally for the PGE_2-mediated release of luteinizing hormone-releasing hormone (51). However, the converse has also been suggested, that PGE_2 may be thermogenic through NE because the destruction by 6-hydroxydopamine of the noradrenergic nerve terminals in the POA of rabbits attenuated the rise in T_c produced by intravenous LPS (38, 41). On the other hand, no mutual interaction between NE and PGE_2 was found in monkeys (49). The present data would indicate that, in guinea pigs, PGE_2 is the thermogenic agent during the second phase of intravenous LPS fever, produced consequent to the activation of α_2-ARs by NE released in the POA. The in vitro release of PGE_2 by NE-stimulated brain tissue and the rapidly increased production of PGE_2 by the microdialysis of NE in the POA of conscious guinea pigs have been demonstrated previously (31, 65). It is, in fact, well documented both in the peripheral and central nervous systems that the stimulation of noradrenergic neurons induces the postsynaptic release of PGE_2, which then limits the further presynaptic release of NE, thereby modulating the activity of noradrenergic neurons (30, 45).

As already noted, the increase in preoptic NE that attended the febrile response of the present guinea pigs to intravenous LPS and, previously, also that of other species to intraperitoneal LPS and IL-1β was coincident with its early rather than its late phase. This would suggest that the fever-triggering message evoked in the periphery that signals its release in the POA was very quickly conveyed centripetally. In view of the rapidity of this transmission, we deduce that it was conveyed neurally. Indeed, ample data have implicated the vagus, especially its hepatic branch afferents, as the carrier of peripheral pyrogenic signals to the brain stem (60, 63; for reviews, see Refs. 19 and 61) and identified PGE_2 released by Kupffer cells stimulated by LPS-activated complement component 5a as this fever signal (40, 54; for reviews, see Refs. 7, 8, 36, 57). Other data have corroborated that signals can proceed from the brain stem to the POA by way of noradrenergic projections originating in the A1 and A2 regions of the medulla oblongata (25) and arriving in the POA via the ventral noradrenergic bundle (27, 29, 71). Furthermore, subdiaphragmatic vagotomy blocks

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intraperitoneal IL-1β-induced hypothalamic NE activation in rats (26, 34, 73) and mice (74).

From these and other well-substantiated findings that the rises in T_c and preoptic PGE_2 levels induced by peripherally administered pyrogens are prevented by the intra-POA administration of low doses of COX-2 inhibitors, it would seem highly likely that the PGE_2 collected in the POA in the present experiments was generated inside rather than outside the BBB. Indeed, the capacity of brain tissue to generate PGE_2 is well established (for reviews, see Refs. 5 and 66). Moreover, PGE_2 infused in the internal carotid artery of conscious guinea pigs could not be detected in the POA and caused a fall rather than a rise of T_c (64). It follows from these results, therefore, that, since its formation was consequent to its induction by locally released NE, the signal that evoked it was, in the first instance, the very same as that which initially evoked the release of NE in the POA, that is, it was a nervous signal transmitted from the liver via the vagus. This contradicts an alternative view, that the fever-producing PGE_2 that acts in the POA originates in the periphery and is transported to it by the bloodstream; it, being lipophilic, then either diffuses across the BBB (47, 58) or enters the POA through the leaky OVLT (56). However, direct evidence that blood-borne PGE_2 passes in the brain and, especially, that PGE_2 derived from the blood in this way raises T_c is controversial (48, 64). Indeed, because PGE_2 is generally considered to be a paracrine rather than an endocrine mediator and, as an organic anion at physiological pH, enters cells poorly by simple diffusion, it would seem improbable a priori that the very rapid T_c rise in response to intravenous LPS should depend on its blood-borne transport from peripheral sources to the POA and ready passage across the BBB. In support, the in vivo expression in rat hypothalami of prostaglandin transporter, a principal carrier of PGs from the blood in cerebral endothelial cells, is not affected by intraperitoneal LPS challenge (37). Moreover, PGE_2-inactivating enzymes are scarce in the hypothalamus and not upregulated in response to peripheral LPS (35; for review, see Ref. 36) so that, to mitigate its central effects, PGE_2 is usually transported from the brain into the blood (4, 17). An alternative pathway of signal transduction from the periphery to the brain, that the fever-mediating PGE_2 is produced by the cerebral endothelial cells themselves (for reviews, see Refs. 46 and 57), although based on well-substantiated evidence that circulating LPS and pyrogenic cytokines induced by it upregulate the expression of COX-2 in cerebral endothelial cells (14, 15), is attended by the difficulty that the hypothalamic expression of multidrug resistance-associated protein 4, which actively transports PGs out of cells, also is not affected by LPS (37).

In view of the delay imposed by its synthetic process, the prompt elevation of PGE_2 in the POA during the first phase of fever can therefore not be attributed to its COX-2-mediated formation. The possibility that COX-1 or COX-1v mediated this PGE_2 production is negated by the present findings that neither SC-560 nor acetaminophen microdialyzed in the POA prevented the development of fever. The noninvolvement of COX-1 and COX-1v in fever production has also been shown by other workers (for review, see Ref. 7). The involvement of COX-2-dependent PGE_2 is similarly excluded since the intra-POA microdialysis of MK-0663 did not inhibit the first febrile rise and since, moreover, it is in any case, as demonstrated herein, released in the POA in correlation with the second phase of fever. It is possible, therefore, that the early increase in preoptic PGE_2 was mediated by a COX-independent mechanism, possibly by the nonenzymatic isoprostane pathway of free radical-catalyzed peroxidation of arachidonic acid to 8-iso-PGE_2 (for review, see Ref. 3). The free radicals in this case could be generated by the auto-oxidation of NE and/or be nitric oxide; the latter is also released locally in the POA after LPS administration (24). The finding that this PGE_2, however, was not integral to the initiation of the febrile response nor, indeed, to its continuation is novel and intriguing as regards LPS-induced fever, although other fevers caused by certain cytokines have been reported previously to occur independently of central PGE_2 (see Ref. 24).

In conclusion, these results are consistent with the view that NE released in the POA in response to the vagally conveyed pyrogenic message of PGE_2 generated by complement component 5a-stimulated Kupffer cells mediates the characteristic biphasic T_c rises evoked in conscious guinea pigs by the present moderate dose (2 μg/kg body wt) of intravenous LPS. The following two, successive mechanisms of action are hypothesized: *1*) rapid induction of the first T_c rise by activation of α_1-ARs without the mediation of PGE_2 and *2*) subsequent development of the second T_c rise by simultaneous stimulation of α_2-ARs, consequently activating (after the delay imposed by the de novo synthesis of the relevant enzymes) the production and release of COX-2/mPGES-1-dependent PGE_2 in the POA. It should be emphasized that the present conclusions concern specifically the initiating, not the sustaining, sequence of the febrile response of guinea pigs to intravenous LPS. Thus they do not contradict the participation in the late phase of this response of any of the various humoral pyrogen signaling pathways that have been proposed for this and other species.

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5. Závěr

Tělesná teplota představuje jeden ze základních fyziologických faktorů, které u živočichů prošly dlouhým fylogenetickým vývojem, během něhož byla teplota těla zpočátku výrazně ovlivňována prostředím (poikilotermní organismy), později se vytvořila významná teplotní nezávislost živočichů na prostředí (homoiotermní organismy). Výsledkem tohoto vývoje je schopnost u některých živočichů, včetně člověka udržovat relativně stálou hodnotu teploty organismu, na úrovni, která je definována jako tzv. bod tepelné rovnováhy. Tohoto stavu se dociluje díky permanentně probíhající termoregulaci, procesu, jenž umožňuje udržovat teplotu teplokrevných živočichů v požadovaném rozmezí prostřednictvím souhry řady specializovaných buněk, které tvoří vlastní architekturu celého termoregulačního systému. Termoregulační systém je tvořen termoreceptory, aferentními dráhami, termoregulačním centrem v hypotalamické části CNS, eferentními dráhami a efektorovými orgány. Tyto jednotky v souhře humorálních, neurohumorálních a nervových regulací vytvářejí podmínky pro efektivní termoregulaci. Na základě souhry periferie a centra je tak zajištěna nejenom neustálá detekce teplotního stavu periferie a jádra, ale souběžně i rovnováha mezi tvorbou nebo ztrátou tepla v organismu. Je logické, že termoregulace podléhá změnám, tedy teplota těla může být zvýšená, či naopak snížená, a to v důsledku mnoha faktorů. Teplotní změny pak vyvolají změny metabolismu organismu, respektive ovlivňují jeho imunitní systém. Typickou reakcí tohoto typu je febrilní reakce – horečka. Horečka jako významný rys řady, především infekčních nemocí, byla předmětem pozornosti již od starověku. Febrilní reakce je řízena centrální nervovou soustavou přes endokrinní, neurologické, imunologické a behaviorální mechanismy. Na rozdíl od jiných typů změn tělesné teploty, horečku doprovázejí různé poruchy chování, metabolické změny, změnay funkcí systémů a změny v

imunitní odpovědi. Horečka a febrilní reakce významně přispívají k patogenezi, klinickému obrazu a průběhu mnoha nemocí a chorob. Na rozdíl od hypertermie znamená horečka regulovaný vzestup teploty vyvolaný exogenními či endogenními pyrogeny syntetizovanými jako reakce na infekční případně jiné onemocnění, přičemž organismus po zvýšení teploty udržuje teplotu tělesného jádra na takto nově nastavené úrovni do doby, než signál v podobě endogenního či exogenního pyrogenu je přítomen. Mezinárodní unie (International Union of Physiological Sciences Commission for Thermal Physiology - IUPS Thermal Commission) v roce 2001 definovala horečku jako stav zvýšené teploty jádra, která je často, ale ne nezbytně, součástí obranné reakce mnohobuněčných organismů vůči invazi mikroorganismů nebo neživé hmoty rozpoznané hostitelem jako patogenní. Studium horečnaté reakce a možností jejich farmakologického ovlivnění je i v současné době předmětem zájmu mnoha badatelských týmů.

Vytyčený cíl této práce jsme postupně plnili v jednotlivých experimentech shrnutých v této práci tak, abychom přispěli k hlubšímu porozumění patofyziologie endotoxinem vyvolané horečky. Hlavní zaměření experimentální části akcentovalo periferní rozpoznání endotoxinu s následným zpracováním imunitního signálu v termoregulačním centru – preoptické oblasti hypotalamu. Základní hypotézou pro vznik horečky bylo po dlouhou dobu generování endogenních pyrogenů (IL-1 beta, IL-6, TNF alfa) v reakci na exogenní stimulus. Základní pilíř léčby horečky představuje mimo jiné její symptomatická léčba pomocí nesteroidních antirevmatik, jejichž mechanismus účinku je spojen s více či méně selektivní inhibicí cyklooxygenáz, enzymů esenciálních pro tvorbu prostaglandinu E_2. Z tohoto důvodu jsme se zaměřili na jeho roli v celém procesu vzniku a regulace horečky, a to zejména ve spojení se třemi základními oblastmi:

Význam PGE_2 Kupfferových buněk a makrofágů při periferní signalizaci endotoxinem mediované horečky; význam sleziny v regulaci horečky a centrální mediace signálu v preoptické oblasti hypotalamu

V našich experimentech sledujících periferní signalizaci po stimulaci LPS jsme prokázali asociaci inciálního vzestupu tvorby prostaglandinu E_2 s časovou sousledností zvýšení tělesné teploty po intravenózním podání LPS, čímž se nabízí alternativní koncept k cytokiny mediovanému přenosu imunitního signálu. Z našich dat vyplývá, že tento iniciální nárůst hladin prostaglandinu E_2 se uskutečňuje prostřednictvím imuno-neuro humorální odpovědi zprostředkované komplementem, zatímco nárůst hladin TNFalfa, IL-1beta a IL-6 ve srovnání s prostaglandinem E_2 nastupuje po léčbě lipopolysacharidem a CVF později, přibližně za 30-45 minut, a není tak bezprostředně asociován s iniciálním vzestupem horečky po podání LPS. Zjistili jsme, že aplikace LPS a kobřího jedu spouští okamžitou produkci prostaglandinu E_2 během prvních 5 minut po jejich aplikaci. Zatímco zvýšené hladiny PGE_2 se stabilizují po 15 minutách a přetrvávají zvýšené po léčbě lipopolysacharidem po celou dobu trvání experimentu (60min), zvýšené hladiny prostaglandinu E_2 způsobené aplikací CVF se vrátí ke svým hodnotám před léčbou po 15 minutách. Aplikace lipopolysacharidu 3 hodiny po předcházející léčbě kobřím jedem způsobila zpoždění odpovědi tvorby prostaglandinu E_2 vlivem vyčerpání komplementu předcházejícím působením kobřího jedu. Novým poznatkem e rozlišení rychlé, komplement dependentní, a pomalé, pravděpodobně na cytokinech závislé produkci prostaglandinu E_2 iniciované podáním lipopolysacharidu, která byla již dříve postulována.

V navazující práci jsme pak asociovali produkci prostaglandinu dominantně s Kupfferovými buňkami. Výsledky této práce ukázaly, že současná elevace PGE_2 spojená s nárůstem teploty tělesného jádra po intravenózním podání LPS je narušena u skupiny zvířat předléčených gadoliniem, tedy u skupiny s deplecí Kupfferových buněk. Došlo také k

významnému zpomalení clearance LPS cirkulujícího v systémovém oběhu. Na druhou stranu produkce cytokinů na léčbu samotným gadoliniem nebyla nikterak ovlivněna s výjimkou TNF-α, jehož hladiny byly dvojnásobné u skupiny zvířat předléčených gadoliniem a následně stimulovaných intravenózním podáním lipopolysacharidu. Můžeme tedy uzavřít, že v souladu s ostatními autory, se nám podařilo in vivo potvrdit předchozí pozorování asociace časného nárůstu plasmatických hladin PGE_2 a vzniku horečky vyvolané LPS. Jako buněčný typ zodpovědný za produkci PGE_2 jsme identifikovali Kupfferovy buňky a zároveň jsme prokázali disociaci horečnaté odpovědi a up-regulace produkce tzv. endogenních cytokinů.

Vzhledem k dříve postulovanému regulačnímu vlivu sleziny jsme se zaměřili na popis její role v kaskádě horečky experimentálně navozené podáním LPS. Zjistili jsme, že splenektomie vede k augmentaci odpovědi, tedy ke vzniku horečky u nízké dávky, která nezpůsobila horečku u kontrolní skupiny zvířat a k vyššímu vzestupu teploty po podání LPS proti příslušným kontrolám. Tento nárůst teploty u splenektomovaných zvířat proti pozitivním kontrolám byl nezávislý na cestě podání a době experimentu (7 vs 30 dní) po provedené splenektomii. Dále jsme zjistili, že se změnila kinetika vychytávání LPS Kupfferovými buňkami. Zatímco splenektomie neovlivnila první výskyt LPS v Kupfferových buňkách, u splenektomovaných zvířat byl zjištěn intenzivnější signál LPS a prodloužila se doba, po kterou bylo LPS v Kupfferových buňkách detekovatelné. Toto pozorování bylo nezávislé na cestě podání a době experimentu po splenektomii. V návaznosti na uvedený experiment se splenektovanými zvířaty jsme identifikovali, že se jedná o aktivní regulační proces, a to prostřednictvím reprodukce efektu augmentace horečnaté odpovědi po splenektomii u zvířat s podvazem splenické žíly. Aktivní role sleziny pak byla ověřena podáním „splenického extraktu“ získaného homogenizací a následnou centrifugací sleziny ze zvířat předléčených LPS v intervalech 5,

15 a 30 min po léčbě. Podání „splenického extraktu vedlo k oslabení zvýšené febrilní reakce na intraperitonálně podané LPS u splenektovaných zvířat a to až na úroveň odpovědi pozitivní kontrolní skupiny. Při bližší identifikaci látky zodpovědné za regulační funkci sleziny můžeme uzavřít, že jsme pozorovali větší atenuaci febrilní odpovědi u morčat po podvazu splenické žíly po zpětném podání splenického extraktu obsahujícím lipidickou složku. V souladu s naším zjištěním, že slezina aktivně moduluje LPS navozenou horečku, Steiner et al. postulovali, že tento vliv může být mediován prostřednictvím COX-1 spojeného s prostaglandin D synthasou a takto generovaného PGD_2 (Steiner et al., 2009). Aplikace PGD_2 byla totiž v minulosti asociována s hypotermií (Ueno et al., 1982) a navíc výšená hladina této syntázy byla právě dříve pozorována ve slezině (Jowsey et al., 2001).

V části centrální mediace pyrogenního signálu jsme se zaměřili na propojení role prostaglandinu E_2, norepinefrinu a aktivace noradrenergní signalizace. Naše experimenty ukazují, že α_1- a α_2-agonista AR aplikovaný do preoptické oblasti CNS morčete evokuje zřetelnou teplotní odpověď. $Alfa_1$-agonista produkuje rychlou, PGE_2 nezávislou teplotní odpověď, zatímco α_2-AR stimulace působí časný propad teploty a pozdní COX-2/PGE_2 dependentní teplotní vzestup. Tyto reakce jsou v nervovém systému indukovány presynaptickým působením norepinephrinu prostřednictvím α-receptorů s následnou modulací izoenzymu COX-2. V navazující práci jsme ověřili zapojení LPS stimulace na výše uvedenou adrenergní signalizaci. V souladu s předchozími pozorováními jsme zjistili, že IV. podání LPS trojnásobně zvýší bazální hodnoty NE v preoptické oblasti, která koreluje s typickým dvoufázovým průběhem LPS navozené horečky po jeho intravenosním podání. Dále jsme zjistili, že hladiny PGE_2 v preoptické oblasti přímo korelují s průběhem teplotních změn. Prazosin, α_1- AR antagonista, výrazně zpozdil nástup a snížil nárůst teploty v první i druhé fázi febrilní odpovědi, což nebylo asociováno s poklesem PGE_2.

Johimbin (α_2- AR antagonista) podaný do preoptické oblasti experimentálního zvířete neovlivnil průběh nástupu a výšku dosažené febrilní odpovědi, ale potlačil druhý vzestup horečnaté odpovědi tak, že teplota přetrvávala na původní hodnotě z první fáze beze změn až do konce experimentu. Aplikace COX-1 selektivních inhibitorů (SC-560, acetaminophen) neovlivnila ani hodnoty PGE_2 v preoptické oblasti ani změny teplot v čase po podání LPS. Na druhou stranu podání MK-0663 (COX-2 selektivního inhibitoru) blokoval sekundární vzestup teploty a nárůst hladin PGE_2 v preoptické oblasti vyvolaný IV podáním LPS. Obě práce sledující centrální signalizaci potvrdily význam NE v preoptické oblasti a asociovaly jeho efekt s rychlým vzestup T_c aktivací α_1-AR bez účasti PGE_2 a dále s vývojem druhého teplotního vzestupu simultánní stimulací α_2-receptorů s tvorbou a uvolněním COX-2 dependentní PGE_2 v preoptické oblasti.

V souladu s cílem práce tak prezentované výsledky přispěly k ozřejmení periferní a centrální signalizace LPS navozené horečky v experimentálním modelu u morčat.

6. Seznam zkratek

ADP	adenosin difosfát
A-cholin	acetylcholin
AITC	alylisothiokyanát
AMP	adenosin monofosfát
AP	area postrema
AR	α_1-adrenoceptor
ATB	antibiotika
ATP	adenosin trifosfát
AVA	arteriovenózní anastomosy
AVP	arginin vasopresin
BAT	brown adipose tissue
cAMP	cyklický adenosin monofosfát
C5a	komplement 5a
CD	cluster of differentiation receptor
CNS	centrální nervový systém
COX-1	cyklooxygenáza 1
COX-2	cyklooxygenáza 2
CVF	cobra venom factor (kobří jed)
CVOs	Circumventrikulární orgány
DMH	dorzomediální hypotalamus
EP_3	prostaglandinový receptor E
FITC-LPS	fluoresceinem označený lipopolysacharid
G+, G-	gramm positivní, negativní
GIT	gastrointestinální trakt
H^+	vodíkový proton

IL	interleukin
IML	intermediolaterální sloupec spinální míchy
INF-α	interferon - alfa
INF-β	interferon - beta
INF-γ	interferon - gama
LPS	lipopolysacharidy
MF	makrofág
MIP-1	macrophage-inflammatory protein-1
mPGES-1	mikrosomalní prostaglandin E synthasa-1
NE	norepinephrin
NO	oxid dusnatý
NTS	nucleus tractus solitarius
OVLT	cévní orgán laminae terminalis
P	preganglilové neurony
PAMPS	patogen associated molecular patterns
PFPF	předem pyrogenní faktory
PGD_2	prostaglandin D_2
PGE_2	prostaglandinu E_2
PH	zadní hypotalamus
POA	preoptické oblasti předního hypotalamu
RPA	raphé /peripyramidální oblast medully
SFO	subfornikální orgán
SG	sympatická ganglia
T_c	teplota jádra
TLR	toll like receptors
TNF	tumor necrosis factor
TRP	transient receptor potential

TRPA	transient receptor potential ankyrin
TRPM	transient receptor potential melastatin
TRPV	transient receptor potential vanilloid
UCP-1,2,3	rozpojovací protein-1,2,3
UCPs	uncoupling proteiny (rozpojovací proteiny)
α-MSH	α-melanocyty stimulující hormon

7. Souhrn

Termoregulace představuje jeden ze základních fyziologických faktorů umožňující udržovat stálou teplotu homoiotermních organismů, a tedy i nezávislost jejich aktivity na teplotě prostředí. Horečka je pak jednou z forem takto realizované termoregulace, a to většinou jako reakce na různé patofyziologické podněty. Cílem naší práce bylo přespět k objasnění patofyziologie a periferní a centrální signalizace prostřednictvím experimentálního modelu LPS (lipopolysacharid) navozené horečky u morčat. Podařilo se nám prokázat asociaci iniciálního vzestupu tvorby prostaglandinu E_2 (PGE_2) s časovou souslednosti zvýšení tělesné teploty po intravenózním podání LPS, jeho asociaci s komplementem a disociaci od plasmatických hladin endogenních pyrogenů jako TNF-alfa, IL-1beta a IL-6. Dále jsme identifikovali Kupfferovy buňky jako perifení zdroj PGE_2 produkce a primárního rozpoznání LPS u našeho experimentálního modelu. V rámci periferní signalizace LPS mediované horečky jsme identifikovali aktivní regulační funkci sleziny prostřednictvím splenického faktoru lipidického charakteru, při jehož nedostatku dochází k augmentaci febrilní odpovědi na LPS. V rámci centrální regulace horečky jsme potvrdili základní roli norepinefrinu, jehož koncentrace rostou v souladu s febrilní odpovědí na LPS. Prostřednictvím selektivních antagonistů α_1- a α_2-receptorů a COX-1 a COX-2 inhibitorů jsme identifikovali, že LPS podmíněný nárůst preoptických hladin norepinefrinu je spojen s rychlým vzestup teploty tělesného jádra prostřednictvím aktivace α_1-AR bez účasti PGE_2 a dále s vývojem druhého teplotního vzestupu prostřednictvím simultánní stimulace α_2-receptorů asociovanou s tvorbou a uvolněním COX-2 dependentní PGE_2.

8. Summary

Thermoregulation is one of the fundamental physiological factors allowing homeotherm organisms to maintain a constant temperature which leads to independence of their activity on the ambient temperature. Fever is one of the examples of thermoregulation usually asociated with different pathophysiological stimulations. The aim of our study was to contribute to the clarification of pathophysiology and peripheral and central signaling of LPS (lipopolysaccharide)-induced fever in an experimental model using guinea pigs. We have demonstrated an association of initial prostaglandin E_2 (PGE_2) rise with a time course of core body temperature elevation after intravenous administration of LPS, its association with complement cascade and dissociation from plasma levels of endogenous pyrogens such as TNF-alfa, IL-1beta and IL-6. Further we have identified Kupffer cells as the peripheral cell source of PGE_2 production and the primary site of LPS recognition in our experimental model. We have also identified an active regulatory role of spleen through splenic factor of lipidic nature associated with peripheral signaling of LPS-mediated fever, which depletion leads to augmentation of febrile response to LPS. In agreenment with previous evidence we have confirmed the essential role of norepinephrine for the central signaling of fever and association of its concentrations with the time course of the febrile response to LPS. With the help of selective α_1-and α_2-receptor antagonists and COX-1 and COX-2 inhibitors we have identified that LPS-mediated increase of preoptic norepinephrine concentrations has been associated with a rapid rise of body core temperature via activation of α_1-AR without the participation of PGE_2 and further with the secondary temperature elevation mediated by simultaneous stimulation of α_2-receptors associated with the COX-2 dependent production and release of PGE_2.

9. Přílohy

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