Szakmai Beszámoló

I. A PUMA-G receptor agyi vérkeringésben betöltött szerepével kapcsolatos vizsgálatok

Első feladatunk az agyi véráramlás-mérési technika egérre történő adaptálása volt. Mivel e területen szegényesek az irodalmi adatok, előzetes kísérleteket végeztünk a mérési rendszer optimalizálására. Ennek során a következő feladatokat oldottuk meg:

1.) *Megfelelő anesztézia-protokoll kiválasztása.* Pentobarbitál alkalmazásakor azt tapasztaltuk, hogy 70 μg/g ip. dózisban nem elegendő a műtéti beavatkozáshoz, nagyobb mennyiségben alkalmazva viszont gyakran okoz légzés-, illetve szív-megállást. Ketamin/xylazin anesztézia hatására jelentősen csökkent az egerek artériás vérnyomása, ami kifejezetten előnytelen az agyi vérkeringés vizsgálata szempontjából. Az izofluránnal létrehozott inhalációs narkózis során azt tapasztaltuk, hogy bár az anesztézia mélysége megfelelő, az általános vérkeringési paraméterek pedig stabilak voltak, az agyi erek hypercapniára adott válaszreakciója majdnem teljesen eltűnt, aminek oka az volt, hogy az anesztézia már önmagában egy nagyfokú, NO által közvetített agyi vazodilatációt okozott. Az uretánnak ilyen hatását nem tapasztaltuk, de 2 mg/g dózisban ez is kisebb mértékű vérnyomás-csökkenést okozott, alacsonyabb dózisban viszont nem csillapította kellőképpen a műtéti fájdalmat. Végül az alábbi protokollt találtuk optimálisnak és használtuk a további kísérletek során: műtét során 1,5 % izoflurán majd a mérések előtt 1,3 mg/g uretán iv. az izoflurán fokozatos elhagyása mellett.

2.) *Általános vérkeringési paraméterek (vérnyomás és szívfrekvencia) monitorozása, valamint intravénás anyagbeadás*. Mivel az egerek ereinek kanülálására alkalmas műanyag csövek nem vásárolhatók, kidolgoztuk, hogyan lehet PE50-es csövekből hőlégfúvó segítségével megfelelő vaszkuláris kanülöket készíteni.

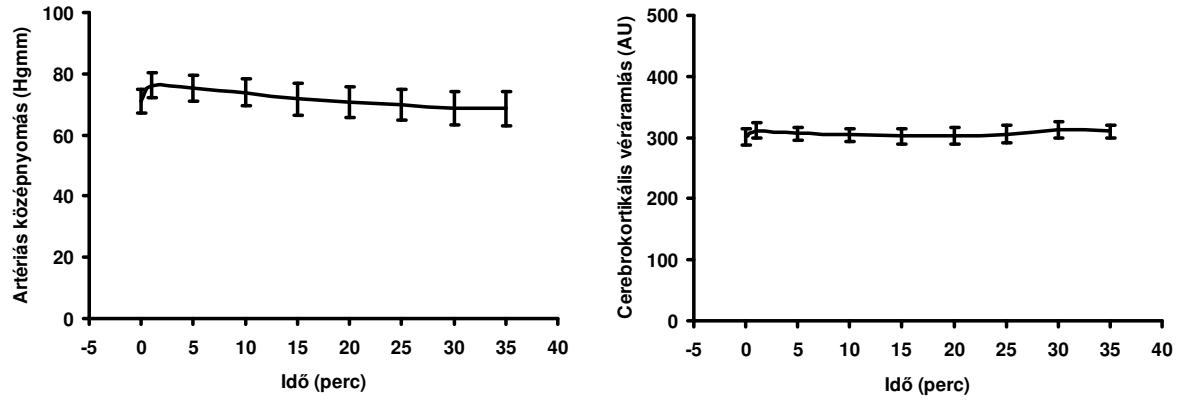
3.) *Hőmérséklet-szabályozás.* Azt tapasztaltuk, hogy az anesztézia a patkányokhoz képest az egerekben sokkal súlyosabb hőmérséklet-szabályozási defektust okoz, ezért beszereztünk és beüzemeltünk egy automatikus testhőmérséklet-szabályozó készüléket.

4.) *Agyi véráramlás-mérés.* Előkísérletekben kiválasztottuk a legalkalmasabb lézer-Doppler véráramlásmérő fejet az egerek parietális agykérgében történő mérésekhez. Az eredetileg patkányra tervezett sztereotaxikus készüléket adaptáltuk egérre.

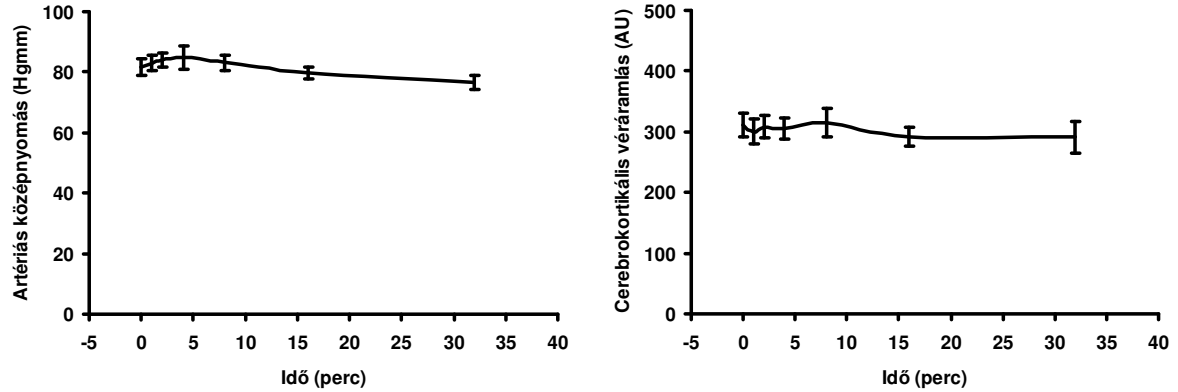
A fent említett metodológiai problémák megoldása elengedhetetlen feltétele volt annak, hogy kísérleteinket standardizált körülmények között, a légzési, a sav-bázis és a vérkeringési paraméterek fiziológiás tartományában végezzük.

Ezután megkezdtük a nikotinsav agyi vérkeringésre kifejtett hatásának és ebben a PUMA-G receptorok szerepének a vizsgálatát. Először azt teszteltük, hogy 20, 50 és 200 μg/g iv. dózisban alkalmazott nikotinsav hogyan befolyásolja az agykérgi véráramlást. Azt tapasztaltuk, hogy a nikotinsav iv. alkalmazva nem okozott szignifikáns változást sem a szisztémás vérkeringésben, sem pedig az agyi véráramlásban (1. ábra).

Feltételeztük, hogy az iv. nikotinsav hatástalanságának hátterében az állhat, hogy nem képes átjutni a vér-agy gáton. Ezért megismételtük kísérleteinket nikotinsav (0,1 és 1 μg/g) intracerebroventrikuláris (icv.) alkalmazásával is. Azt tapasztaltuk, hogy a nikotinsav icv. alkalmazva sem okozott szignifikáns változást sem a szisztémás vérkeringésben, sem pedig az agyi véráramlásban (2. ábra).

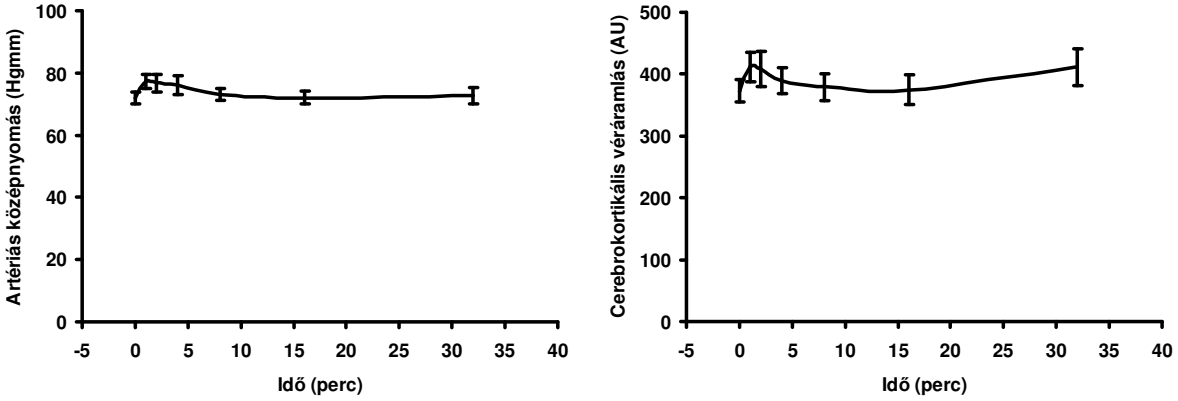


1. ábra. Az artériás középnyomás és az agykérgi véráramlás PUMA-G$^{+/+}$ egerekben nikotinsav (200 μg/g, iv.) adása előtt (0 min.) és után különböző időpontokban (n=7).



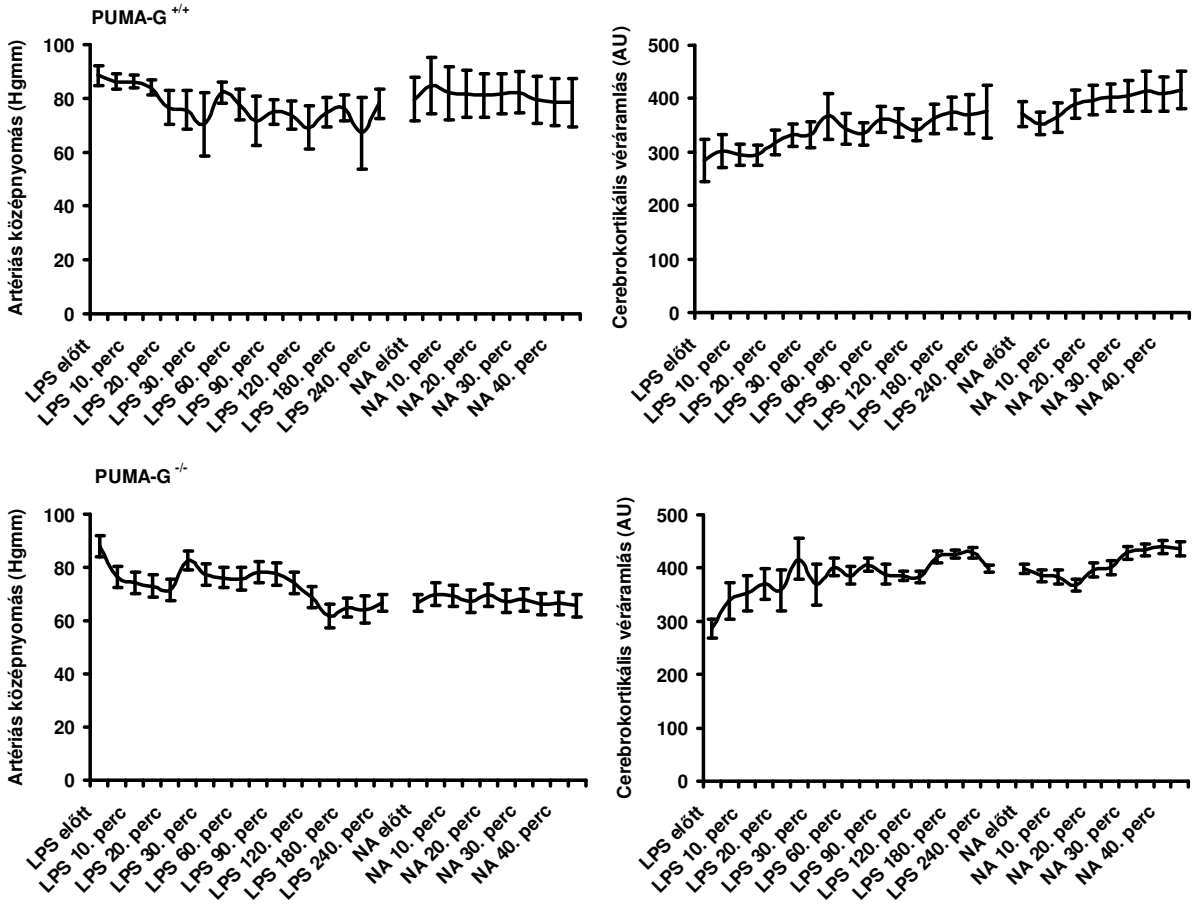
2. ábra. Az artériás középnyomás és az agykérgi véráramlás PUMA-G$^{+/+}$ egerekben nikotinsav (1 μg/g, icv.) adása előtt (0 min.) és után különböző időpontokban (n=10).

Ezután megvizsgáltuk, hogy ha a PUMA-G receptor expresszióját fokozzuk (a kísérlet előtt 24 órával ip. adott 100 ng E. coli lipopoliszachariddal), kialakul-e a nikotinsav hatására a szisztémás vagy agykérgi vérkeringés bármilyen változása. Azt tapasztaltuk, hogy 200 μg/g iv. nikotinsav ilyen körülmények között alkalmazva sem okozott szignifikáns változást a szisztémás, ill. agykérgi vérkeringésben (3. ábra).



3. ábra. Az artériás középnyomás és az agykérgi véráramlás lipopoliszachariddal előkezelt PUMA-G$^{+/+}$ egerekben nikotinsav (200 μg/g, iv.) adása előtt (0 min.) és után különböző időpontokban (n=11).

Végül azt vizsgáltuk, hogy a nagy dózisban (1 μg/g) icv. adott lipopoliszacharid (LPS) hatására kialakuló agyi hyperemia kialakulásában szerepe van-e a PUMA-G receptornak. Az LPS adását követően 240 percig folyamatosan regisztráltuk a vérnyomást és agykérgi véráramlást, majd 200 μg/g nikotinsavat adtunk iv. és méréseinket további 35 percen keresztül folytattuk. LPS hatására a vérnyomás enyhe csökkenése ellenére az agykérgi véráramlás fokozódását tapasztaltuk, mely tovább folytatódott a nikotinsav adása után is. A vérkeringési paraméterek változásai azonban nem különböztek PUMA-G$^{-/-}$ állatokban a PUMA-G$^{+/+}$ kontrollokban tapasztaltakhoz képest (4. ábra).



4. ábra. Az artériás középnyomás és az agykérgi véráramlás PUMA-G$^{+/+}$ (felső ábrák, n=6) és PUMA-G$^{-/-}$ (alsó ábrák, n=4) egerekben lipopoliszacharid (1 μg/g) majd nikotinsav (200 μg/g, iv.) adása előtt és után.

Eredményeink összességében arra utaltak, hogy a PUMA-G receptor nem játszik szignifikáns szerepet az agykérgi véráramlás szabályozásában sem élettani sem pedig kórélettani körülmények között.

II. A PUMA-G receptor bőrben játszott szerepével kapcsolatos vizsgálatok

Az eredeti munkatervben szereplő kísérleteken kívül vizsgálatokat végeztünk a nikotinsav bőr-véráramlásra gyakorolt hatásával kapcsolatban is. Ennek célja a véráramlás-fokozódást közvetítő sejtek azonosítása volt. Korábbi munkáinkban igazoltuk, hogy a PUMA-G receptor kifejeződik a bőrben található immunsejtekben és ezek közül valamelyik sejttípus közvetítheti a nikotinsav hatására kialakuló vazodilatációt. Leírtuk továbbá, hogy a hízósejtek nem játszanak szerepet a folyamatban.

Újabb vizsgálatainkra azáltal nyílt lehetőség, hogy az utóbbi években létrehoztak olyan egértörzseket, melyekben a humán diftéria-toxin receptor expresszálódik sejttípus-specifikus promoterek (CD11b, CD11c illetve Langerin) irányításával. Diftéria toxin szisztémás adásával a receptort expresszáló sejtek (makrofágok, dendritikus sejtek illetve epidermális Langerhans-sejtek) depletálhatók. Megállapítottuk, hogy sem a makrofágok sem pedig a dendritikus sejtek deplécióját nem befolyásolja a nikotinsav hatására kialakuló véráramlás-fokozódást (flush reakciót) a bőrben. Ezzel szemben a Langerhans-sejtek deplécióját majdnem teljesen eltüntette a nikotinsav okozta flush-t, de nem befolyásolta a prosztaglandin D_2 (PGD_2) véráramlás-fokozó hatását. PUMA-$G^{+/+}$ Langerhans sejtekben nikotinsav hatására fokozódott az intracelluláris Ca^{2+} koncentráció, mely hatás elmaradt PUMA $G^{-/-}$ sejtekben. Végezetül igazoltuk, hogy az epidermális Langerhans-sejtekben expresszálódnak a flush-reakció közvetítéséért felelős PGD_2-t és PGE_2-t szintetizáló enzimek.

Eredményeink hozzásegítenek a nikotinsav-kezelés legkellemetlenebb mellékhatását közvetítő folyamatok feltárásához, egyúttal pedig felhívják a figyelmet a Langerhans-sejtek eddig ismeretlen élettani/kórélettani szerepére a bőr véráramlásának szabályozásában.

Ismert, hogy a nikotinsav zsírsejteken kifejtett antilipolitikus hatását a PUMA-G receptor közvetíti. Mivel a sebocyták is igen komplex lipidszintetikus tevékenységgel jellemezhetőek, megvizsgáltuk, hogy a nikotinsav miként befolyásolja ezen sejtek faggyútermelését.

Transzgenikus riporter-egerek segítségével, valamint in situ hibridizációval igazoltuk a PUMA-G receptort kódoló gén expresszióját a bőr faggyúmirigyeiben és a szebózus fityma-mirigyben. Vad típusú (PUMA-$G^{+/+}$) felnőtt hím egerek ivóvízébe 6 héten keresztül 0,3 % nikotinsavat adagolva azt tapasztaltuk, hogy a fityma-mirigy triglicerid-tartalma szignifikánsan ($p=0,012$) lecsökkent (0,28±0,06 %) a kontrollokéhoz (0,70±0,11 %) viszonyítva. Ezzel szemben PUMA-$G^{-/-}$ egerekben hasonló nikotinsav-kezelést követően a triglicerid-tartalom (0,91±0,12 %) nem változott meg a kezeletlen állatokban mért szinthez (0,79±0,14 %) képest. Eredményeink szerint tehát a nikotinsav a PUMA-G receptor által közvetített mechanizmussal csökkenti a sebocyták zsírtartalmát egérben, ami felveti a PUMA-G receptor agonisták alkalmazásának lehetőségét az acne és seborrhea kezelésére.

III. Az agyi vérkeringés szabályozásával kapcsolatos vizsgálatok

Endocannabinoidok cerebrovaszkuláris hatásainak vizsgálata

Megvizsgáltuk, hogy miként befolyásolják az endogén cannabinoid vegyületek az agykéreg véráramlását fiziológiás körülmények között, valamint kombinált hypoxia/hypercapnia (H/H) során. Altatott felnőtt hím Wistar patkányokban az agykérgi véráramlást (CBF) a parietális kéregben laser-Doppler módszerrel mértük. Kísérleteink első szakaszában az endocannabinoidok felvételét gátló AM404 (10 mg/kg iv.) hatását vizsgáltuk a nyugalmi CBF-re. Kísérleteink második szakaszában azt vizsgáltuk, hogy a CB1-receptor antagonista AM251 miként befolyásolja az 5%O_2-20%CO_2-75%N_2 gázkeverékkel kiváltott CBF-emelkedést.

AM404 hatására egy átmeneti CBF-fokozódást tapasztaltunk. Az áramlásfokozódással egyidejűleg csökkent az állatok légzésszáma, fokozódott az artériás CO_2 és csökkent az O_2 tenzió, arra utalva, hogy az AM404 elsődleges hatása légzésdepresszió volt. Ezután megvizsgáltuk az AM251 hatását a mesterségesen kiváltott H/H CBF-fokozó hatására. A CBF-emelkedést mind 3, mind pedig 10 mg/kg iv. AM251 szignifikánsan fokozta, míg a vehikulum hatástalannak bizonyult.

Eredményeink arra utalnak, hogy hypoxia és hypercapnia során az endocannabinoid felszabadulás véráramlás csökkenést okoz, aminek hátterében az excitatorikus glutamáterg neurotranszmisszió preszinaptikus gátlását feltételezzük.

Az endogén szénmonoxid cerebrovaszkuláris hatásainak vizsgálata

Az endogén szénmonoxid (CO) hemből képződik a hemoxigenáz (HO) enzimek által katalizált reakció során. Kísérleteinkben a CO-nak az agyi erek tónusára és az agyszövet perfúziójára kifejtett hatásait vizsgáltuk. Az általában is igen magas HO aktivitást mutató agyszöveten belül a hypothalamusban kifejezetten nagymértékű az enzim expressziója, ezért először e régióban végeztük vizsgálatainkat. A CO közvetlen hatásai mellett a nitrogén monoxid szintetáz (NOS)- és és ciklooxigenáz (COX)-reakcióutakkal való kölcsönhatásaira is próbáltunk fényt deríteni.

Igazoltuk, hogy a konstitutív endogén CO képződés egyrészt tónusosan gátolja a hypothalamikus NOS aktivitást, másrészt pedig stimulálja a PGE_2–felszabadulást és e két úton keresztül indirekt módon képes befolyásolni a hypothalamus véráramlását. Nyugalmi körülmények között e két hatás egyensúlyban van, azonban NO-hiányos állapotban a PGE_2 által közvetített vazodilatátor hatás fokozottan érvényesül és hozzájárul a hypothalamus véráramlásának fenntartásához.

Más agyi régiókban, pl. a parietális agykéregben az endogén CO NOS-t gátló hatása dominál és így véráramlás-csökkenést okoz. Leírtuk továbbá, hogy a más kutatócsoportok által korábban újszülött malacok vizsgálatával nyert kísérleti adatokkal ellentétben a HO–CO reakcióút felnőtt patkányokban nem járul hozzá szignifikánsan a hipoxia-, ill. hiperkapnia-okozta agykérgi véráramlás-fokozódáshoz.

Szfingolipid mdiátorok szerepe az a. carotis tónusának szabályozásában

További kísérleteket végeztünk a szfingolipid mediátorok hatásaival kapcsolatban az arteria carotis válaszkészségére. A szfingomielinből szfingomielináz (SM) által katalizált reakció során képződő ceramid és a belőle szintetizálódó szfingolipid mediátorok az utóbbi évek kutatásai szerint számos élettani és kórélettani folyamatban játszanak fontos szerepet. Nemrégiben fokozott SM aktivitást írtak le endotoxémiás és krónikus szívelégtelenségben szenvedő betegek vérében és felmerült ennek kóroki szerepe az e betegségekben megfigyelhető ér-diszfunkció kialakulásában. Kísérleteink célja kettős volt: egyrészt a neutrális SM (nSM) hatásának vizsgálata az arteria carotis válaszkészségére, másrészt pedig a reaktív oxigén-szabadgyökök (ROS) szerepének tisztázása e hatás közvetítésében. Vad típusú (WT), szuperoxid dizmutázt túlexpresszáló transzgenikus (SODtg) és a NAD(P)H oxidáz 2-es izoformáját nem expresszáló NOX2ko egerekből izolált arteria carotis gyűrűk válaszkészségét határoztuk meg izometriás körülmények között miográffal. Az érfal sejtjeinek ROS termelését dihidroethidinnel történő festéssel detektáltuk.

Ceramiddal történő inkubáció az érfal valamennyi rétegében fokozott ROS felszabadulást okozott, és ez a hatás a simaizomban volt a legkifejezettebb. A különböző genotípusú erek nyugalmi kontrakciós válaszai nem különböztek egymástól, azonban az acetilkolin (ACh) által kiváltott endothelium-függő relaxáció szignifikánsan erősebb volt a SODtg erekben jelezve, hogy a nyugalmi ROS-termelés konstitutívan gátolhatja az NO-függő érválaszokat. Mind a WT mind pedig NOX2ko erekben nSM csökkentette a fenilefrin kontrakciós és az ACh relaxációs hatását. A SODtg erekben elmaradt az nSM-okozta kontrakció-csökkenés, azonban az ACh relaxációs hatása gyengébb volt nSM jelenlétében. Eredményeink alapján azt a következtetést vontuk le, hogy az nSM ROS által közvetített, de NOX2-től független módon csökkenti az érsimaizom kontrakciós válaszait és ezáltal hozzájárulhat az adrenerg vazokonstriktorokkal szembeni rezisztencia kialakulásához szeptikus sokk késői fázisában.

Mellékletek

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Hypersensitivity to Thromboxane Receptor Mediated Cerebral Vasomotion and CBF Oscillations during NO-deficiency in Rats

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Dedicated to the memories of Eörs Dóra (1943-1992) and Arisztid G. B. Kovách (1920-1996).

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ABSTRACT

Background: Low frequency (4-12 cpm) spontaneous fluctuations of the cerebrovascular tone (vasomotion) and oscillations of the cerebral blood flow (CBF) have been reported in several diseases associated with endothelial dysfunction. Since endothelium-derived nitric oxide (NO) suppresses platelet aggregation as well as vascular release of thromboxane A_2, NO-deficiency is often associated with activation of thromboxane receptors (TP). In the present study we hypothesized that in the absence of NO, hypersensitivity of the TP-receptor mediated cerebrovascular signaling pathway promotes vasomotion.

Methodology/Principal findings: In isolated rat middle cerebral arteries the TP-receptor agonist U-46619 in a concentration of 100 nM, which was without any effect under physiological conditions, induced sustained vasomotion after inhibition of the NO synthesis. Similarly, intravenous administration of 1 μg/kg U-46619 to anesthetized rats failed to induce any changes of the systemic or cerebral circulatory parameters under physiological conditions but aggravated CBF-oscillations after NO-blockade.

Conclusion/Significance: These results indicate that hypersensitivity of the TP-receptor mediated signaling pathway is a pathogenic factor in the development of cerebral high-amplitude vasomotion which may propagate to vasospasm in pathophysiological states associated with NO-deficiency.

INTRODUCTION

Low frequency (4-12 cpm) fluctuations of the cerebral oxygen availability in mammals were reported for the first time by Davis and Bronk in 1957 [1] and confirmed later in primates including humans (for review see: [2]). These fluctuations are termed "spontaneous" to indicate their independence of cardiac or respiratory cycles. In 1981 Dóra and Kovách [3] showed similar oscillations in cerebrocortical NADH fluorescence which lagged by 2 sec behind the cortical vascular volume suggesting that the vascular event initiated the metabolic one. The concept that the low frequency metabolic oscillations of the brain are induced by variations of the cerebral blood flow (CBF) is also supported by observations in isolated cerebral vessels showing vasomotion in vitro [4-5]. Renewed interest in CBF oscillations was generated by studies in humans with laser-Doppler flowmetry [6-7], transcranial Doppler sonography [8], functional MRI [9] and near infrared spectroscopy [10].

Vasomotional activity reflected by CBF oscillations has been reported in subarachnoid hemorrhage (SAH) before the onset of cerebral vasospasm and during recovery [11]. This indicates that overactivation of the mechanism(s) responsible for vasomotion may be involved in the pathogenesis of spastic contractions and the enhancement of vasomotional activity may predict the development of acute vasospasm. On the other hand, vasodilation (induced by hypercapnia or volatile anesthetics) promptly and reversibly suspends low frequency CBF oscillations [12-13] and the cerebrovascular endothelium also supresses vasomotion by releasing NO [2]. In accordance, enhanced CBF oscillations have been reported in pathophysiological states associated with

decreased bioavailability of NO, such as SAH [11], traumatic brain injury [14] and hypertension [15].

Recent studies in isolated cerebral arteries have indicated the role of the thromboxane pathway in the functional changes induced by inhibition of NO synthesis [5,16-17]. This interaction is particularly interesting in the light of recent observations indicating that in NO-deficiency other prostanoids (e.g. prostacyclin) may also induce vascular smooth muscle effects via activation of the thromboxane (TP) receptor [18] and cerebrovascular expression of TP-receptors is enhanced after SAH [19]. In the present study we hypothesized that in NO-deficiency a hypersensitivity of the cerebrovascular TP-receptor mediated signaling pathway develops resulting in enhanced vasomotion and blood flow oscillations in the cerebral cortex.

METHODS

The experiments were performed in adult male Wistar rats (300-400 g) under the guidelines of the Hungarian Law of Animal Protection (243/1988) and all procedures were approved by the Semmelweis University Committee on the Ethical Use of Experimental Animals (590/99 Rh). The animals were anesthetized with urethane (1.5 g/kg intraperitoneally), the depth of anesthesia was regularly controlled during the experiments by checking the corneal or plantar nociception reflex and additional urethane was administered intravenously (iv.) as necessary. The animals were spontaneously breathing through an intra-tracheal cannula. Catheters were inserted into both femoral arteries (for systemic arterial blood pressure measurement and for blood sampling) and

into the left femoral vein (for drug administration). Body temperature was kept constant between 36-38 °C with a controlled heating pad.

Systemic arterial pressure was recorded continuously on a polygraph (Model 7E, Grass, Quincy, MA, USA). Measurement of cerebrocortical blood flow (CoBF) has been performed by laser-Doppler (LD) flowmetry as described in detail elsewhere [20]. The head of the animals was fixed in a stereotaxic head holder with the nose 5 mm down from the interaural line. The skull of the parietal region was exposed and the bone was thinned over the parietal cortex on both sides with a microdrill, so that the lamina interna of the skull remained intact. Two LD probes were placed above the thinned skull at a 12°-angle to the vertical to provide an optimal view of the cortex (4 mm caudal from bregma, 5 mm lateral from midline). CoBF was measured with a two-channel blood flow monitor (MBF3D, Moor Instruments, UK) and was recorded continuously. The LD monitor was calibrated before each individual experiment with a constant movement latex emulsion. The laser light was in the infrared range (780 nm) and penetrated about 1 mm into the brain covering approximately 7 mm^2 of the parietal region, so that the data acquired mostly represented the characteristics of the blood flow in the parietal cortex [20].

Animals were randomly assigned to two *in vivo* experimental groups. In the first (control) group systemic and cerebral circulatory parameters, as well as blood gas and acid-base values were determined before as well as for 75 minutes after an iv. bolus injection of 1 ml/kg vehicle (saline). Thereafter the animals received the thromboxane receptor agonist U-46619 in a dose of 1 μg/kg iv., which in preliminary experiments was below the threshold of inducing any systemic or cerebral circulatory changes. The second group received first N^G-nitro-L-arginine methyl ester (L-NAME) in a dose of 100 mg/kg iv. for

the inhibition of NO synthesis and 75 minutes later U-46619 as described above. Previous studies have verified that L-NAME in the dose used in our present study effectively inhibits cerebral NOS activity [21]. The final measurements were performed in both experimental groups 25 minutes after the administration of U-46619.

The *in vitro* experiments were performed in middle cerebral arteries (MCAs) supplying the parietal cortex, the site of the *in vivo* CoBF measurements. MCA segments were prepared from adult male Wistar rats as previously described [22] and studied in a conventional myograph system (610M, Danish Myo Technology A/S, Aarhus, Denmark). Effects of 100 nM U-46619 were determined on the isometric vascular tension in intact vessels and after NOS-blockade induced by 100 μM L-NAME.

The Discrete Fourier transform (spectrum) of the time series obtained in vivo (CoBF) or in vitro (vascular tone) was calculated by Fast Fourier Algorithm (FFT) [23]. The calculations were executed in the Matlab environment which uses an adaptive version of the FFT, called FFTW [24]. The DC (zero frequency) component was eliminated from the spectrum by subtracting the mean value of time series from the samples and this way generating zero-mean time series. In order to identify the largest frequency component of the spectrum the interesting region was gated by an appropriate frequency window.

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Values are presented as mean ± SEM; n represents the number of animals or MCA segments. Statistical analysis was performed using repeated measures ANOVA followed by a Tukey post-hoc test. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Baseline physiological parameters were within the normal range in both *in vivo* experimental groups (Table 1.). Neither iv. administration of saline nor that of the TP-receptor agonist U-46619 in a dose of 1 μg/kg induced any significant changes in acid-base, blood gas or systemic circulatory parameters in the control group (Table 1.). Furthermore, neither the average CoBF (Table 1.) nor its Fourier spectrum (Figure 1.) changed after the administration of saline or U-46619 in control animals. These observations confirmed that U-46619 in this dose has no significant effect on the systemic and cerebrocortical circulation under physiological conditions.

In the "NOS blockade" group iv. administration of L-NAME had no significant effect on acid base or blood gas parameters but increased systemic blood pressure and decreased heart rate. These changes developed within 25 minutes after L-NAME and remained unaltered later even after the administration of U-46619 (Table 1.). The CoBF reduced by more than 30 % within the first 25 min of L-NAME administration but did not change further until the completion of the experiments (Table 1.). Low frequency CoBF oscillations, which were absent under resting conditions, developed after the administration of L-NAME with a dominant frequency of 144 ± 2 mHz and magnitude of 5.9 ± 0.7 AU (Figure 2.). U-46619 significantly ($p=0.017$) increased the magnitude of these oscillations to 8.8 ± 0.9 AU without changing the dominant frequency (144 ± 3 mHz) (Figure 2.).

Experiments with the isolated MCAs showed similar results to the *in vivo* observations. In the control group no spontaneous vasomotion could be detected before or after administration of saline or 100 nM U-46619 (Figure 1.), indicating that U-46619 in this concentration fails to induce any significant effect on the vascular tension under physiological conditions. Administration of L-NAME alone did not change the vascular tone, and failed to induce vasomotion, except in one vessel out of ten (Figure 2.). In contrast, stimulation of the TP-receptors with 100 nM U-46619, which was without any effect in control vessels, induced strong vasomotional activity in all NOS-blocked MCAs (Figure 2.)

DISCUSSION

Our results indicate that activation of TP-receptors increases cerebral vasomotion and CBF oscillations in NO-deficiency. This observation is particularly interesting in the light of the large number of pathologic conditions associated with diminished release and/or biological effectiveness of NO, such as atherosclerosis, diabetes, hypertension, ischemia/reperfusion and SAH. Since NO suppresses platelet aggregation as well as vascular release of TXA_2, NO-deficiency is often associated with activation of TP-receptors. Therefore, experimental conditions of the present study mimicked features of cerebrovascular diseases. Vasomotion is often enhanced in these pathophysiological states although it is still a question of debate whether CBF oscillations represent the last attempt of the cerebral circulation to prevent neuronal hypoxia or it is already the first

sign of disrupted regulation. Both of these interpretations can be supported by the observations that CoBF oscillations precede the onset of vasospasm in SAH [11].

NO reportedly suppresses the synthesis of TXA_2 [25] and inhibition of NOS was recently reported to enhance TXA_2 release from cerebrovascular endothelial cells [17]. This interaction between NO and TXA_2 may explain why did NOS blockade by itself induce CoBF oscillations in vivo but failed to induce vasomotion in vitro, where the endogenous TXA_2 was diluted in the large volume (8 ml) of the organ baths. However, the weak exogenous stimulation of the TP-receptors by 100 nM U-46619, which was without any effect under physiological conditions, induced strong vasomotion in the absence of NO indicating a hypersensitivity of the vessels to TXA_2. Therefore, cerebral vasomotion and CoBF oscillations appear to be potentiated but not directly induced by the activation of TP-receptors.

In a recent study we have shown that both the $G_{q/11}$ and the $G_{12/13}$ heterotrimeric G proteins are involved in the mediation of TXA_2-induced vasoconstriction [26]. However, the vascular effects of weak TP-receptor stimulation, like those applied in the present study, are primarily induced by the $G_{12/13}$-mediated activation of the small G protein RhoA and Rho-kinase (Németh and Benyó, unpublished observations), the main signaling pathway of calcium-sensitization. Since vasomotion is induced by calcium waves in the smooth muscle [27], TP-receptor mediated calcium-sensitization can enhance the resultant changes of the vascular tension, which is the most plausible explanation of our findings.

Why is this TP-receptor mediated mechanism enhanced in NO-deficiency? As noted above, the increased release of endogenous TXA_2 by the cerebrovascular endothelium [17] can only partly explain our observations. In the cerebral circulation constitutive NO-synthesis maintains a basal vasodilator tone, partly by inhibition of the vasoconstrictor TXA_2-pathway [16]. TP-receptors are targets of cyclic GMP-dependent kinase (PKG) which induce desensitization by phosphorylation at Ser^{331} of the C-tail domain of the receptor [28-29]. Furthermore, NO also interferes with the signaling pathway coupling TP-receptor activation to the calcium sensitization of the vascular smooth muscle. For instance, NO reportedly inhibits calcium sensitization by PKG-mediated inhibition of RhoA [30-31] and telokin [32], as well as by inhibition of RhoA activation through protein kinase A (PKA)-dependent phosphorylation of $G\alpha_{13}$ [33]. PKG can also inhibit calcium sensitization by phosphorylating directly the myosin phosphatase targeting subunit (MYPT1) at Ser^{695} resulting in the reduction of phosphorylation at the adjacent inhibitory Thr^{696} site [34-36], the target of Rho-kinase [37]. Therefore, in the absence of NO not only the TP-receptors but also these downstream signaling pathways will be released from the tonic inhibitory influence of NO resulting in sensitization of the smooth muscle contractile machinery to fluctuations induced by calcium waves.

In conclusion, our results indicate that hypersensitivity of the TP-receptors and/or related downstream signaling pathways in the cerebrovascular smooth muscle contributes to the development of cerebral vasomotion and consequent blood flow oscillations after inhibition of NO synthesis. Pharmacological inhibition of this enhanced reactivity may be beneficial to prevent vasospasm in cerebrovascular disorders associated with NO-deficiency.

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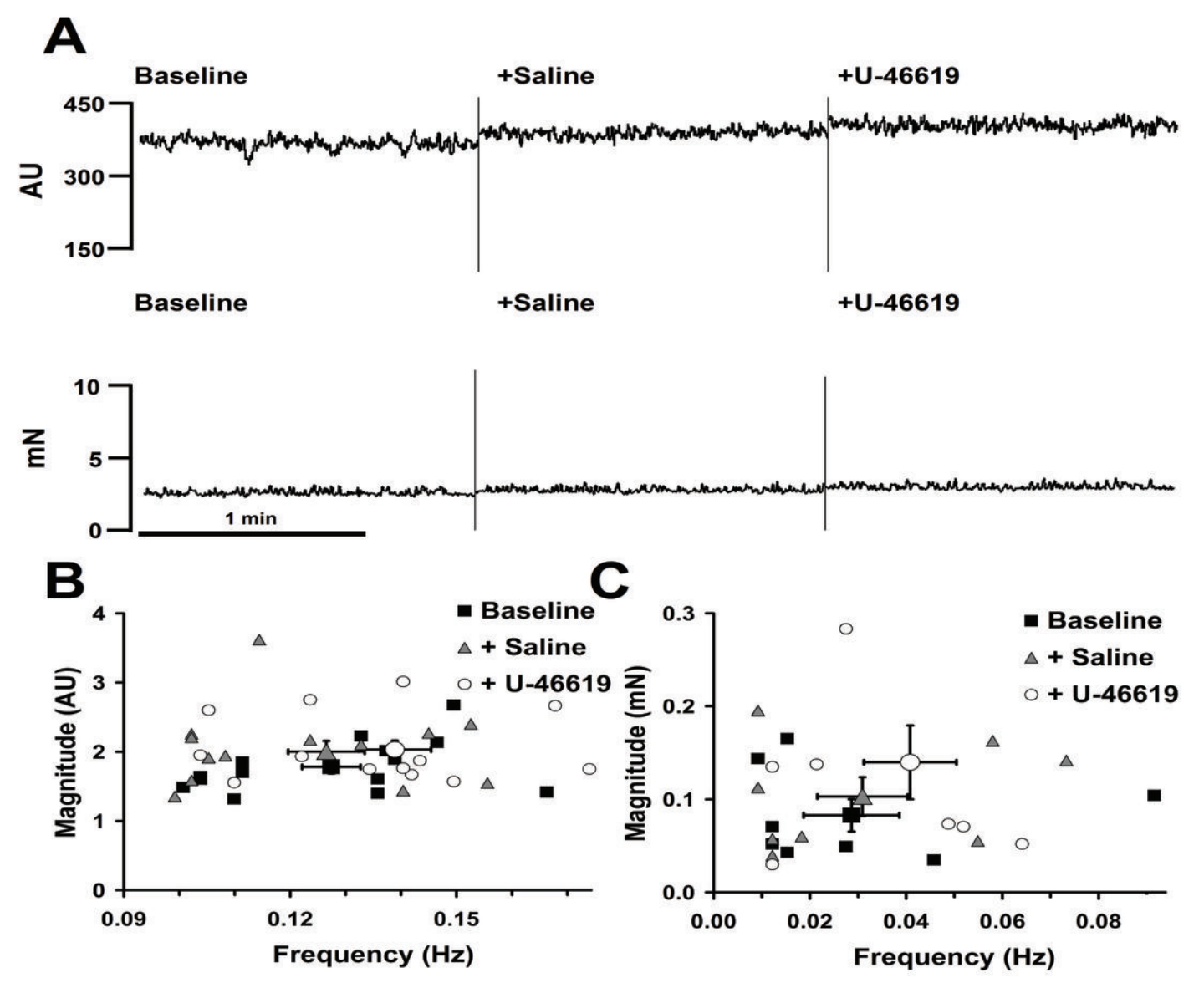
FIGURE LEGENDS

Figure 1. Weak activation of thromboxane receptors doesn`t alter cerebrocortical blood flow and cerebrovascular tension under physiological conditions. A Original recordings of the cerebrocortical laser-Doppler flux *in vivo* (upper panels, scale in Arbitrary Units) and isometric tension of the MCA *in vitro* (lower panels, scale in mNewtons) before (left panels) and after (middle panels) vehicle (saline) treatment as well as after administration of the TP-receptor agonist U-46619 (right panels). Quantitative analysis of slow wave oscillations *in vivo* (**B**) and *in vitro* (**C**) with discrete Fourier transformation. The dominant frequency and the corresponding magnitude of the Fourier spectrum are shown on the figure for the individual experiments. Large symbols indicate the averages ± SEM.

Figure 2. Weak activation of thromboxane receptors induces strong vasomotion in the absence of NO. A Original recordings of the cerebrocortical laser-Doppler flux *in vivo* (upper panels, scale in Arbitrary Units) and isometric tension of the MCA *in vitro* (lower panels, scale in mNewtons) before (left panels) and after (middle panels) the inhibition NOS activity with L-NAME as well as after administration of the TP-receptor agonist U-46619 (right panels). Quantitative analysis of slow wave oscillations *in vivo* (**B**) and *in vitro* (**C**) with discrete Fourier transformation. The dominant frequency and the corresponding magnitude of the Fourier spectrum are shown on the figure for the individual experiments. Large symbols indicate the averages ± SEM. Note that the "Magnitude" scales in **B** and **C** are different from those in **Figure 1B** and **1C**.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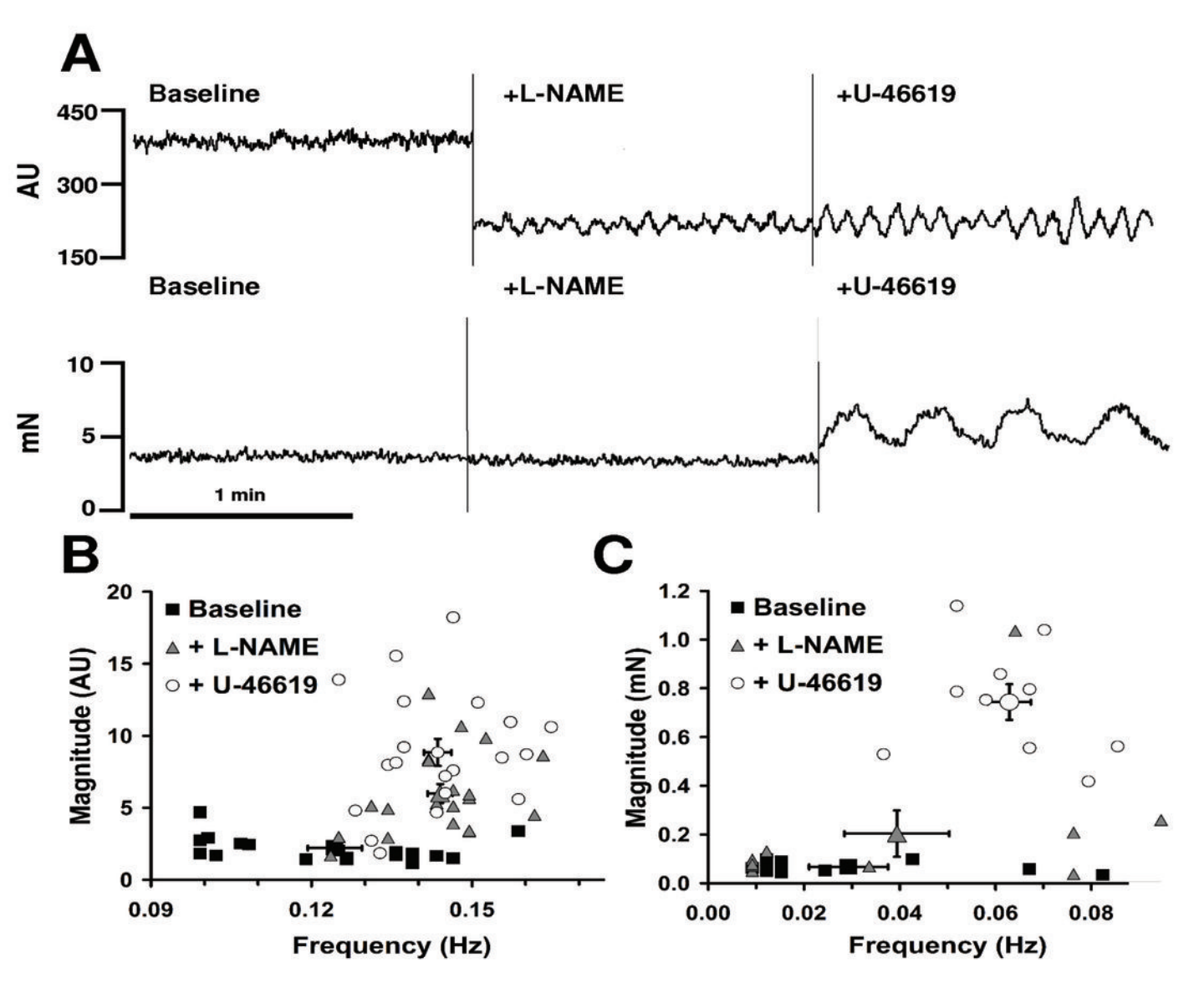
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Frequency (Hz)

Table 1. Cerebrocortical blood flow and physiological parameters in the two in vivo experimental groups at different time points of the experiments.

	Exp. group	0 min.	25 min.	50 min.	75 min.	100 min.
Cerebrocortical Blood Flow (AU)	*Control*	399.1 ± 24.0	424.6 ± 30.7	449.0 ± 38.5	446.2 ± 33.4	438.3 ± 31.0
	NOS blocked	416.2 ± 13.0	285.7 ± 11.1***†††	271.8 ± 9.6***†††	286.0 ± 11.8***†††	274.4 ± 10.6***†††
Mean Arterial Pressure (mmHg)	*Control*	97.9 ± 6.4	96.0 ± 4.0	92.1 ± 3.6	94.2 ± 6.2	95.4 ± 5.4
	NOS blocked	100.0 ± 9.3	156.3 ± 11.0***†††	156.6 ± 11.9***†††	155.3 ± 8.9***†††	147.6 ± 8.0***†††
Heart Rate (bpm)	*Control*	410 ± 22	409 ± 47	400 ± 38	402 ± 41	386 ± 46
	NOS blocked	408 ± 46	385 ± 48	383 ± 48*	382 ± 49*	375 ± 14**
$PaCO_2$ (mmHg)	*Control*	41.0 ± 6.8	41.8 ± 4.1	43.2 ± 3.4	41.7 ± 5.2	45.7 ± 6.6
	NOS blocked	43.7 ± 4.6	44.4 ± 2.9	42.6 ± 3.2	41.8 ± 4.4	41.2 ± 4.4
PaO_2 (mmHg)	*Control*	96.9 ± 11.5	95.1 ± 10.5	90.9 ± 8.8	95.0 ± 11.1	97.7 ± 16.7
	NOS blocked	99.0 ± 13.6	97.9 ± 10.7	99.3 ± 10.1	102.3 ± 11.8	94.9 ± 11.4
O_2 Saturation (%)	*Control*	96.5 ± 1.3	96.9 ± 0.7	96.1 ± 0.7	96.5 ± 1.0	96.5 ± 1.2
	NOS blocked	96.5 ± 1.5	96.4 ± 0.7	96.8 ± 0.8	97.1 ± 1.1	96.4 ± 1.3
pH	*Control*	7.34 ± 0.04	7.36 ± 0.04	7.34 ± 0.03	7.35 ± 0.03	7.33 ± 0.04
	NOS blocked	7.32 ± 0.05	7.31 ± 0.03†	7.33 ± 0.04	7.34 ± 0.03	7.34 ± 0.04
Standard Base Excess (mmol/l)	*Control*	-3.7 ± 2.8	-2.0 ± 1.9	-3.1 ± 1.9	-2.1 ± 1.1	-1.9 ± 1.1
	NOS blocked	-2.2 ± 2.0	-3.4 ± 2.6	-3.4 ± 2.0	-3.4 ± 1.9	-3.5 ± 1.9

L-NAME and U-46619 were injected after the “0 min.” and “75 min.” measurements, respectively.

The *n* was 7 in the control and 10 in the NOS blocked experimental groups. *, ** and *** indicate significant differences vs. the “0 min.” value ($p<0.05$, 0.01 and 0.001, respectively). † and ††† indicate significant differences vs. the corresponding value in the control group ($p<0.05$ and 0.001, respectively).

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ACCELERATED COMMUNICATION

Nicotinic Acid-Induced Flushing Is Mediated by Activation of Epidermal Langerhans Cells

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ABSTRACT

The antidyslipidemic drug nicotinic acid (niacin) has been used for decades. One of the major problems of the therapeutical use of nicotinic acid is a strong cutaneous vasodilation called flushing, which develops in almost every patient taking nicotinic acid. Nicotinic acid-induced flushing has been shown to be mediated by the nicotinic acid receptor GPR109A and to involve the formation of vasodilatory prostanoids. However, the cellular mechanisms underlying this short-term effect are unknown. Here, we show that epidermal Langerhans cells are essential for the cutaneous flushing response induced by nicotinic acid. Langerhans cells respond with an increase in $[Ca^{2+}]_i$ to nicotinic acid and express prostanoid synthases required for the formation of the vasodilatory prostanoids prostaglandin E_2 and prostaglandin D_2. Depletion of epidermal Langerhans cells but not of macrophages or dendritic cells abrogates nicotinic acid-induced flushing. These data unexpectedly identify epidermal Langerhans cells as essential mediators of nicotinic acid-induced flushing and may help to generate new strategies to suppress the unwanted effects of nicotinic acid. In addition, our results suggest that Langerhans cells besides their immunological roles are also involved in the local regulation of dermal blood flow.

Nicotinic acid (niacin) is the oldest lipid-modifying drug and induces a unique spectrum of changes in lipid and lipoprotein levels (Carlson, 2005). Besides its ability to decrease triglyceride and low-density lipoprotein cholesterol levels, it has the strongest HDL cholesterol-elevating activity among the currently available lipid modifying drugs. Because a low HDL cholesterol level is an independent risk factor for cardiovascular diseases, the HDL cholesterol elevating effect of nicotinic acid has recently led to a renewed interest in this drug (Carlson, 2006; Kontush and Chapman, 2006; Offermanns, 2006), and there is good evidence indicating a beneficial effect of nicotinic acid alone or in combination with statins (Coronary Drug Project Research Group, 1975; Brown et al., 2001; Taylor et al., 2004).

The major problem of oral nicotinic acid treatment is the occurrence of a strong flushing phenomenon associated with cutaneous vasodilation, which occurs in virtually all patients and severely influences patients' compliance. It has been shown recently that the nicotinic acid receptor GPR109A (HM74A in humans, PUMA-G in mice) mediates not only the short-term metabolic effects of nicotinic acid but also the flushing response (Tunaru et al., 2003; Benyo et al., 2005). However, the cells and molecular mechanisms mediating nicotinic acid-induced flushing are unclear.

The flushing response of nicotinic acid can be inhibited by pretreatment with cyclooxygenase inhibitors (Andersson et al., 1977; Svedmyr et al., 1977; Eklund et al., 1979; Kaijser et al., 1979), and the levels of several vasodilatory prostanoids like prostaglandin E_2 (PGE_2) and prostaglandin D_2 (PGD_2) and their metabolites are elevated after administration of nicotinic acid (Eklund et al., 1979; Nozaki et al., 1987; Mor-

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ABBREVIATIONS: HDL, high density lipoprotein; DT, diphtheria toxin; DTR, diphtheria toxin receptor; LDF, laser-Doppler flow; PGE_2, prostaglandin E_2; PGD_2, prostaglandin D_2; mPGES-1 and mPGES-2, type 1 and type 2 PGE_2 synthases; MHC, major histocompatibility complex; PE, phosphatidylethanolamine; GFP, green fluorescent protein; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

row et al., 1989; Stern et al., 1991). These observations have led to the hypothesis that prostanoids are involved in nicotinic acid-induced flushing. Pharmacological and genetic evidence has been provided recently that PGD_2 acting through the PGD_2 receptor and PGE_2 acting via type 2 PGE_2 receptor and type 4 PGE_2 receptor mediate the nicotinic acid-induced flushing response (Benyo et al., 2005; Cheng et al., 2006).

The cells required for nicotinic acid-induced and prostanoid-mediated flushing, however, remain elusive. The ability of topically applied nicotinic acid to induce locally restricted hyperemia (Wilkin et al., 1985; Morrow et al., 1992) and the fact that the nicotinic acid receptor GPR109A is expressed in macrophages and other immune cells (Schaub et al., 2001) suggest that immune cells of the skin are involved in the flushing response. This is supported by data demonstrating that MHC class II-positive skin cells express GPR109A and that the lack of nicotinic acid-induced flushing response in GPR109A-deficient mice can be restored by the transplantation of wild-type bone marrow (Benyo et al., 2005). We therefore systematically studied the potential involvement of various cell types in the cutaneous effects of nicotinic acid. By conditional ablation of defined subpopulations of immune cells and by the visualization of the local effects of nicotinic acid, we show in the present study that epidermal Langerhans cells respond to nicotinic acid and mediate the flushing response.

Materials and Methods

Mice. CD11b-DTR and CD11c-DTR mice were purchased (The Jackson Laboratory, Bar Harbor, ME), and Langerin-DTR transgenic mice have been described previously (Bennett et al., 2005). GPR109A-deficient mice were kindly provided by Klaus Pfeffer (Universität Düsseldorf, Düsseldorf, Germany). Mice were housed in temperature-controlled facilities on a 12-h light/dark cycle with ad libitum food and water access. All experimental procedures were performed in accordance with institutional guidelines of the University of Heidelberg (Heidelberg, Germany).

Measurement of Flush. Cutaneous blood flow was determined as described previously (Benyo et al., 2005). In brief, anesthetized mice were placed on their left side on a controlled heating pad, and blood flow in the ear was determined with a laser-Doppler probe (407; Perimed AB, Stockholm, Sweden). Original LDF recordings were averaged for 1-s intervals, and the baseline LDF was determined before injection of the tested compound. All data are presented as mean ± S.E.M., and n indicates the number of experimental animals. Statistical analysis of differences between flushing responses before and after diphtheria toxin (DT) treatment was performed by Student's paired t test, and $p < 0.05$ was considered significant. Nicotinic acid was prepared in 5% (2-hydroxypropyl)-β-cylodextrin (Sigma, St. Louis, MO), and the pH of the solutions was adjusted to 6.9 to 7.1 with 1 M NaOH. Prostaglandin D_2 (Cayman Chemical Co., Ann Arbor, MI) was dissolved in dimethyl sulfoxide and diluted 20 times with saline.

DT Treatment. After control experiments, the CD11b-DTR, CD11c-DTR, and Langerin-DTR transgenic animals were treated with 25, 4, and 16 ng/g diphtheria toxin (Sigma), respectively, as described previously (Jung et al., 2002; Bennett et al., 2005; Duffield et al., 2005). DT was injected intraperitoneally 24 and 48 h before retesting of the flushing responses. Wild-type littermate animals were treated with the same protocol and served as controls. Between two experiments the animals were allowed to recover for at least 1 week.

Isolation of Epidermal Sheets and Immunohistochemistry. Mouse ears were dissected, split into ventral and dorsal sheets, and incubated for 50 min in RPMI 1640 medium and 10 mM EDTA. The epidermis was removed from the dermis using fine forceps and was fixed on ice for 1 h in 4% paraformaldehyde/PBS. After permeabilization with 0.2% Triton/PBS for 10 min at 25°C and two washes with PBS for 10 min, the epidermal sheets were incubated with 10% goat serum for 30 min. Polyclonal rabbit anti-PGE synthase 1, polyclonal rabbit anti-PGE synthase 2 and monoclonal mouse PGD_2 synthase (all from Cayman Chemical) were each mixed with PE-labeled anti-MHC II-IA/IE (BD PharMingen, San Diego, CA) in 10% goat serum/PBS and incubated with epidermal sheets overnight at 4°C on a rotator. Secondary FITC-labeled anti-rabbit and anti-mouse antibodies were applied together with DAPI in 1.5% horse serum for 2 h on a rotator. After washing, epidermal sheets were mounted and analyzed by fluorescence microscopy.

Fluorescence Activated Cell Sorting. Splenocytes were isolated by collagenase digestion and passing through a 40-μm nylon mesh. Red blood cells were removed using Lympholyte-M (Cedarlane Laboratories, Burlington, NC). Splenocytes were stained with FITC-anti-CD11c and PE-labeled anti-F4/80 (BD PharMingen). Peritoneal macrophages were obtained by peritoneal lavage and stained with PE-labeled anti-F4/80 (BD PharMingen). All flow cytometric analyses were performed using the FACSCalibur and Cell Quest software (BD Biosciences, San Jose, CA).

Measurement of Intracellular [Ca^{2+}]. Epidermal sheets of Langerin-GFP-DTR or GPR109A-deficient animals were incubated in RPMI 1640 medium with 6 μM Fura-2/acetoxymethyl ester/pluronic acid (Invitrogen, Carlsbad, CA) and in the case of Puma-G knockout together with FITC-labeled anti MHC II-IA/IE (BD PharMingen) for 45 min. The epithelial sheets were placed dermal side up in a perfusion chamber and immobilized with thin silk threads tied to a steel ring. Epithelial sheets were superfused with 900 μl of RPMI 1640 medium at a flow rate of 3.5 ml/min, and stimulation was achieved by pipetting 100 μl of a 10× concentrated solution into the chamber. Excitation light was provided by a monochromator (TILL Photonics, Gräfelfing, Germany), and the fluorescence emission was captured by a cooled charge-coupled device camera (Image QE; TILL Photonics) on an upright microscope (Olympus BX51WI, Tokyo, Japan). The calcium concentration was visualized in Langerhans cells that were identified by GFP or FITC fluorescence, respectively. Ratio images were collected at intervals every 500 ms. Imaging was controlled and analyzed with Tillvision Software 4.0 (TILL Photonics).

Results

As reported previously, nicotinic acid-induced cutaneous vasodilation can be observed and quantified in the mouse ear using laser-Doppler flowmetry (Benyo et al., 2005; Cheng et al., 2006). Intraperitoneal injection of nicotinic acid results within a few minutes in a biphasic increase in the blood flow that lasts for approximately 40 min. Based on the fact that transplantation of wild-type bone marrow into GPR109A-deficient mice can restore the ability of nicotinic acid to induce flushing (Benyo et al., 2005), we tested the potential involvement of various cutaneous immune cells in the nicotinic acid-induced flushing response. Because nicotinic acid does not induce an increase in histamine levels (Morrow et al., 1989), and because mast cell-deficient mice still respond with flushing to nicotinic acid (Benyo et al., 2005), mast cells are obviously not required for the flushing phenomenon. Besides mast cells, dermal macrophages and dermal dendritic cells are the major immune cells present in the dermis. To examine their potential involvement in nicotinic acid-induced flushing, we conditionally depleted macrophages and dendritic cells using recently developed transgenic mouse lines in which either the CD11c promoter element drives the

expression of the human DT receptor in dendritic cells (Jung et al., 2002) or in which diphtheria toxin receptor (DTR) expression is restricted to macrophages by using the CD11b promoter element (Duffield et al., 2005). Murine cells are insensitive to DT because their DTR homolog does not bind DT. In both CD11b-DTR and CD11c-DTR mice we were able to show that the systemic administration of DT led to the depletion of macrophages and dendritic cells, respectively (Fig. 1A, B, F, and G). When DT-treated CD11b-DTR and CD11c-DTR mice were injected with nicotinic acid, a normal biphasic flushing response could be observed, which was indistinguishable from the flushing response induced by nicotinic acid before DT treatment (Fig. 1, C–E and H–J). This clearly indicates that neither macrophages nor dendritic cells in the dermis or elsewhere in the body are involved in the nicotinic acid-induced flushing response.

Another major immune cell type present in the skin is epidermal Langerhans cells, which densely populate the epidermal layer of the skin. By using a mouse line expressing DTR under the control of the Langerhans cell-specific langerin promoter (Bennett et al., 2005), we were able to study the potential role of Langerhans cells in nicotinic acid-induced flushing. DT treatment of Langerin-DTR mice resulted in ablation of the Langerhans cell population from the epidermis of mice, whereas dermal dendritic cells were not affected (Bennett et al., 2005) (Fig. 2, A and B). It is interesting that depletion of Langerhans cells was accompanied by abrogation of nicotinic acid-induced flushing, whereas Langerin-DTR animals before DT treatment showed normal distribution of epidermal Langerhans cells and normal flushing response to nicotinic acid (Fig. 2, C–E). Wild-type mice pretreated with DT under the same conditions as Langerin-DTR transgenic mice exhibited a normal flushing response (data not shown). Treatment of Langerin-DTR animals with DT did not principally affect the ability of these animals to respond with cutaneous vasodilation to other stimuli as, shown

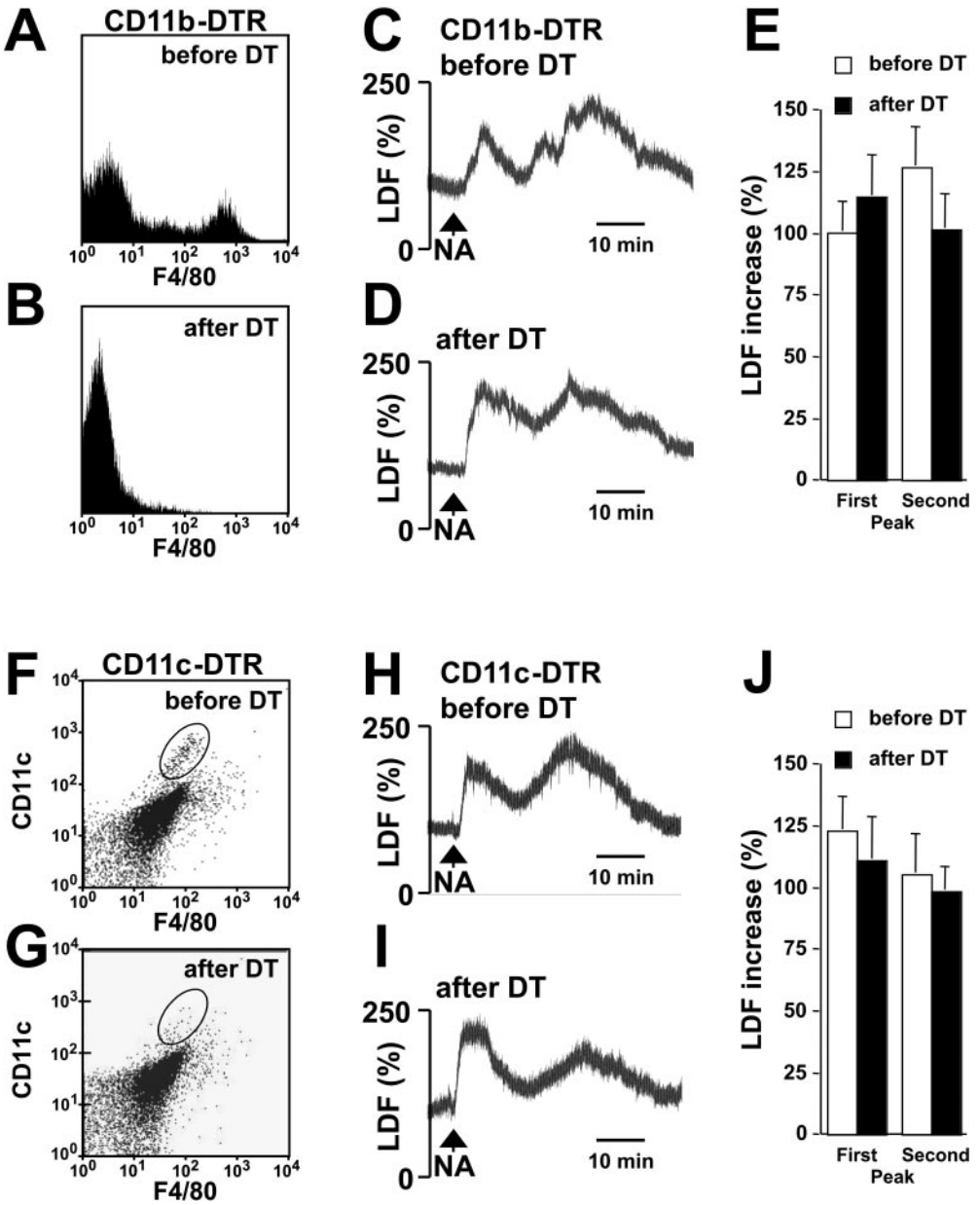

Fig. 1. Effect of macrophage and dendritic cell depletion on nicotinic acid-induced flushing. CD11b-DTR (A–E) and CD11c-DTR transgenic mice (F–J) were analyzed before or after intraperitoneal injection of DT. A, B, F, and G, verification of macrophage and dendritic cell depletion. Cells harvested by peritoneal lavage (A and B) or splenocytes (F and G) were analyzed by fluorescence-activated cell sorting. C–E and H–J, original laser-Doppler flow recordings and quantitative analysis of the percentage of LDF increase in response to intraperitoneal administration of 200 μg/g nicotinic acid in untreated or DT-treated CD11b-DTR (C–E) and CD11c-DTR mice (H–J).

by the normal flushing response to the intraperitoneal injection of PGD_2 (Fig. 2, F–H). Thus, epidermal Langerhans cells are specifically required for the nicotinic acid-induced cutaneous vasodilation and are positioned upstream of PGD_2 release.

To test whether Langerhans cells functionally respond to nicotinic acid, we prepared epidermal sheets from ears of Langerin-DTR mice, which express enhanced green fluorescent protein fused to DTR (Bennett et al., 2005), and loaded epidermal cells with the Ca^{2+} indicator Fura-2/acetoxymethyl ester. At the same time, Langerhans cells were visualized by fluorescence microscopy (Fig. 3A). Exposure of Fura-2-loaded epidermal sheets to 100 μM nicotinic acid resulted in a transient increase in $[Ca^{2+}]_i$ of Langerhans cells (Fig. 3, B and C). Fura-2-loaded Langerhans cells in epidermal sheets from GPR109A-deficient mice, which were identified by staining with an anti-MHCII antibody, did not respond with any increase in $[Ca^{2+}]_i$ to the application of nicotinic acid but still responded to the Ca^{2+} ionophore ionomycin (Fig. 3D).

Because PGD_2 and PGE_2 have been shown to be principal mediators of the nicotinic acid-induced flushing response, we tested whether epidermal Langerhans cells express prostaglandin D_2 and prostaglandin E_2 synthases. Staining of epidermal sheets from mouse ears with specific antibodies directed against murine PGD_2 synthase and types 1 and 2 PGE_2 synthases (mPGES-1 and mPGES-2) together with an antibody against mouse MHCII showed the presence of both PGD_2 synthase and type 2 PGE synthase, whereas we were unable to detect mPGES-1 (Fig. 4). The mPGES-2 enzyme seemed to be exclusively expressed in epidermal Langerhans cells, whereas prostaglandin D_2 synthase was also detected in other cells of the epidermis (Fig. 4).

Discussion

The nicotinic acid-induced flushing response is a phenomenon that was first described shortly after the discovery of nicotinic acid as a vitamin (Spies et al., 1938; Goldsmith and Cordill, 1943). The ability of nicotinic acid to induce a strong cutaneous vasodilation has always been an obstacle in the clinical use of nicotinic acid as a lipid-modifying drug. Despite the pharmacological importance of this phenomenon, the underlying mechanism is still not clear. Based on the recent observation that bone marrow-derived immune cells in the skin mediate the nicotinic acid-induced flushing response, we systematically studied the involvement of individual cell populations like dermal macrophages, dermal dendritic cells, and epidermal Langerhans cells, which are all present at relatively high numbers in human and mouse skin (Lenz et al., 1993; Dupasquier et al., 2004). Our data clearly indicate that the nicotinic acid-induced flushing response is mediated by epidermal Langerhans cells, whereas dermal dendritic cells and macrophages are not required. Depletion of Langerhans cells alone is able to prevent nicotinic acid-induced flushing.

Previous studies have shown that PGD_2 and PGE_2 are critically involved in the nicotinic acid-induced flushing response (Benyo et al., 2005; Cheng et al., 2006). To test

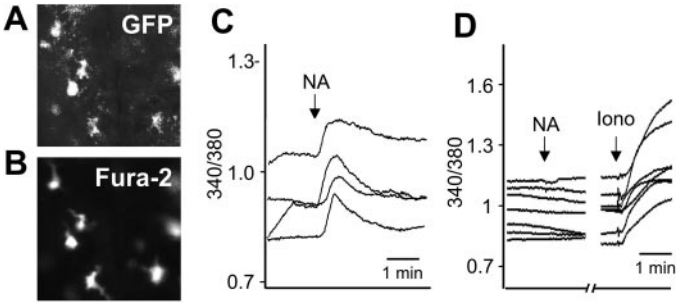

Fig. 3. Epidermal Langerhans cells respond to nicotinic acid. Langerhans cells were identified in epidermal sheets of untreated Langerin-DTR mice by enhanced green fluorescent protein fluorescence (A), and sheets were loaded with Fura-2 and exposed to 100 μM nicotinic acid (B and C). D, representative traces demonstrating the effect of nicotinic acid (100 μM) and ionomycin (Iono, 1 μM) on $[Ca^{2+}]_i$ on MHCII-positive cells of epidermal sheets prepared from GPR109A-deficient mice. Values on the *y*-axis indicate the measured 340/380-nm fluorescence ratio as an indicator of the free intracellular $[Ca^{2+}]$.

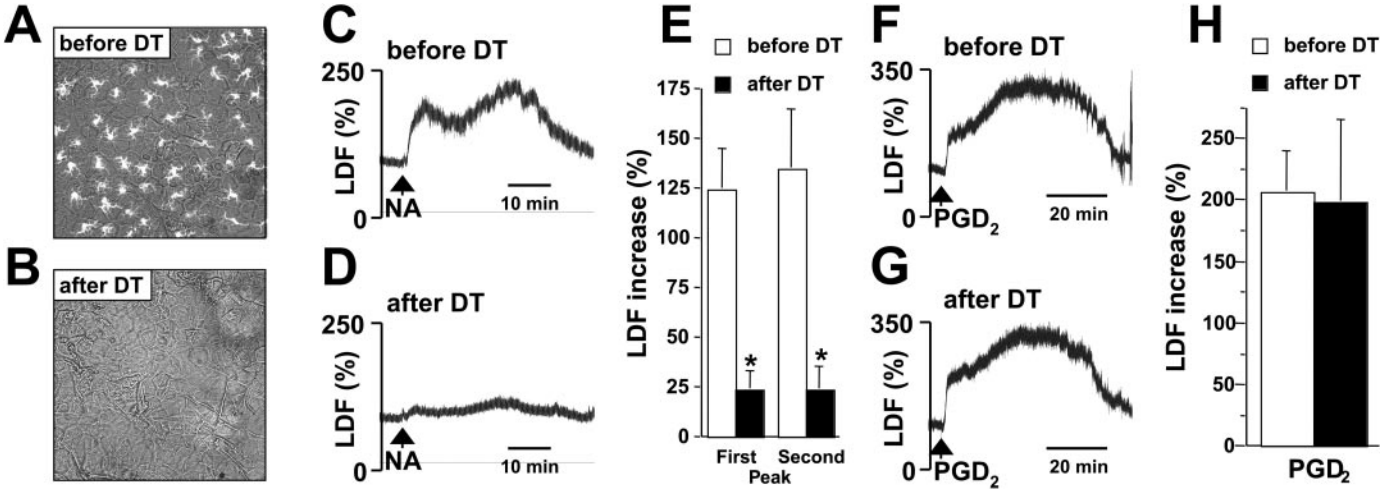

Fig. 2. Nicotinic acid-induced flushing requires the presence of epidermal Langerhans cells. Langerin-DTR transgenic mice were analyzed before or after intraperitoneal injection of DT. A and B, verification of Langerhans cell depletion. Shown is an overlay of the light microscopical image and the fluorescence image of epidermal sheets prepared from untreated (A) and DT-treated (B) Langerin-DTR mice, which were stained with PE-labeled anti-MHCII antibodies. C–E, original laser-Doppler flow recordings and quantitative analysis of the percentage of LDF increase in response to intraperitoneal administration of nicotinic acid in untreated or DT-treated Langerin-DTR mice. F–H, original laser-Doppler flow recordings and quantitative analysis of the percentage of LDF increase after intraperitoneal administration of 2 μg/g PGD_2 in untreated or DT-treated Langerin-DTR mice.

whether Langerhans cells are principally able to synthesize both prostanoids, we tested them for the expression of PGD_2 and PGE_2 synthases. We found both PGD_2 synthase and the constitutive type 2 PGE_2 synthase (mPGES-2) to be expressed in epidermal Langerhans cells. The inducible type 1 PGE synthase form (mPGES-1) (Kudo and Murakami, 2005) could, however, not be detected. This is consistent with earlier findings showing that Langerhans cells are a major source of prostanoids in the epidermis and that their main prostanoid product is PGD_2 whereas they are also able to synthesize PGE_2 (Ruzicka and Aubock, 1987; Rosenbach et al., 1990).

Our finding that Langerhans cells respond to nicotinic acid with a transient increase in the cytoplasmic Ca^{2+} concentration suggests that activation of G_i through the nicotinic acid receptor results in a G$\beta\gamma$-mediated phospholipase C activation, a classic response of immune cells to the activation of G_i-coupled receptors (Exton, 1996; Rhee, 2001). The transient increase in the cytoplasmic Ca^{2+} concentration is a major trigger of the activation of phospholipase A_2 and subsequent formation of arachidonic acid. Arachidonic acid is then further metabolized by the ubiquitously expressed type 1 cyclooxygenase and both PGD_2 and PGE_2 synthases, which are present in Langerhans cells. The release of PGD_2 and PGE_2 from nicotinic acid-activated Langerhans cells then results in vasodilation in the dermal papillae of the upper dermis layer, which are just adjacent to the epidermis-dermis junction close to the localization of Langerhans cells.

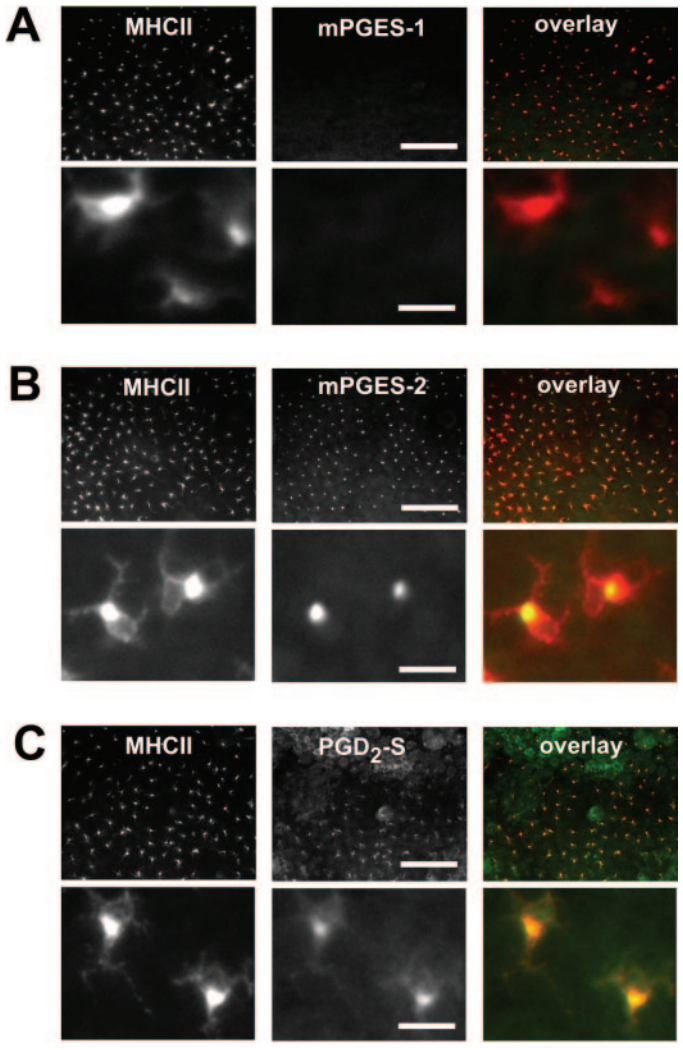

Fig. 4. Expression of prostanoid synthases in epidermal Langerhans cells. Epidermal sheets from wild-type mice were stained with antibodies against MHCII and mPGES-1 (A) or mPGES-2 (B) or PGD_2 synthase (C, PGD_2-S). Scale bars: top, 50 μm; bottom, 5 μm.

The nicotinic acid-induced flushing response is subject to a tolerance phenomenon that can be observed within days after repeated administration of nicotinic acid (Olsson, 1994). Tolerance is unlikely to develop on the basis of receptor desensitization because the metabolic effects are stable even after long-term administration of nicotinic acid (Olsson, 1994). It is more likely that the downstream mechanisms specific for the nicotinic acid-induced flushing response are involved. We could rule out the possibility that tolerance to nicotinic acid-induced flushing is due to any translocation of Langerhans cells, because repeated administration of nicotinic acid did not change the number of Langerhans cells per epidermal area, although the vasodilatory effect had seized (data not shown). It is therefore more likely that the nicotinic acid-induced prostanoid formation in Langerhans cells undergoes tachyphylaxis. This would be consistent with the observation that the development of tolerance to nicotinic acid-induced flushing is accompanied by a reduced formation of prostanoids (Stern et al., 1991). It has been described in the past that patients with atopic dermatitis show a severely reduced flushing response to topical application of nicotinic acid esters (Uehara and Ofuji, 1977; English et al., 1987). Thus, activation of Langerhans cells under certain pathological conditions like atopic dermatitis (Leung et al., 2004) may lead to a resistance of Langerhans cells to nicotinic acid-induced prostanoid formation.

The observed link between the activation of epidermal Langerhans cells and the regulation of blood vessel diameter in the upper dermis raises the question of whether this mechanism, in addition to its pharmacological significance, has any physiological or pathophysiological role. There are numerous transient and long-term forms of skin alterations that go along with erythema. It will be interesting to study the role of Langerhans cells not only in immunological responses of the skin but also in the local regulation of dermal blood flow.

Our data clearly show that epidermal Langerhans cells play a central role in the nicotinic acid-induced flushing response by mediating the formation of vasodilatory prostanoids in response to activation by nicotinic acid. This study provides new insight into the mechanism underlying the clinical problem of nicotinic acid-induced flushing and may provide the basis for new approaches aiming at the reduction of this unwanted side effect. In addition, our data point to an interesting new function of epidermal Langerhans cells in the regulation of the local blood flow in the dermis, whose potential physiological and pathophysiological role remains to be further characterized.

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BRAIN RESEARCH

Research Report

Adaptation of the hypothalamic blood flow to chronic nitric oxide deficiency is independent of vasodilator prostanoids

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Prostanoid

ABSTRACT

The aim of our study was to investigate the adaptation of the hypothalamic circulation to chronic nitric oxide (NO) deficiency in rats. Hypothalamic blood flow (HBF) remained unaltered during chronic oral administration of the NO synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 1 mg/ml drinking water) although acute NOS blockade by intravenous L-NAME injection (50 mg/kg) induced a dramatic HBF decrease. In chronically NOS blocked animals, however, acute L-NAME administration failed to influence the HBF. Reversal of chronic NOS blockade by intravenous L-arginine infusion evoked significant hypothalamic hyperemia suggesting the appearance of a compensatory vasodilator mechanism in the absence of NO. In order to clarify the potential involvement of vasodilator prostanoids in this adaptation, cyclooxygenase (COX) mRNA and protein levels were determined in the hypothalamus, but none of the known isoenzymes (COX-1, COX-2, COX-3) showed upregulation after chronic NOS blockade. Furthermore, levels of vasodilator prostanoid (PGI_2, PGE_2 and PGD_2) metabolites were also not elevated. Interestingly, however, hypothalamic levels of vasoconstrictor prostanoids (TXA_2 and $PGF_{2\alpha}$) decreased after chronic NOS blockade. COX inhibition by indomethacin but not by diclofenac decreased the HBF in control animals. However, neither indomethacin nor diclofenac induced an altered HBF-response after chronic L-NAME treatment. Although urinary excretion of PGI_2 and PGE_2 metabolites markedly increased during chronic NOS blockade, indicating COX activation in the systemic circulation, we conclude that the adaptation of the hypothalamic circulation to the reduction of NO synthesis is independent of vasodilator prostanoids. Reduced release of vasoconstrictor prostanoids, however, may contribute to the normalization of HBF after chronic loss of NO.

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1. Introduction

Acute inhibition of nitric oxide (NO) synthesis causes sustained hypertension accompanied by a marked reduction of the cardiac output and regional blood flow, including the perfusion of the heart (Shah and MacCarthy, 2000) and brain (Faraci and Heistad, 1998). During chronic NO synthase (NOS) blockade, however, the coronary and the cerebral blood flows return to the normal level although the vasoconstriction in other organs remains unchanged (Benyó et al., 1995; Huang et al., 1995; Ito et al., 1995; Kelly et al., 2000; Puybasset et al., 1996; Tayama et al., 1998). In accordance, endothelial NOS (eNOS) knockout mice show normal blood flow in the heart (Gödecke et al., 1998) and brain (Atochin et al., 2003) indicating that the circulation of these organs is able to adapt to chronic NO deficiency. In case of the coronary circulation, it is well established that a vasodilator prostanoid, presumably prostacyclin, may function as a compensatory factor after the chronic loss of NO (Beverelli et al., 1997, Lacza et al., 2003; Marcelín-Jiménez and Escalante, 2001; Puybasset et al., 1996). The mechanism of the adaptation of the cerebral circulation to chronic NO deficiency is far less understood. Vasodilator prostanoids, released constitutively from brain microvessels (Gecse et al., 1982; Kis et al., 1999; Kövecs et al., 2001; Parfenova et al., 2002; Peri et al., 1995), are apparently involved in the maintenance of the resting cerebral blood flow in some species including humans and rats (Busija, 2002; Leffler, 1997) and enhancement of their influence may serve as a reserve regulatory mechanism in NO deficiency. We have observed a significant increase of the prostacyclin and prostaglandin E_2 levels in the cerebrospinal fluid of rats after acute NOS inhibition (Lacza et al., 2001b and unpublished observations), which may indicate the activation of a prostanoid mediated compensatory mechanism in NO deficiency. In a recent study, pial arteriolar relaxations in response to acetylcholine or hypercapnia were improved during chronic as compared to acute NOS blockade in female rats and the improvement of the vasoreactivity was reversible by indomethacin indicating the augmentation of the prostanoid contribution to these vasodilator responses in chronic NO deficiency (Xu et al., 2003).

The aim of our present study was therefore to clarify the role of COX pathways in the adaptation of the cerebral circulation to NO deficiency. We have focused on the hypothalamic circulation, since in our earlier studies the hypothalamic blood flow (HBF) was shown to normalize within 1 week after the onset of the chronic NOS blockade (Benyó et al., 1995). Furthermore, in this brain region relatively high COX expression has been demonstrated (O'Banion, 1999; Kis et al., 2004; Simmons et al., 2004) and prostanoid release reportedly contributes to the maintenance of the resting HBF of rats under physiological conditions (Dahlgreen et al., 1981; Gerozissis et al., 1983; McCulloch et al., 1982).

2. Results

During the 1-week pretreatment period, the daily water intake was not different between control ($n=54$) and L-NAME-treated ($n=48$) animals (101±7 vs. 96±4 ml/kg/day, respectively). In animals subjected to NOS blockade, the average L-NAME consumption was 96±4 mg/kg/day. After 1 week of L-NAME pretreatment, the animals showed marked hypertension (Table 1), but the HBF remained unchanged (0.86±0.04 vs. 0.86±0.04 ml/g/min in controls) in spite of the markedly reduced hypothalamic NOS activity (1.19±0.16 vs. 4.85±1.19 pmol citrulline/mg protein/min, $p=0.003$). Baseline arterial blood gas and acid–base parameters showed no significant differences between the control and the L-NAME pretreated experimental groups (Table 1).

Acute NO blockade achieved by intravenous injection of high dose (50 mg/kg) L-NAME induced marked hypertension (Fig. 1A) and reduction of the HBF (Fig. 1B) in control animals ($n=11$) but had no significant effect after chronic NOS blockade ($n=7$) indicating the appearance of an NO-independent mechanism in the maintenance of the HBF during chronic NO deficiency (Figs. 1A and B).

Intravenous L-arginine administration (30 mg/kg initial bolus followed by 10 mg/kg/min infusion) was without any significant effect on the mean arterial blood pressure (MABP), HBF, arterial blood gas tensions or pH in control animals ($n=11$, Figs. 2A and B). This observation is consistent with our previous report in cats (Kovách et al., 1992) and indicates that

Table 1 – Baseline physiological parameters in the different experimental groups

Experimental group (acute treatment)	I. L-NAME		II. L-Arginine		III. Diclofenac		IV. Indomethacin		V. Vehicle	
Subgroup (chronic pretreatment)	Control ($n=11$)	L-NAME ($n=7$)	Control ($n=12$)	L-NAME ($n=12$)	Control ($n=8$)	L-NAME ($n=10$)	Control ($n=17$)	L-NAME ($n=13$)	Control ($n=6$)	L-NAME ($n=6$)
MABP (mmHg)	91±3	133±6***	98±4	140±7***	93±6	130±3***	94±4	125±3***	91±5	120±5*
PO_2 (mmHg)	93±2	98±4	87±2	84±2	101±2	100±3	95±2	99±3	102±4	97±2
O_2 sat. (%)	96.6±0.3	97.4±0.3	95.7±0.2	95.7±0.2	97.4±0.2	97.3±0.3	96.5±0.3	96.6±0.3	96.9±0.4	96.7±0.3
PCO_2 (mmHg)	39.5±1.5	44.2±1.4	41.9±0.6	37.9±1.1	42.3±1.0	42.3±1.0	44.8±0.7	41.2±1.1	43.7±3.2	42.9±1.3
pH	7.37±0.02	7.40±0.01	7.35±0.01	7.38±0.01	7.38±0.01	7.38±0.01	7.33±0.01	7.31±0.01	7.32±0.03	7.33±0.02

Mean arterial blood pressure (MABP) as well as O_2 and CO_2 tensions (PO_2 and PCO_2), O_2 saturation and pH of arterial blood samples were determined before acute treatment with the respective drug or vehicle. * and *** indicate significant differences vs. the corresponding "control" values ($p<0.05$ and 0.001, respectively).

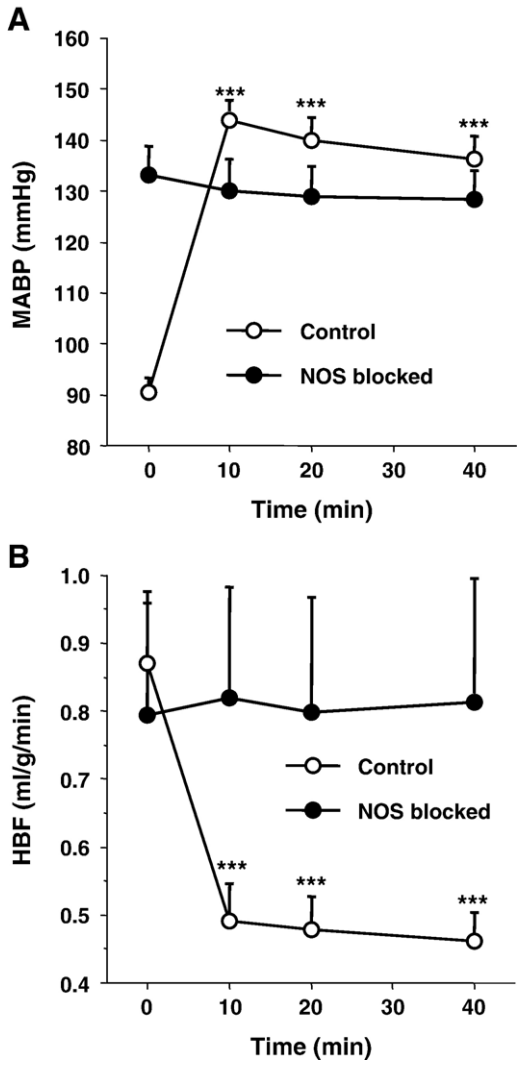

Fig. 1 – Acute L-NAME administration induces marked hypertension and reduction of the hypothalamic blood flow in control but not in chronically NOS blocked animals. Mean arterial blood pressure (A) and hypothalamic blood flow (B) before (0 min) as well as 10, 20 and 40 min after intravenous injection of 50 mg/kg L-NAME in control (open circles, $n=11$) and chronically L-NAME pretreated rats (filled circles, $n=7$). $^{***}p<0.001$ vs. "0 min".

L-arginine availability is not a rate-limiting factor of the resting NO production. L-Arginine, however, was shown to reverse L-NAME-induced cerebrovascular NOS-inhibition in a previous study (Sándor et al., 1994), and therefore we analyzed its effect also in chronically L-NAME pretreated animals ($n=12$). L-Arginine infusion normalized the MABP (Fig. 2A) and at the same time evoked marked hypothalamic hyperemia (Fig. 2B), without changing arterial blood gas or acid–base parameters (data not shown). Since reversal of the NOS blockade by L-arginine elevated the HBF over its normal level (in spite of the reduction of the MABP to its physiological level), it was reasonable to hypothesize that a compensatory vasodilator mechanism has been activated in the hypothalamic vasculature during chronic NO deficiency. The potential involvement of COX isoenzymes and vasodilator prostanoids in this adaptation process has been addressed in the second part of the study.

During chronic NOS blockade, the urinary concentrations of prostacyclin and prostaglandin E_2 metabolites increased significantly, indicating the activation of COX pathways in the systemic circulation (Fig. 3). In the hypothalamus of control rats, RT-PCR revealed the constitutive expression of all three known COX isoenzymes. Chronic NOS blockade, however, did not induce upregulation of any of the mRNAs (Fig. 4A). Consistently, the COX-1 and COX-2 protein levels were not significantly different in L-NAME pretreated and control animals (Fig. 4B). Furthermore, levels of vasodilator prostanoids in the hypothalamus did not change after chronic NOS blockade (Table 2). Interestingly, however, the concentration of

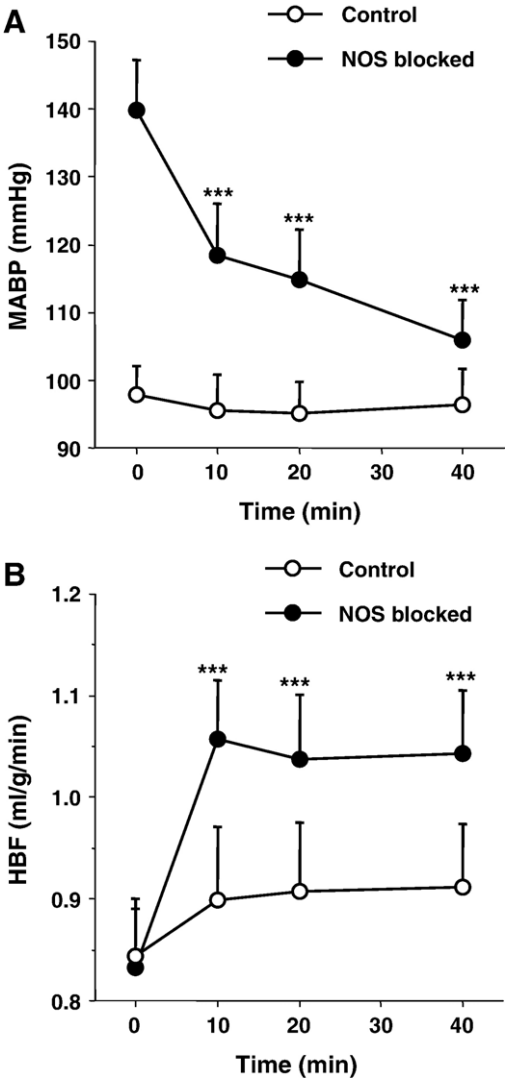

Fig. 2 – L-Arginine administration normalizes the blood pressure and elevates the hypothalamic blood flow over its physiological level in chronically NOS blocked animals. Mean arterial blood pressure (A) and hypothalamic blood flow (B) before (0 min) as well as 10, 20 and 40 min after the onset of intravenous L-arginine infusion in control (open circles, $n=12$) and chronically L-NAME pretreated rats (filled circles, $n=12$). $^{***}p<0.001$ vs. "0 min".

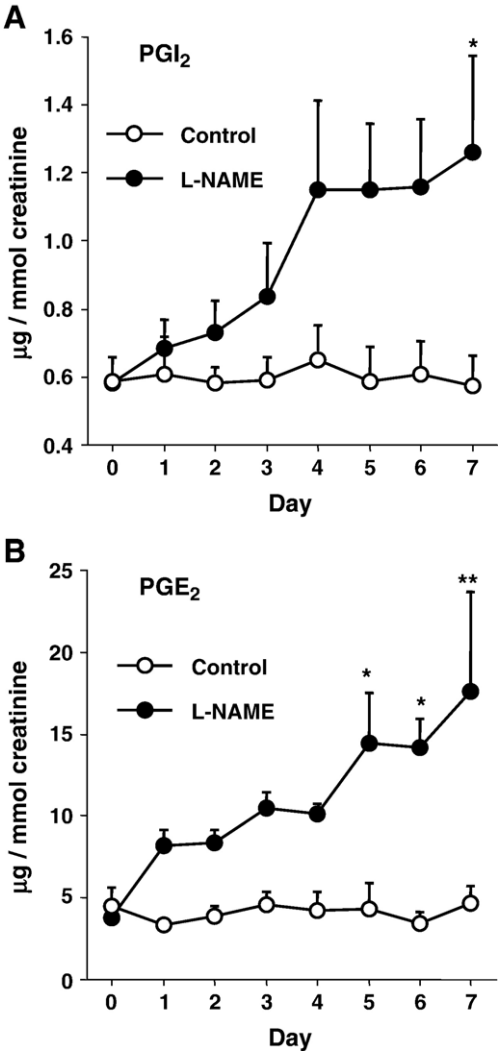

Fig. 3 – Chronic L-NAME administration increases the production of vasodilator prostanoids. Urinary concentrations of prostacyclin (PGI_2, panel A) and prostaglandin E_2 (PGE_2, panel B) metabolites in control (open circles, $n=5$) and in L-NAME-treated (filled circles, $n=5$) rats before ("Day 0") and for 1 week after the onset of the treatment. Values are normalized with the urinary creatinine content. $^{*}p<0.05$, $^{**}p<0.01$ vs. "Day 0".

vasoconstrictor prostanoid metabolites decreased significantly in the hypothalamus of L-NAME pretreated animals (Table 2).

COX inhibition by diclofenac (10 mg/kg iv) did not affect HBF or other physiological variables in control ($n=8$) or L-NAME-treated animals ($n=10$, Fig. 5A). In contrast, indomethacin (5 mg/kg iv) significantly reduced the HBF in control rats ($n=17$, Fig. 5B) without changing the MABP or arterial blood gas and acid–base parameters (data not shown). However, the HBF-decreasing effect of indomethacin was not enhanced in L-NAME pretreated animals ($n=13$, Fig. 5B). The vehicle of indomethacin (2 ml/kg saline iv) did not influence either the HBF or any other measured variables (data not shown). These results altogether indicate that the adaptation of the hypothalamic circulation to the reduction of NO synthesis is independent of vasodilator prostanoids. Reduction of vasoconstrictor prostanoid release, however, may significantly contribute to the normalization of the HBF after the loss of NO.

3. Discussion

Chronic NOS blockade in normotensive rats is a well established model of specific vascular dysfunction and hence of early vascular disease, since several pathophysiological states including essential hypertension, atherosclerosis and vasospasm are associated with some degree of deficiency of NO production or action (Faraci and Heistad, 1998). In case of endothelial damage or other NO-depleting situations, alternative vasodilator mechanisms may be upregulated in order to compensate for the loss of NO. Characterization of these compensatory mechanisms may help to improve therapeutic strategies in cardiovascular diseases.

Previous studies have indicated the ability of the cerebral circulation to adapt to chronic NO deficiency. First, it has been demonstrated that the resting cerebral blood flow,

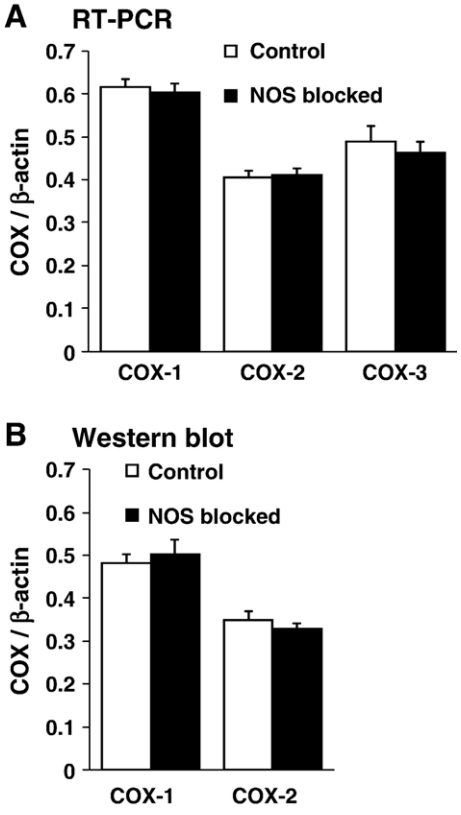

Fig. 4 – Chronic NOS blockade does not influence the mRNA and protein levels of COX isoenzymes in the hypothalamus. (A) Semiquantitative RT-PCR indicating equal expression of COX-1, COX-2 and COX-3 mRNA in the hypothalamus of control (open bars, $n=4$) and chronically L-NAME pretreated (filled bars, $n=4$) rats. Densitometric analysis of the RT-PCR results normalized by β-actin. (B) Western blot analysis indicating equal expression of COX-1 and COX-2 proteins in the hypothalamus of control (open bars, $n=6$) and chronically L-NAME pretreated (filled bars, $n=6$) rats. Densitometric analysis of the Western blot results normalized by β-actin.

Table 2 – Prostanoid concentrations (pg/mg tissue) in the hypothalamus of control and L-NAME pretreated rats

	Control ($n=9$)	L-NAME ($n=9$)
6-Keto-Prostaglandin $F_{1\alpha}$	9.8±1.9	9.3±2.3
Prostaglandin E_2	13.6±1.8	11.6±2.2
Prostaglandin D_2	11.6±1.3	12.2±2.9
Thromboxane B_2	40.2±4.2	26.1±4.1*
Prostaglandin $F_{2\alpha}$	37.7±3.9	18.6±3.9**

* $p<0.05$.
** $p<0.01$ vs. the corresponding "control" values.

which is significantly reduced after acute NOS inhibition, returns to its physiological level during chronically diminished NO synthesis (Atochin et al., 2003; Benyó et al., 1995; Huang et al., 1995; Kelly et al., 2000). Second, chronic NOS blockade influences less severely the cerebral vasodilator responses evoked by hypercapnia, acetylcholine or NMDA than the acute blockade (Pelligrino et al., 1996; Wang et al., 1994; Xu et al., 2003). In the present study, although we were unable to identify the compensatory mechanism(s) responsible for the normalization of the hypothalamic blood flow, we have clarified three important aspects of the adaptation during chronic NO deficiency.

First, our observation that acute NOS blockade by L-NAME infusion induced a dramatic HBF decrease in control but not in chronically L-NAME-treated animals clearly indicates that during chronic NO deficiency an NO-independent vasodilator mechanism is involved in the maintenance of the HBF. This conclusion is not obvious since the constitutive NOS activity of the hypothalamus is only reduced by approximately 90% during chronic oral L-NAME treatment (Benyó et al., 1995). Taken into account that in case of diminished NO production the cerebrovascular reactivity to NO increases (Kovách et al., 1992; Moncada et al., 1991), the remnant 10% NOS activity could contribute significantly to the maintenance of the cerebral blood flow. Indeed, acute L-NAME treatment was shown to reduce the blood flow in the neocortex, hippocampus and striatum of rats subjected to chronic NOS blockade (Kelly et al., 1995), indicating that the remnant NO production contributes to the maintenance of the blood flow in these brain regions. Our results, however, indicate the importance of an NO-independent mechanism in the normalization of the hypothalamic blood flow during chronic NOS blockade.

Second, the observation that reversal of the chronic NOS blockade by L-arginine infusion elevates the HBF over its normal level (in spite of the reduction of the MABP to its physiological level) suggests the appearance of a vasodilator mechanism, which is activated to compensate the loss of NO. This has to be a very effective mechanism, since the high regional blood flow increase during simultaneous arterial pressure decrease indicates a remarkable reduction of the cerebrovascular resistance in the hypothalamus. Numerous observations in different experimental systems indicated that prostacyclin may act as a backup vasodilator in chronic NO deficiency. For instance, elevated plasma levels of prostacyclin and increased urinary excretion of the prostacyclin metabolite 6-keto-prostaglandin $F_{1\alpha}$ were reported after chronic inhibition of NOS in rats (Cao et al., 1999; Danielson and Conrad, 1996; Henrion et al., 1997; Tomida et al., 2003). Furthermore, the endothelium-dependent relaxation by bradykinin was sensitive to indomethacin in coronary arteries of dogs subjected to chronic NOS blockade but not in controls (Puybasset et al., 1996). A bradykinin-induced increase in prostacyclin production was greater in coronary arteries taken from nitro-L-arginine-treated dogs, which difference was attributable to the upregulation of the endothelial COX-1 isoform during chronic inhibition of NOS (Beverelli et al., 1997, Puybasset et al., 1996).

The third conclusion of our present study is, however, that prostacyclin or other vasodilator prostanoids are not involved in the adaptation of the hypothalamic circulation to chronic NO deficiency. Although we have observed increased urinary

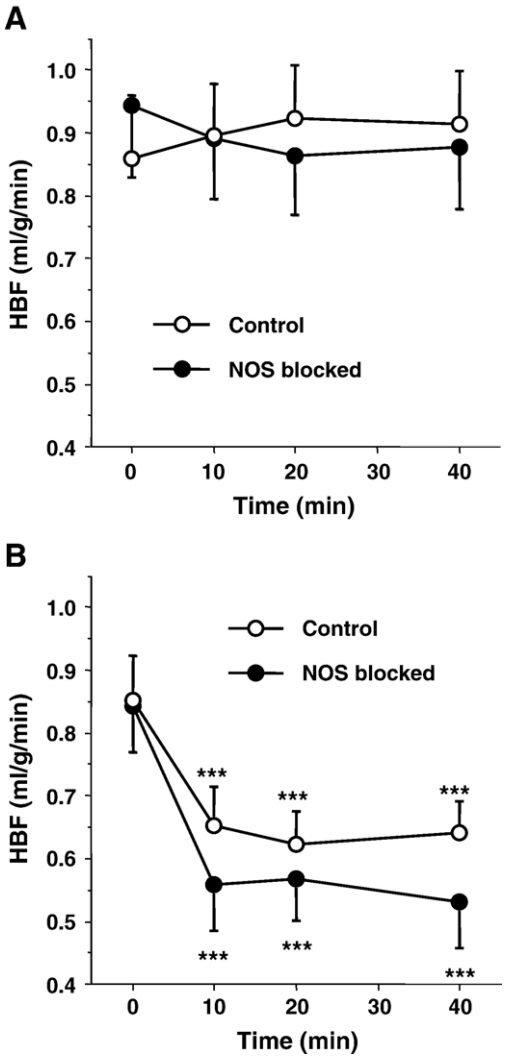

Fig. 5 – The effect of COX-inhibition by diclofenac or indomethacin on the hypothalamic blood flow is not impaired in animals subjected to chronic NOS blockade. Hypothalamic blood flow before (0 min) as well as 10, 20 and 40 min after intravenous injection of 10 mg/kg diclofenac (A) or 5 mg/kg indomethacin (B) in control (open circles, $n=8$ and 17 on panels A and B, respectively) and chronically L-NAME pretreated rats (filled circles, $n=10$ and 13 on panels A and B, respectively). ***$p<0.001$ vs. "0 min".

excretion of vasodilator prostanoid metabolites during chronic NOS blockade, the hypothalamic levels of these prostanoids remained unchanged. Furthermore, none of the COX isoforms showed an increased expression in the hypothalamus of rats subjected to chronic L-NAME treatment. In accordance, the influence of indomethacin or diclofenac on the HBF was not changed after chronic NOS blockade. All these data clearly indicate that neither prostacyclin nor any other vasodilator prostanoids play a significant role in the adaptation of the hypothalamic circulation to chronic NO deficiency.

We have observed a significant reduction in the hypothalamic levels of vasoconstrictor prostanoids after chronic NOS blockade. This finding is particularly interesting in the light of our previous observations showing that the cerebral vasoconstriction induced by inhibition of the resting NO synthesis is partly due to the increased reactivity of the cerebral vessels to TXA_2 (Benyó et al., 1998; Lacza et al., 2001a). During chronic NOS blockade, however, the reduction of vasoconstrictor prostanoid release may compensate this vascular hyperreactivity and contribute to the normalization of the cerebral vascular resistance and blood flow.

A further interesting finding of the present study is the difference between the effects of indomethacin and diclofenac on the resting HBF. Similar differences between the effectiveness of these two COX inhibitors were reported previously: indomethacin but not diclofenac inhibited the cerebral hyperemic response to hypercapnia (Quintana et al., 1988) and to acetazolamide-induced extracellular acidosis (Wang et al., 1993) in rats. Both compounds, in doses used in our present and the above cited studies, were shown to inhibit prostanoid production in the rat brain (Abdel-Halim et al., 1978). However, indomethacin was also reported to inhibit cerebrovascular prostacyclin receptors, which effect could explain its stronger influence on the resting HBF (Parfenova et al., 1995). The difference of the relative potencies of these inhibitors towards the different COX isoenzymes, i.e., the higher affinity of indomethacin to COX-1 and diclofenac to COX-2 (Chandrasekharan et al., 2002; Mitchell et al., 1994), is also a possible explanation for the different HBF-effects. Recent observations that COX-1-deficient mice show diminished blood flow in some brain regions including the hypothalamus (Niwa et al., 2001) also suggest that this isoenzyme mediates the release of vasodilator prostanoid(s) influencing the resting cerebrovascular tone.

In conclusion, the present study demonstrates that during chronic NO deficiency the hypothalamic blood flow remains unaltered and activation of one or more vasodilator mechanisms appear to be responsible for this adaptation. Constitutive prostanoid release, presumably by COX-1, contributes to the maintenance of the hypothalamic blood flow under physiological conditions, but the influence of this mechanism is not enhanced during chronic NO deficiency. Although the enhanced urinary excretion of PGI_2 and PGE_2 metabolites indicates COX activation in the systemic circulation during chronic NOS blockade, we conclude that the adaptation of the hypothalamic circulation to the reduction of NO synthesis is independent of vasodilator prostanoids. Reduced release of vasoconstrictor prostanoids, however, may significantly contribute to the normalization of HBF after chronic loss of NO.

4. Experimental procedures

All investigations were performed in adult male Wistar rats (b.w. 300–400 g) under the guidelines of the Hungarian Law of Animal Protection (243/1988) and approved by the Semmelweis University Committee on the Ethical Use of Experimental Animals (590/99 Rh). During the pretreatment period, the animals were housed individually and drinking either normal tap water or a 1 mg/ml L-NAME solution for 1 week.

The in vivo experiments were carried out in 102 rats anesthetized with Urethane (1.3 g/kg ip; Sigma, St. Louis, MO, USA) and spontaneously breathing via a trachea cannula. Catheters were inserted into both femoral arteries (to measure blood pressure and for blood sampling) and into the left femoral vein for drug administration. The skull was fixed in a stereotaxic head-holder. Body temperature was kept constant between 36 and 38 °C with a controlled heating lamp. Systemic arterial pressure was continuously recorded on a polygraph (Model 7E, Grass, Quincy, MA, USA). Hypothalamic blood flow (HBF) was determined by using Aukland's H_2-gas clearance method (Aukland et al., 1964), as described (Horváth et al., 2003). Briefly, a 100-μm in diameter Teflon-coated Pt electrode with a 1-mm bare tip was introduced stereotactically into the ventromedial hypothalamic area. H_2 washout curves were produced by H_2-gas inhalation and were recorded on the polygraph. Bi-exponential analysis of the washout curves by a computer program based on the Marquardt algorithm (Marquardt, 1963) was used to calculate the HBF values.

Blood gas parameters (PCO_2, PO_2, O_2 saturation) and pH in femoral arterial samples were measured by a Radiometer Blood Gas Analyzer (ABL-300, Copenhagen, Denmark) at the times of HBF determinations. Mean arterial blood pressure (MABP) was determined at the same time. After the last measurements, the anesthetized animals were rapidly exsanguinated and hypothalamic tissue samples were excised and frozen rapidly. Total hypothalamic NOS activity was measured on the basis of the formation of labeled citrulline from labeled L-arginine (Nagy et al., 2000, Sándor et al., 1994). Tissue samples were homogenized in brain homogenizing solution containing 50 mM Tris–HCl (pH 7.4), 0.3 M sucrose, 0.1 mM EDTA, 1 mM dithioerythritol and 1 mM phenyl-methyl-sulfonylfluorid (PMSF, protease inhibitor). Homogenates were added to the samples containing 5 mM HEPES, 1 mM NADPH, 10 μM tetrahydrobiopterin, 30 μM calmodulin, 2.5 mM $CaCl_2$ at pH 7.4 and reaction was initiated by adding 20 μM ^{3}H-arginine (all final concentrations). After 30 min incubation, the reaction was stopped, samples were put onto 2-cm Dowex-50×8 resin columns and eluates were mixed with 5-ml dioxane-based scintillation fluid and measured in a Beckman TriCarb liquid scintillation spectrometer. Protein contents were determined by the biuret reaction, after precipitating the samples with $HClO_4$ and redissolved in NaOH. NOS-specific activities were calculated in picomole citrulline formed per minute per milligram protein units.

For the determination of hypothalamic tissue prostanoid levels (prostaglandin D_2, E_2 and $F_{2\alpha}$, as well as the stable prostacyclin and thromboxane A_2 metabolites 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2, respectively), two groups of animals, pretreated with the same protocol as described

above, were rapidly exsanguinated in deep ether anesthesia. Hypothalamic tissue samples were frozen rapidly and kept at −75 °C until further analysis. In order to measure prostanoid concentrations, the hypothalamus was homogenized in 4 volumes of water (weight/volume) with an UltraTurrax and afterwards with a glass potter. Deuterated internal standards were added and prostanoids were extracted with 1 ml ethyl acetate/hexane (7:3, v/v) as described (Schweer et al., 1994). The sample was evaporated and reconstituted in 1 ml water. Further derivatization and gas chromatography/triple quadrupole mass spectrometry (GC/MS/MS) conditions were described previously (Schweer et al., 1994).

For the determination of COX mRNA expression by RT-PCR, total RNA was isolated from hypothalamic tissue samples by SV Total RNA Isolation System (Promega, Madison, WI, USA). We used the same primer sets to detect COX-1, COX-2 and β-actin mRNA as previously described (Kis et al., 2003). For COX-3 detection, the sense primer (5′-CAGAGTCATGAGTCGTGAG; 1376773–1376791 bases of GenBank NW_047653.1) was designed to bind to intron 1 and the antisense (5′-AGAGGGCAGAATGCGAGTAT; 501–520 bases of GenBank S67721) to bind to exon 5 of the rat COX-1 gene, as previously described (Kis et al., 2004). The expected length of the RT-PCR product was 573 base pairs. Our COX-3 primer set distinguishes between COX-1 and COX-3 and also distinguishes between COX-3 and "partial" COXs (PCOX-1a, PCOX-1b) because our antisense primer binds to exon 5 which is lacking in PCOXs (Chandrasekharan et al., 2002). The conditions of the PCR reactions were set according to the instructions of the Qiagen OneStep RT-PCR Kit. Briefly, the RT reaction was performed at 50 °C for 30 min, followed by the initial PCR activation step at 95 °C for 15 min. Thirty-seven PCR cycles were performed with the following parameters: denaturation 30 s at 94 °C, annealing 45 s at 58 °C and extension at 72 °C for 1 min. The last step of the process was a final extension at 72 °C for 10 min. The COX PCR products were identified by the size and the sequence. Automated DNA sequencing of the PCR products was performed on an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Western blotting method was described in details elsewhere (Szabó et al., 2004). Briefly, the hypothalamic tissue samples were prepared as described above and protein content of the homogenate was determined by biuret reagent and then the samples were heated in a buffer containing 10% glycerol, 2.5% 2-mercaptoethanol, 5% SDS and 0.1% bromophenol blue marker dye. After heat treatment, equal protein amounts (70 μg in 10 μl) were applied onto the gel. After the electrophoretic separation, samples were transferred to nitrocellulose membranes. Samples were then blocked with 3% BSA for 24 h at 4 °C and tested for COX-1 and COX-2 isoenzymes using primary antibodies (Cayman, Ann Arbor, MI, USA, 120 min, room temperature, 1:500 dilution). Anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Sigma, 1:2000 dilution) were then added for 30 min and finally membranes were treated with enhanced chemiluminescence solution (Amersham, Buckinghamshire, UK) to develop chemiluminescent bands that were visualized on an X-ray film (Medifort, Forte, Vác, Hungary). Bands were converted to computer-edited files. Positive controls were used for both isoenzymes and molecular mass standard was also run.

In order to measure the urinary excretion of prostacyclin and prostaglandin E_2 metabolites, the animals were housed individually in metabolic cages (Techniplast, Buguggiate, Italy). Urine samples were collected daily from control and L-NAME-treated animals. The concentration of urinary prostacyclin metabolites (2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$ and 6-keto-prostaglandin $F_{1\alpha}$) was determined by a commercial enzyme immunoassay kit (Assay Designs Inc., Ann Arbor, MI, USA). The concentration of the urinary PGE_2 metabolite 11α-hydroxy-9,15-dioxo-2,3,4,5,20-pentanor-19-carboxyprostanoic acid was measured by GC/MS/MS as described previously (Schweer et al., 1994). Urinary prostanoid concentrations were normalized with the concentration of creatinine, which was measured by the Metra creatinine assay kit (Quidel, San Diego, CA, USA).

L-NAME (Sigma), indomethacin (Merck) and diclofenac (Sigma) were given in intravenous doses of 50, 5 and 10 mg/kg, respectively, dissolved in 2 ml/kg saline. Intravenous L-arginine (Sigma, dissolved in saline) administration consisted of a 30 mg/kg initial bolus followed by 10 mg/kg/min infusion with a rate of 0.1 ml/min.

All values are presented as mean ± SEM; *n* represents the number of animals. Statistical analysis was performed using ANOVA for repeated measurements or one-way ANOVA followed by Tukey's post hoc test. For comparison of two groups, Student's unpaired *t*-test was used. A *p* value of less than 0.05 was considered to be statistically significant.

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Influence of the heme-oxygenase pathway on cerebrocortical blood flow

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Heme-oxygenase (HO)-derived carbon monoxide (CO) is generated in the cardiovascular and in the central nervous systems. Endogenous CO exerts direct vascular effects and has also been shown to inhibit nitric oxide synthase (NOS). In the current study, the heme-oxygenase blockade [zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG), 45 μmol/kg intraperitoneally] decreased cerebral CO production and increased cerebrocortical blood flow (CBF) in anesthetized rats. This latter effect was abrogated by the NOS blockade (50 mg/kg L-NAME intravenously). Furthermore, inhibition of CO production had no effect on stepwise hypoxia/hypercapnia-stimulated increases in CBF. Our results indicate that endogenous CO reduces the resting CBF via inhibition of NOS but fails to influence the CBF response to hypoxia and hypercapnia in adult rats. *NeuroReport* 18:1193–1197 © 2007 Lippincott Williams & Wilkins.

Keywords: carbon monoxide, cerebral circulation, heme oxygenase, hypercapnia, hypoxia, nitric oxide synthase

Introduction

Heme oxygenase (HO) catalyzes the degradation of monomeric free heme to generate carbon monoxide (CO) [1–4]. Previously thought to be simply a waste product, endogenously formed CO is now known to serve as a messenger in numerous physiological and pathophysiological processes [1–4]. In the cardiovascular system, CO is an activator of soluble guanylate cyclase and can promote the relaxation of vascular smooth muscle, activate large-conductance calcium-activated K^+ channels and inhibit vascular smooth muscle cell proliferation [4]. In the central nervous system, CO plays an important role in synaptic plasticity, learning and memory processes, as well as in the regulation of hypothalamic neuropeptide release [1].

Expression of HO isoenzymes in neural and vascular tissues is well documented [5,6]. It is also known that freshly isolated cerebral microvessels, as well as microvascular endothelial cells, produce CO. The generation of CO is increased by stimulation of the ionotropic glutamate receptors [6,7]. The role of endogenous CO, however, in the regulation of the adult cerebral circulation is not well understood [3]. In newborn pigs, both exogenous CO and the HO substrate, heme, induce pial arterial dilatation; and the latter can be inhibited by the HO blocker chromium mesoporphyrin (CrMP) [5]. In addition, hypoxia-induced vasodilation decreases, whereas hypocapnia-induced vasoconstriction increases after topical application of CrMP [5,8]. Interestingly, pial arteriolar dilation in response to hypercapnia remains unaltered after topical CrMP treatment [5], but gets reduced after systemic administration of another HO inhibitor: tin protoporphyrin [9]. Cerebrospinal fluid (CSF) levels of endogenous CO increase during seizures [9,10], and seizure-induced pial arteriolar dilatation and the cerebral blood-flow increase are attenuated by HO inhibitors in piglets and adult male rats, respectively [10–12]. Glutamate receptor-mediated dilation of newborn pig pial arterioles also involves the HO pathway [7,13]. It appears that endogenous CO dilates piglet cerebral arterioles by augmenting the coupling of Ca^{2+} sparks to K_{Ca} channels in smooth muscle cells [14].

Although the majority of the cerebrovascular studies have been carried out in newborn models, cerebral vasoregulatory mechanisms are known to change substantially during maturation. Furthermore, recent studies confirm that CO-synthesizing and nitric oxide-synthesizing enzymes are colocalized in cerebral vessels and perivascular neuronal cells [6,15]. As CO has been shown to inhibit nitric oxide synthase (NOS) activity [16], it raises the possibility of interactions between these pathways in cerebral circulation. We, therefore, wanted to determine the influence of the HO pathway on the cerebrocortical blood flow (CBF) in adult animals in the presence and absence of a functional NOS system, under resting and combined hypoxic/hypercapnic conditions.

Materials and methods

Experiments were performed using adult male Wistar rats (body weight 300–400 g), under the guidelines of the

Hungarian Law of Animal Protection (243/1988), and with approval from Semmelweis University Committee on Ethical Use of Experimental Animals (590/99 Rh). During the in-vivo experiments, the animals were anesthetized with urethane (1.3 g/kg intraperitoneally, Sigma), while spontaneously breathing via a fitted trachea cannula. Indwelling catheters were inserted into the right femoral artery for blood pressure measurements and sampling and into the left femoral vein for drug administration. Femoral arterial blood gases ($PaCO_2$, PaO_2, O_2 saturation) and acid–base parameters (pH, standard base excess) were measured with an ABL-300 Radiometer Blood Gas Analyzer (Brønshøj, Denmark). CBF was measured with a two-channel laser-Doppler (LD) monitor (MBF 3D, Moor Instruments, Devon, UK) as described previously [17], with infrared laser light (780 nm) penetrating 1 mm into the brain, spanning 7 mm^2 of parietal cortex.

In the first part of the study, the effect of HO blockade on the CBF was studied under physiological (normoxic/normocapnic) conditions. The first experimental group served as a vehicle-treated control and CBF was determined before and after an intraperitoneal injection of 3 ml saline. In the second group, ZnDPBG [zinc deuteroporphyrin 2,4-bis glycol, Frontier Scientific (Logan, Utah, USA), 45 μmol/kg intraperitoneally] was applied. This had previously been shown to effectively inhibit HO activity in the rat brain [18]. Animals in the third and fourth experimental groups were pretreated with the NOS inhibitor L-NAME (N^G-nitro-L-arginine methyl ester, Sigma, 50 mg/kg intravenously). Thirty minutes later the third and fourth groups received saline or ZnDPBG, respectively. CBF was determined in all experimental groups before as well as 15, 30 and 45 min after the administration of vehicle or ZnDPBG.

To verify the inhibitory effect of ZnDPBG on brain HO activity *in vivo*, matched series of animals were treated with either ZnDPBG or vehicle as described above. Thirty minutes later, innate cerebral CO generation was determined using solid-phase gas chromatography (custom built Peak Performer 1 RCP; Peak Laboratories LLC, Mountain View, California, USA) as described in detail elsewhere [8,19]. Briefly, isolated midbrain tissues from each animal were individually sonicated in Krebs' buffer and divided into eight aliquots. For each animal midbrain sample, head space CO was measured in quadruplicate vials after being maintained at 2°C or the remaining matched aliquots after being incubated at 37°C for 60 min. The differences between the incubated and cold vials were expressed as μmol CO generated/kg of wet tissue per hour. ZnDPBG induced a reduction of cerebral HO activity from 4.58 ± 0.87 to 2.47 ± 0.36 μmol/kg per hour ($P = 0.025$).

In the second part of the study, the effect of HO blockade on the cerebrocortical hyperemic response to hypoxia/hypercapnia (H/H) was examined. H/H was induced in a stepwise manner by the administration of different gas mixtures (5%O_2–20%CO_2–75%N_2 for producing moderate H/H and 20%CO_2–80%N_2 for producing severe H/H, respectively) with a constant flow of 3 l/min through a 5 ml chamber connected to the trachea, at atmospheric pressure. CBF was recorded continuously and peak CBF values were determined during the two 10-min long steps of H/H. After the first moderate and severe H/H challenge, the animals were divided into two experimental groups receiving either saline or ZnDPBG intraperitoneally. Thirty minutes later the moderate and severe H/H was repeated in both groups and peak CBF values were determined from the continuous recording of CBF.

The in-vitro experiments were performed in the middle cerebral arteries (MCAs) supplying the parietal cortex, the site of our in-vivo CBF measurements. MCA segments were prepared from adult male Wistar rats as described previously [20] and studied in a conventional myograph system (Danish Myo Technology A/S, Aarhus, Denmark). The effects of 10 μM ZnDPBG or 10 μM bradykinin (as positive control) were tested after precontraction of vessels by 10 μM prostaglandin $F_{2\alpha}$.

Results

Effects of heme-oxygenase blockade under normoxic/normocapnic conditions

Arterial blood gas and acid–base parameters were within the physiological range (PaO_2: 90–105 mmHg, O_2 saturation: 95–97%, $PaCO_2$: 40–45 mmHg, pH: 7.32–7.36, standard base excess: −3–0 mM) during the experiments. L-NAME pretreatment increased mean arterial blood pressure (from 102.2 ± 3.0 to 146.3 ± 4.1 mmHg, $P < 0.001$), decreased heart rate (from 424 ± 12 to 373 ± 12 beats/min, $P = 0.012$) and reduced CBF (from 359 ± 18 to 258 ± 13 AU, $P < 0.001$).

Administration of ZnDPBG, which inhibits CO formation, increased CBF, but saline vehicle alone had no effect (Fig. 1). Inhibition of NO formation by L-NAME pretreatment

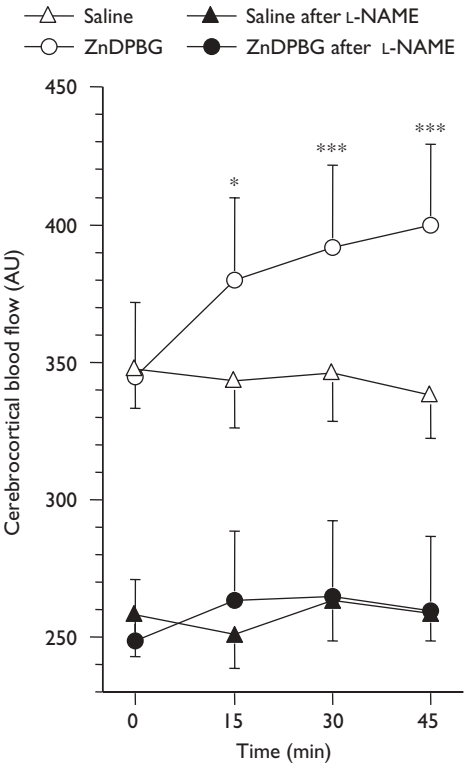

Fig. 1 Cerebrocortical blood flow (CBF) before (0 min) as well as 15, 30, and 45 min after intraperitoneal injection of saline (triangles) or 45 μmol/kg zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG; circles) in naïve (open symbols) or in N^G-nitro-L-arginine methyl ester (L-NAME) pretreated (filled symbols) rats. *$P = 0.016$, ***$P < 0.001$ vs. '0 min', with repeated measures analysis of variance and Tukey's *post hoc* test, $n = 12$–16. AU, arbitrary unit.

completely blocked ZnDPBG-induced elevations of CBF (Fig. 1). Under in-vitro conditions, 10 μM ZnDPBG had no effect on the tension of isolated MCA segments; this effect cannot be attributed to endothelial damage as functionally intact endothelium was confirmed by relaxation to 10 μM bradykinin (79±5% of the precontraction induced by prostaglandin $F_{2\alpha}$).

Effects of heme oxygenase blockade on the hypoxia and hypercapnia induced increase of cerebrocortical blood flow

Before the initial H/H challenge, baseline cardiovascular, arterial blood gas and acid–base parameters were within physiological ranges in both (later ZnDPBG-treated or saline-treated) experimental groups. During moderate H/H, PaO_2 was reduced to 60–65 mmHg, $PaCO_2$ was increased to 80–85 mmHg and pH decreased to 7.10–7.15 (Fig. 2a). Despite no change in MAP, CBF increased in both experimental groups by ~45% (Fig. 2b). During severe H/H, PaO_2 was reduced to 45–50 mmHg, $PaCO_2$ was increased to 95–100 mmHg and pH decreased to 7.05–7.10 (Fig. 2a); CBF increased in both experimental groups by ~65% (Fig. 2c), whereas MAP remained unchanged.

The second stepwise H/H challenge, after ZnDPBG or saline, induced similar changes in the blood-gas tensions and pH as the initial challenge had done (Fig. 2a), without affecting MAP (data not shown). Most important, neither ZnDPBG nor saline treatment had any effect on CBF responses to moderate (Fig. 2b) or severe (Fig. 2c) H/H, when compared with the pretreatment values.

Discussion

Our current study provides evidence for the interaction between the HO and NOS pathways in the regulation of the CBF that had been described in the other vascular beds [19,21,22]. The CBF increase after administration of ZnDPBG and its inhibition with L-NAME pretreatment indicate that constitutive CO release tonically suppresses the NO production and consequently reduces blood flow in the cerebral cortex.

The interactions between CO and NO are well documented. Endogenous CO is known to relax smooth muscle, but in the presence of a functional endothelium CO-induced inhibition of NOS can promote vasoconstriction [4,21]. Systemic inhibition of CO formation, with CrMP, has no effect on hindlimb vascular resistance but promotes a marked and sustained vasoconstriction after L-NAME pretreatment [19]. Delta-aminolevulinic acid, a heme precursor that promotes CO formation, causes vasoconstriction in intact isolated rat gracilis muscle arterioles, but is converted to vasodilation by removal of the endothelium and by pretreatment with L-NAME to inhibit NO formation [22]. These earlier studies provide evidence that CO-induced constriction arises from the suppression of endothelial NO production. Furthermore, L-NAME increased the reduction of renal blood flow and augmented the contraction of isolated renal interlobular arteries in response to the HO inhibitor stannous mesoporphyrin [23].

It has been suggested that HO-derived CO can be a primary regulator of NO production [16], and a growing body of evidence continues to support that contention. Ishikawa *et al.* [15] have demonstrated that HO-2 is

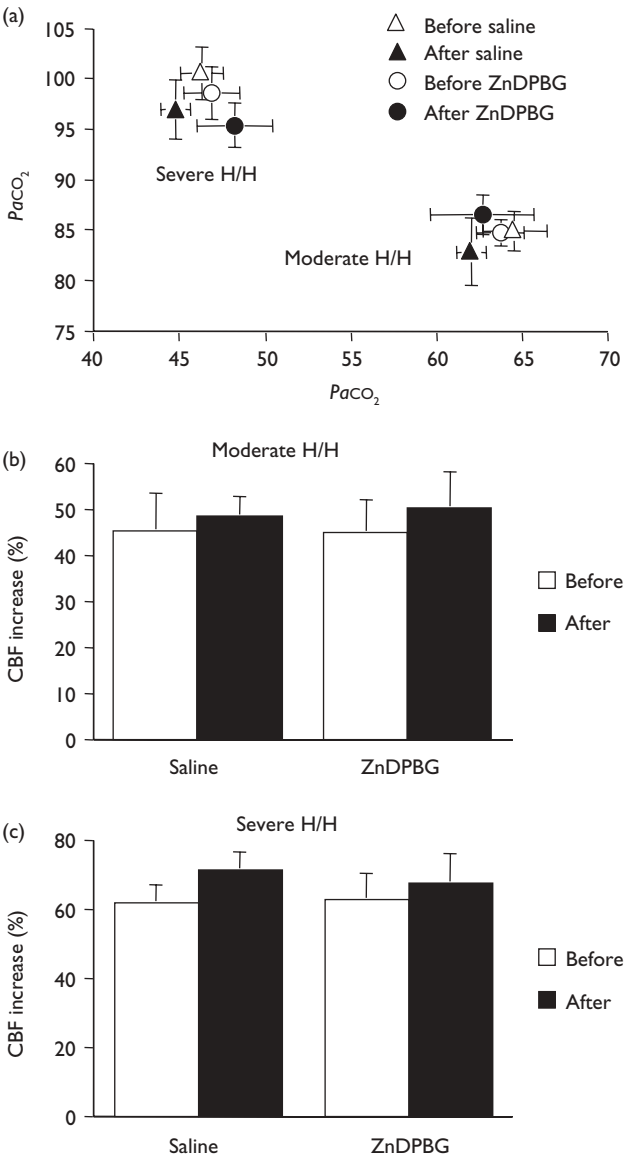

Fig. 2 Arterial blood-gas tensions (a) as well as changes of the cerebrocortical blood flow (CBF) during moderate (b) and severe (c) hypoxia/hypercapnia before (open symbols/bars) and after (filled symbols/bars) intraperitoneal injection of saline [triangles on (a)] or 45 μmol/kg zinc deuteroporphyrin 2,4-bis glycol [ZnDPBG; circles on (a)].

colocalized with endothelial NOS (eNOS) in the cerebrovascular endothelium as well as with neuronal NOS (nNOS) in neurons and arachnoid trabecular cells, suggesting that the colocalization of the CO-generating and NO-generating pathways are sufficient for interaction. Tricarbonyldichlororuthenium (II) dimer, a CO-releasing molecule, was shown to reduce NO release from cultured endothelial cells. In addition, HO blockade by zinc protoporphyrin IX (ZnPP) induced CO-reversible and L-NAME-reversible increase of the cerebrovascular and perivascular NO production by 70–80%. These results are comparable with the ZnDPBG-induced 67% increase of the NOS activity in the rat hypothalamus observed in our previous study [24]. Most important, ZnPP induced a dose-dependent increase of the pial arteriolar diameter, which could be prevented by coadministration of either CO or L-NAME, indicating the

involvement of NO in the mediation of the vasodilation. Our current results confirm and complement these observations by providing direct evidence for the significance of the CO–NOS interaction at the level of cerebrocortical blood perfusion. In our study, however, ZnDPBG failed to relax the isolated middle cerebral artery, whereas Ishikawa *et al.* [15] reported marked pial arteriolar dilatation in response to ZnPP in rats. As, in the latter study, pial arteriolar responses were determined *in vivo*, the most plausible explanation of the discrepancy between the two findings is that reduction of nonvascular CO release or augmentation of nonvascular NO release plays an important role in CBF increase after inhibition of HO.

As HO degrades heme to form CO, iron and biliverdin, alternative interactions might explain the influence of HO activity on cerebrovascular NO functions [25]. It has been described that CO binds directly to NOS and inhibits NO production. Iron released in the course of heme degradation by HO can inhibit de-novo NOS synthesis by inhibiting its nuclear transcription, but our currently reported effect is rapid and is, therefore, unlikely to arise from changes in protein synthesis. Alternatively, HO activity might influence NOS synthesis by changing heme availability and the active site of NOS requires two heme molecules. Finally, the NOS and HO pathways might interact by competing for NADPH as a cofactor required for their enzymatic activity. It has, however, been shown that exogenous CO promotes vasoconstriction and mimics the effects of increased HO activity, suggesting a nicotinamide adenine dinucleotide phosphate-independent mechanism [22].

Our current study also shows that the HO–CO pathway does not play a discernible role in hypoxia/hypercapnia-induced increased CBF in adult rats. This observation is different from that of previous studies in newborn models in which HO blockade inhibited the pial arteriolar dilation in response to hypoxia or hypercapnia [5,9]. As the regulatory processes of the CBF during hypoxia or hypercapnia show marked alterations during maturation, we can only speculate that our contrasting findings arise from age-related differences in the models. Furthermore, we cannot exclude the possibility that endogenous CO can have dual cerebrovascular effects during H/H: (i) it directly facilitates the H/H induced CBF increase, but (ii) its simultaneous inhibitory influence on NO synthesis neutralizes this effect. Further studies can clarify this hypothesis and investigate the role of endogenous CO in the adaptation of the cerebral circulation to chronic hypoxia when the expression of HO-1 is increased in the brain [2].

Conclusions

Our results indicate that endogenous CO exerts a tonic influence on resting CBF by inhibiting NO synthesis. Furthermore, inhibition of the HO–CO pathway increases CBF most likely by increasing neuronal NO production. Finally, the HO–CO pathway does not influence hypoxia and hypercapnia-induced CBF increase in adult rats.

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Carbon monoxide–prostaglandin E_2 interaction in the hypothalamic circulation

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The heme oxygenase (HO)–carbon monoxide pathway was earlier shown to increase hypothalamic blood flow after inhibition of nitric oxide synthesis in rats. We hypothesized that this effect is mediated by prostaglandin E_2 (PGE_2). Inhibition of constitutive HO activity decreased cerebral PGE_2 production and simultaneously increased hypothalamic nitric oxide synthase (NOS) activity without changing hypothalamic blood flow. Furthermore, HO blockade induced cyclooxygenase-dependent decrease and NOS-mediated increase of the hypothalamic blood flow after inhibition of NOS and cyclooxygenase, respectively. Therefore, constitutive carbon monoxide release seems to have two indirect effects on the hypothalamic circulation: vasodilation mediated by PGE_2 and vasoconstriction as a result of NOS inhibition. *NeuroReport* 19:1601–1604 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: carbon monoxide, cerebral circulation, cyclooxygenase, heme oxygenase, hypothalamus, nitric oxide synthase, prostaglandin E_2

Introduction

Carbon monoxide (CO) produced by heme oxygenase (HO) enzymes has been recently recognized as an important mediator in numerous physiological and pathophysiological processes. In the central nervous system, CO plays an important role in synaptic plasticity, learning and memory processes, thermal regulation and modulation of hypothalamic neuropeptide release [1,2]. Furthermore, the HO pathway has been recently implicated as one of the main regulatory components of the cerebral circulation [3,4].

Most of the experimental data regarding the cerebrovascular functions of endogenous CO has been obtained in newborn or juvenile pigs [4]. It has been shown that CO dilates piglet cerebral arterioles by enhancing the coupling of Ca^{2+} sparks to $K_{Ca^{2+}}$ channels in smooth muscle cells. Glutamate activates the HO pathway in cerebral vessels and astrocytes and endogenous CO mediates glutamate and seizure-induced pial arteriolar dilation in newborn pigs. Furthermore, endogenous CO contributes to the cerebral vasodilation in response to hypoxia and hypotension [4].

The role of endogenous CO in the adult cerebral circulation is far less understood, although a recent study indicated that the cerebrovascular effects of the HO pathway might substantially change with age [5]. In adult rats, the cerebrovascular responses to seizures and to the ionotropic glutamate receptor agonist alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid are also mediated, at least in part, by endogenous CO [6,7]. The effects of hypoxia and hypercapnia on the cerebrocortical circulation are, however, independent of the HO pathway [6,8]. Interestingly, the inhibition of constitutive HO activity results in nitric oxide (NO)-mediated pial arteriolar dilation and cerebrocortical hyperemia both in newborn pigs and in adult rats [8–10] indicating that endogenous CO tonically suppresses NO-mediated cerebrocortical vasodilation. In a recent study, we have observed this indirect vasoconstrictor effect of the HO pathway also in the rat hypothalamic circulation but it was compensated by a simultaneous vasodilator action of CO through a yet unidentified mechanism [11]. In this study, we hypothesized that prostaglandin E_2 (PGE_2) mediates the vasodilatory effect of constitutive HO activity on the hypothalamic blood flow of adult rats. Although vasodilator prostanoids are not involved in the mediation of CO-induced cerebrovascular responses in newborn pigs, [4] in a recent study endogenous CO was shown to stimulate the release of PGE_2 from hypothalamic tissue explants of adult rats [12]. Our first aim was to investigate the presence of this interaction *in vivo* by measurement of prostanoid levels in the cerebrospinal fluid after inhibition of constitutive HO activity. Furthermore, we aimed to evaluate the significance of the HO–PGE_2 interaction in the regulation of the resting hypothalamic blood flow.

Methods

The experiments were carried out in adult (300–400 g body weight) male Wistar rats anesthetized with 1.3 g/kg intraperitoneally (i.p.) injected urethane, and spontaneously

breathing through a trachea cannula. Catheters were inserted into both femoral arteries (for systemic arterial blood pressure measurement and for blood sampling) and into the left femoral vein (for drug administration). The skull was fixed in a stereotaxic head-holder. Body temperature was kept constant between 36 and 38°C with a controlled heating lamp.

Systemic arterial pressure was recorded continuously on a polygraph (Model 7E, Grass, Quincy, Massachusetts, USA). Hypothalamic blood flow was determined by using Aukland's H_2-gas clearance method as described elsewhere [13]. Briefly, a teflon-coated platinum electrode of 100 μm diameter with a 1 mm bare tip was introduced stereotactically into the ventromedial hypothalamic area. H_2-wash-out curves were produced by H_2-gas inhalation, and were recorded on the polygraph. Tissue blood flow was calculated from the washout curves by using the initial slope technique, omitting the first 0.5 min. Blood gas values ($PaCO_2$, PaO_2, O_2 saturation) and pH were measured in femoral arterial samples by a Radiometer Blood Gas Analyzer (ABL-300, Copenhagen, Denmark) at the times of blood flow determinations. Mean arterial blood pressure and heart rate were also determined at the same time points. After the last measurements, the anesthetized animals were rapidly exsanguinated and hypothalamic tissue samples were excised, frozen rapidly, and kept at –75°C until the measurement of NOS activity on the basis of labeled citrulline formation from labeled L-arginine as described [11]. Briefly, tissue samples were homogenized in brain homogenizing solution containing 50 mM TrisHCl (pH 7.4), 0.3 M sucrose, 0.1 mM EDTA, 1 mM dithioerythritol, and 1 mM phenyl-methyl-sulfonylfluoride (protease inhibitor). Homogenates were added to the samples containing 5 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, 1 mM nicotinamide adenine dinucleotide phosphate, 10 μM tetrahydrobiopterin, 30 μM calmodulin, 2.5 mM $CaCl_2$ at 7.4 pH and reaction was initiated by adding 20 μM ^{3}H-arginine (final concentrations). After 30 min incubation, the reaction was stopped and samples were put onto 2 cm Dowex-50 × 8 resin columns. Eluates were mixed with 5 ml dioxane-based scintillation fluid and measured in a Beckman liquid scintillation spectrometer (Palo Alto, California, USA). Protein contents were determined by the biuret reaction, and NOS activities were calculated in pmole citrulline formed per minute per milligram protein units.

To determine the effect of HO blockade on cerebral prostanoid production, two groups of animals were anesthetized as described above and treated i.p. with either the HO inhibitor zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG, 45 μmol/kg i.p.) or saline. Forty-five minutes later, cerebrospinal fluid was obtained from the cisterna magna and kept at −75°C until further analysis. Concentrations of prostaglandin (PG) D_2, E_2, and $F_{2\alpha}$, as well as the stable prostacyclin metabolite 6-keto-$PGF_{1\alpha}$ have been determined using gas chromatography/triple quadrupole mass spectrometry as described previously [14].

All chemicals were obtained from Sigma (St Louis, Missouri, USA) except for ZnDPBG, which was purchased from Frontier Scientific Europe Ltd (Carnforth, Lancashire, UK). Earlier studies have verified that diclofenac, ZnDPBG, and N^G-nitro-L-arginine methyl ester in the doses used in this study are effective in the brain for inhibition of cyclooxygenase (COX), HO, and NOS, respectively [8,15,16].

All values are presented as mean ± SEM; *n* represents the number of animals. Statistical analysis was performed using analysis of variance for repeated measurements or one-way analysis of variance followed by Dunnett's post-hoc test. For comparison of two groups, Student's unpaired *t*-test was used. A *P* value of less than 0.05 was considered to be statistically significant.

Results

Inhibition of the HO pathway by ZnDPBG (45 μmol/kg i.p.) resulted in decreased concentrations of PGE_2 in the cerebrospinal fluid without influencing the levels of other prostanoids such as PGD_2, 6-keto-$PGF_{1\alpha}$, and $PGF_{2\alpha}$ (Fig. 1). Thereafter, the effect of ZnDPBG on the hypothalamic blood flow has been determined in two groups of animals with normal PGE_2-synthesizing capacity or after the inhibition of COX enzymes by diclofenac (10 mg/kg intravenously). ZnDPBG had no effect in control animals but significantly increased hypothalamic blood flow after diclofenac (Fig. 2a) without influencing arterial blood gas, acid–base, or systemic circulatory parameters (data not shown). After diclofenac pretreatment, the ZnDPBG-treated rats showed significantly ($P=0.007$) higher hypothalamic NOS activity (5.30 ± 1.05 pmol citrulline/mg protein/min) as compared with saline-treated controls (2.45 ± 0.32 pmol citrulline/mg protein/min). In animals pretreated by both diclofenac and the NOS inhibitor, N^G-nitro-L-arginine methyl ester (50 mg/kg intravenously) and ZnDPBG failed to influence the hypothalamic blood flow (Figure 2a).

Discussion

In contrast to the well-documented role of NO in the cardiovascular system [17], the vascular actions of endogenous CO are poorly understood. Results of this study coupled with our earlier observations, indicate that the resting activity of the HO pathway has a dual influence on the hypothalamic circulation; a vasodilation mediated by PGE_2 and a simultaneous vasoconstriction owing to the reduction of NO synthesis. Under resting conditions these

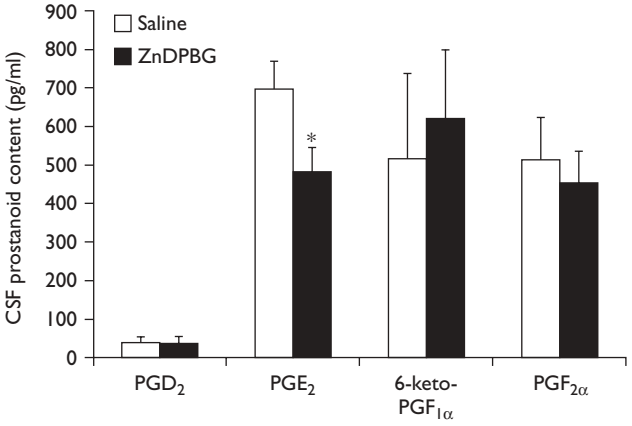

Fig. 1 Prostanoid levels in the cerebrospinal fluid (CSF) after the administration of the heme oxygenase inhibitor zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG) (closed bar, $n=8$), or its vehicle (open bar, $n=9$). Values are mean ± SEM. *$P<0.05$ versus 'saline'. PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

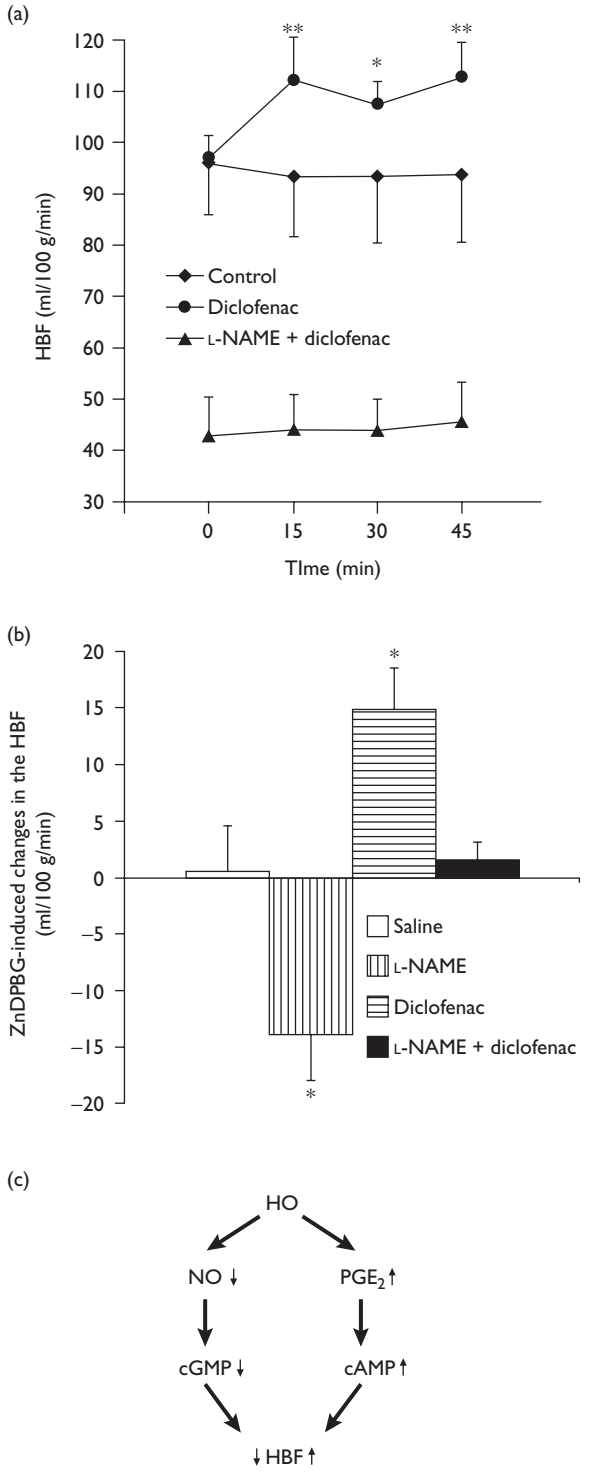

Fig. 2 (a) Hypothalamic blood flow (HBF) in animals without pretreatment (diamond, $n=5$), subjected to cyclooxygenase (COX) blockade (circle, $n=5$), or combined nitric oxide synthase (NOS) and COX blockade (triangles, $n=7$) before (0 min) as well as 15, 30, and 45 min after the administration of the heme oxygenase (HO) inhibitor zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG). Values are mean ± SEM. $^*P<0.05$ and $^{**}P<0.01$ versus '0 min'. (b) Changes in hypothalamic blood flow after administration of the HO inhibitor ZnDPBG in animals pretreated with vehicle (open bar), the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) (vertically stripped bar), the COX inhibitor diclofenac (horizontally stripped bar), and combination of L-NAME and diclofenac (filled bar). Values are mean ± SEM. $^*P<0.05$ versus 'saline'. (c) Proposed mechanism of the dual action of HO on the hypothalamic circulation.

two effects are equally potent and neutralize each other, at least in the hypothalamus. In the cerebral cortex, however, the NO-mediated pathway seems to be dominant and HO blockade results in a NO-mediated pial vasodilation [9,10] and cerebrocortical hyperemia [8]. These observations support the view that the HO pathway has a regionally heterogeneous influence on the cerebral circulation through interaction with the NOS pathway.

The most important new finding of this study is that the vasodilator influence of the HO pathway on the hypothalamic vasculature is mediated by PGE_2. HO cleaves heme moieties liberating equimolar amounts of CO, free iron, and biliverdin, which are converted to bilirubin by biliverdin reductase. From these metabolites, CO and biliverdin are the putative regulators of PGE_2 synthesis, as they have been shown to stimulate the release of PGE_2 from primary cultures of rat hypothalamic cells *in vitro* [12,18]. These observations and a recent report indicating that the HO pathway has no direct influence on the PGE_2 synthesis in cerebral microvessels [19] clearly suggest that brain parenchymal rather than vascular PGE_2 release mediates the vasorelaxant effect of constitutive HO. This would also explain why exogenous CO reportedly relaxes rat pial arteries only under in-vivo conditions [5], but not *in vitro* [20]. Stimulation of neuronal PGE_2 release by the HO pathway, however, may also be involved in physiological functions other than regulation of the hypothalamic blood flow. It has been recently proposed that this interaction may have physiological relevance in thermal regulation and modulation of hypothalamic neuropeptide release [2].

In an earlier study, we have described an indirect influence of the HO pathway on the hypothalamic circulation through inhibition of NO synthesis [11]. Experimental data of this earlier study together with the present observations are summarized in Fig. 2b, indicating that HO blockade by ZnDPBG has no influence on the hypothalamic blood flow under resting conditions but results in either a decreased or an increased blood flow after NOS-blockade and COX-blockade, respectively. After simultaneous NOS-blockade and COX-blockade, ZnDPBG fails to influence the hypothalamic circulation. The proposed pathways mediating the effects of HO metabolites on the hypothalamic vasculature are depicted in Fig. 2c. Although the HO pathway seems to have no direct cerebrovascular effect in adult rats, it can both increase and decrease hypothalamic blood flow through stimulation of PGE_2 release or inhibition of NO release, respectively. Under physiological conditions these pathways are equally potent and neutralize each other. In case of diminished NO production, however, the PGE_2 pathway may dominate and contribute to the maintenance of the hypothalamic blood supply. It is noteworthy that this pathway seems to be independent from the morphological or functional integrity of the endothelium, as CO can be produced by vascular smooth muscle and brain parenchymal cells [21,22] and the secondary PGE_2 release also seems to originate from nonvascular cells (as mentioned above). Therefore, the HO–CO–PGE_2–cAMP pathway is an ideal 'backup' mediator for maintaining the hypothalamic blood perfusion in case of insufficient endothelial NO production, for example in atherosclerosis or hemorrhagic shock. Under these pathophysiological conditions, stimulation of endogenous CO production or administration of CO-releasing substances may have a potential therapeutic value by

preventing hypothalamic ischemia and the consequent disturbances of homeostatic functions.

In case of sustained induction of HO activity, however, the above-described interaction between the HO and COX pathways can be changed substantially. As COX enzymes are heme proteins and the heme prosthetic group is essential for their catalytic activity, heme degradation by HO may negatively influence COX activity. In accordance, HO-1 induction has been shown to reduce COX expression and activity in endothelial cells [23] and in the kidney [24]. Furthermore, HO-1 induction in the endothelium results in increased expression of PG transporter and in enhanced PG clearance [25]. This study indicates that the constitutive HO activity in the brain fails to induce such effects as the PGE_2 levels decrease after HO blockade whereas other prostanoids remain unaltered. In pathophysiological states associated with HO-1 induction in the brain (e.g. subarachnoid hemorrhage), however, the decreased production and enhanced clearance of PGE_2 and PGI_2 together with the CO-mediated inhibition of NOS activity [9,11] may negatively influence the cerebral circulation.

Conclusion

This study identifies PGE_2 as the primary mediator of the vasodilator influence by constitutive HO activity on the hypothalamic circulation in adult rats. Under physiological conditions this effect of CO is counterbalanced by the suppression of NOS. The HO pathway seems to regulate the cerebral circulation of adult rats mainly through interactions with the COX and NOS pathways in a regionally heterogeneous manner.

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Additive effect of cyclooxygenase and nitric oxide synthase blockade on the cerebrocortical microcirculation

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The contribution of vasodilator cyclooxygenase (COX) metabolites to the maintenance of the cerebrocortical blood flow (CBF) has been studied under physiological conditions and in nitric oxide (NO) deficiency. Inhibition of COX decreased resting CBF without changing arterial blood pressure. NO synthase blockade resulted in hypertension and CBF reduction as well as in enhanced cerebral prostacyclin and prostaglandin E_2 production. Despite the increased vasodilator prostanoid release in the absence of NO, the CBF-decreasing effect of COX blockade failed to increase. Therefore, the COX pathway seems to play a similar role under physiological and NO-deficient conditions in the maintenance of the resting CBF. ***NeuroReport*** **20:1027–1031 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Cyclooxygenase (COX) inhibitors are the most widely used pharmaceutical agents worldwide. Unfortunately, apart from their beneficial anti-inflammatory, antipyretic and analgesic effects, COX inhibitors can produce side effects, particularly in the gastrointestinal and cardiovascular systems [1]. While gastrointestinal complications are mainly attributed to the inhibition of the COX-1 enzyme, it is believed that adverse cardiovascular effects (e.g. increased risk of myocardial infarction and stroke) are mainly the consequences of COX-2 blockade and related to the decreased synthesis of vasodilator and antithrombotic prostanoids, such as prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2). In a healthy vasculature, the effects of COX blockade may be less deleterious in the presence of the potent vasodilator and antithrombotic action of endothelium-derived nitric oxide (NO). The release and/or biological half-life of NO, however, can be severely diminished in certain pathophysiological states including arteriosclerosis, diabetes or hyperhomocysteinemia [2]. In NO deficiency, the release and functional importance of vasodilator prostanoids may substantially increase and therefore COX inhibition may result in enhanced cardiovascular complications. For instance, recent reports from our and several other laboratories indicated that vasodilator prostanoids, mainly PGI_2 and PGE_2, play an important compensatory role in the absence of NO in the coronary circulation, and COX blockade aggravates the reduction of coronary blood flow induced by inhibition of the NO synthesis [3–5]. A similar interaction between the NOS and COX pathways in the cerebral circulation is far less studied although it may explain the increased risk of cerebrovascular complications induced by COX inhibitors in diseases associated with decreased availability of NO, such as diabetes or arteriosclerosis [2].

Although the initial intracellular signaling steps of NO-induced and prostanoid-induced smooth muscle relaxation are substantially different (i.e. direct stimulation of guanylyl cyclase and G_s protein coupled receptor mediated activation of adenylyl cyclase, respectively), the prostanoid system can very effectively compensate for the loss of the NO–cGMP pathway [6]. For instance, although cGMP seems to selectively activate protein kinase G (PKG), cAMP activates both protein kinase A (PKA) and PKG. Furthermore, most of the molecular targets of PKG can also be phosphorylated by PKA and the adenylyl cyclase–cAMP–PKA pathway is upregulated in the absence of NO [6]. Therefore, in this study we hypothesized that PGI_2 and/or PGE_2 may have an accentuated role in the maintenance of the cerebral blood flow in NO deficiency. To test this hypothesis we examined whether (i) inhibition of NO synthesis results in enhanced cerebral PGI_2/PGE_2 release and (ii) NO deficiency potentiates the cerebral blood flow changes induced by COX blockade in rats. We performed blood flow measurements in the cerebral cortex, because in a previous study the blood perfusion of this brain region was relatively well preserved after NO synthase (NOS) blockade [7] indicating the presence of effective compensatory mechanism(s).

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Methods

The experiments were performed on 46 adult male Wistar rats (body weight 300–400 g) under the guidelines of the Hungarian Law of Animal Protection (243/1988) and all procedures were approved by the Semmelweis University Committee on the Ethical Use of Experimental Animals (590/99 Rh). The animals were anesthetized with urethane administered intraperitoneally (1.5 g/kg; Sigma), the depth of anesthesia was regularly controlled during the experiments by checking the corneal or plantar nociception reflex and additional urethane was administered intravenously (i.v.) as necessary. The right femoral vein was cannulated for drug administration and the right femoral artery for the measurement of mean arterial blood pressure and to allow sampling for analysis of arterial blood gases. The animals were artificially ventilated through an intratracheal cannula with a mixture of humidified air and oxygen; blood gases were analyzed regularly and kept in the physiological range. Core temperature of the animals was kept at 37°C with a heating blanket and a thermostat.

The measurement of cerebrocortical blood flow (CBF) was performed by laser-Doppler (LD) flowmetry as described in detail elsewhere [8]. The head of the animal was fixed in a stereotaxic head holder with the nose 5 mm down from the interaural line. The skull of the parietal region was exposed and the bone was thinned over the parietal cortex on both sides with a microdrill, so that the lamina interna of the skull remained intact. Two LD probes were placed above the thinned skull at a 12° angle to the vertical to provide an optimal view of the cortex (4 mm caudal from bregma, 5 mm lateral from midline). CBF was measured with a two-channel blood flow monitor (MBF3D, Moor Instruments, UK) and was recorded continuously. The LD monitor was calibrated before each individual experiment with a constant movement latex emulsion. The laser light was in the infrared range (780 nm) and penetrated about 1 mm into the brain covering approximately 7 mm^2 of the parietal region, so that the data acquired mostly represented the characteristics of the parietal cortex [8].

Animals were randomly assigned to four in-vivo experimental groups. In the first (control) group ($n = 5$), systemic and cerebral circulatory parameters, as well as blood gas and acid–base values, were determined both before and after 30 min of an i.v. bolus injection of 1 ml/kg vehicle (saline). The second group ($n = 10$) received i.v. N^G-nitro-L-arginine methyl ester (L-NAME, Sigma, St. Louis, Missouri, USA) at a dose of 50 mg/kg for the inhibition of NO synthesis and the measurements were performed using the same protocol. In the third group of rats ($n = 6$), the effects of COX blockade were determined (induced by 10 mg/kg i.v. indomethacin, Merck, Rahway, New Jersey, USA), whereas in the fourth group ($n = 5$) the combined effects of NOS and COX inhibition were determined.

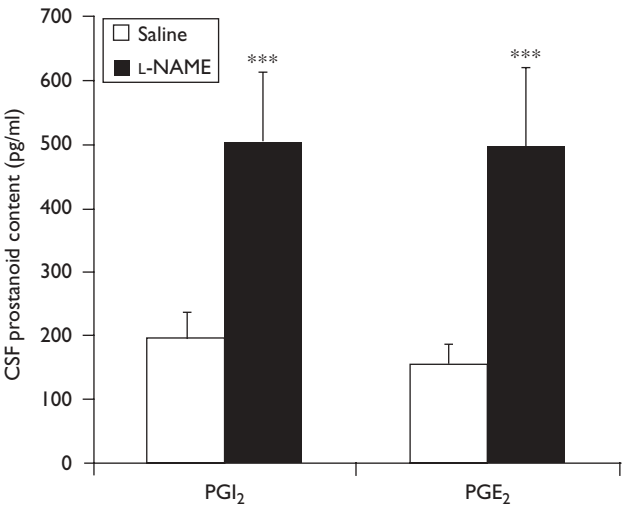
To analyze the effect of NOS blockade on the formation of vasodilator prostanoids in the brain, two additional groups of animals were anesthetized and treated either with vehicle ($n = 11$) or L-NAME ($n = 9$) as described above. Forty-five minutes later, cerebrospinal fluid (CSF) was obtained from the cisterna magna and kept at −75°C until further analysis. Concentrations of PGE_2 and the stable prostacyclin metabolite 6-keto-prostaglandin $F_{1\alpha}$ were determined using chemiluminescence enzyme immunoassay kits (Assay Designs, Ann Arbor, Michigan, USA).

All values are presented as mean ± SEM; n represents the number of experiments. Statistical analysis was performed using analysis of variance followed by Newman–Keuls post-hoc test. For comparison of two groups, Student's unpaired t-test was used. A P value of less than 0.05 was considered to be statistically significant.

Results

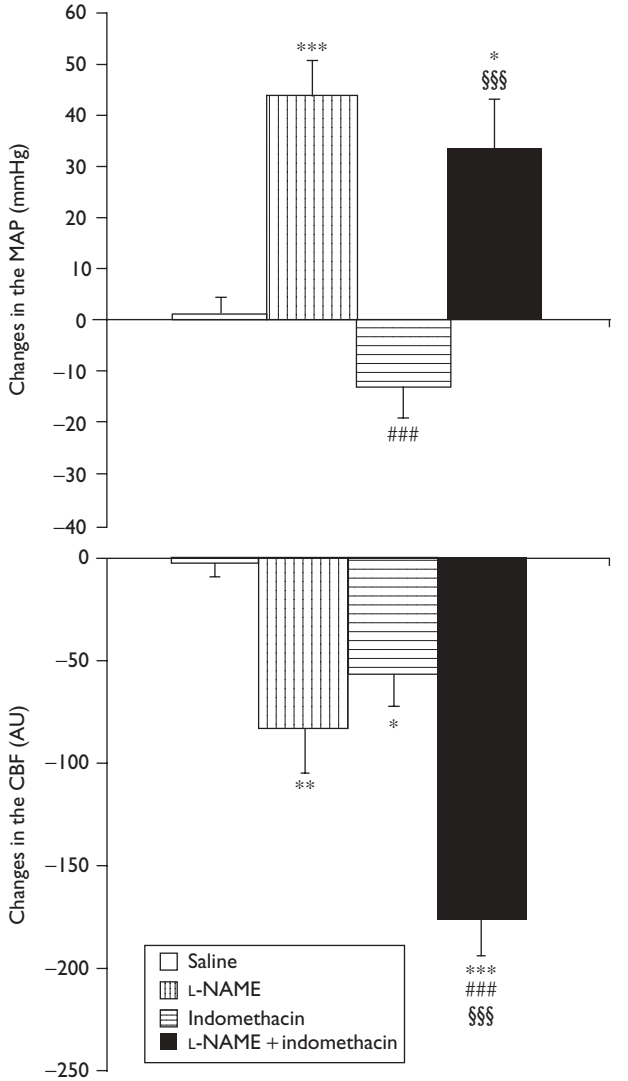
Inhibition of NO synthesis resulted in increased levels of PGE_2 and PGI_2 in the CSF (Fig. 1). At the beginning of the in-vivo experiments, the arterial blood gas and acid–base parameters were within the physiological range (pCO_2 36.9 ± 0.6 mmHg; pO_2 106.3 ± 2.8 mmHg; pH 7.37 ± 0.01) and did not change during the measurements in any of the experimental groups (data not shown). NOS blockade induced marked hypertension and at the same time significant reduction of the CBF

Fig. 1



Prostanoid levels in the cerebrospinal fluid (CSF) after administration of the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) (closed bar, $n = 9$), or its vehicle (open bar, $n = 11$). Values are mean ± SEM. ***$P < 0.001$ vs. saline. PGE_2, prostaglandin E_2; PGI_2, prostacyclin.

Fig. 2



Changes of the mean arterial pressure (MAP, upper panel) and cerebrocortical blood flow (CBF, lower panel) after intravenous administration of saline (open bars, $n=5$), the nitric oxide synthase (NOS) inhibitor L-NAME (vertically striped bars, $n=10$), the cyclooxygenase (COX) inhibitor indomethacin (horizontally striped bars, $n=6$), or simultaneous NOS and COX blockade (closed bars, $n=5$). Values are mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. saline; $^{\#\#\#}P<0.001$ vs. L-NAME; $^{\S\S\S}P<0.001$ vs. indomethacin.

(Fig. 2). Inhibition of COX by indomethacin also decreased CBF but had no influence on the blood pressure (Fig. 2). Combined NOS and COX blockade induced hypertension and more severe CBF reduction than inhibition of any of these pathways alone (Fig. 2). However, two-factor analysis of variance revealed that there was no interaction ($P = 0.25$) between the cerebrovascular effects of the two interventions indicating that NO deficiency did not enhance the CBF-decreasing effect of COX blockade despite the increased release of vasodilator prostanoids.

Discussion

The high vulnerability of central nervous system neurons to hypoxia and the deleterious consequences of ischemic neuronal damage with respect to the survival of the organism require that very precise and effective vasoregulatory mechanisms control the cerebral circulation. Perhaps the most important vasoactive agent involved in the maintenance of the proper blood supply to the brain is NO, both under resting conditions and during functional hyperemia. In NO deficiency, however, other vasoactive mediators may substitute NO to maintain sufficient blood perfusion of the brain. Recently, endogenous CO was proposed to serve as a 'backup' vasodilator in the hypothalamus in the absence of NO [9,10]. However, we have recently shown that in the cerebral cortex CO functions as a vasoconstrictor [8] and therefore it cannot compensate for the loss of NO. Despite this fact, the cerebrocortical blood flow is relatively well preserved in the absence of NO as compared with other brain regions [7], which indicates the presence of effective compensatory mechanisms.

Several lines of evidence indicate that vasodilator prostanoids may be involved in the adaptation of the circulatory system to NO deficiency. For instance, the contractile effect of the COX inhibitor mefenamic acid in rat diaphragmatic arterioles was enhanced significantly after NOS blockade [11]. Furthermore, in this experimental system, simultaneous administration of mefenamic acid and the NOS inhibitor nitro-L-arginine completely prevented the acetylcholine-induced vasodilation, although these drugs were without any significant effect when applied separately [11]. After the inhibition of NOS, elevated plasma levels and increased urinary excretion of PGI_2 and PGE_2 were observed [12,13] and the hypertensive effect of NOS blockade was increased by pretreatment with indomethacin [14]. Nitrovasodilator drugs and NO were shown to suppress vascular prostacyclin release [15]. Therefore, it is not surprising that prostacyclin can very effectively substitute NO during chronic NOS blockade or after deletion of the eNOS gene. Flow-dependent dilation of rat gracilis muscle arterioles and acetylcholine-induced relaxation of mesenteric arteries, the responses of which are normally mediated by endothelium-derived NO, remain unaltered in eNOS knockout mice [16,17]. However, indomethacin, which has no or weak effect on vessels prepared from wild-type animals, almost completely abolishes the above-mentioned responses after eNOS gene deletion [16,17], indicating that prostacyclin or other prostanoids compensate for the loss of NO.

Numerous studies have shown the compensatory role of vasodilator prostanoids in the coronary circulation in NO deficiency. In canine and rabbit coronary arteries, endothelium-dependent relaxations by acetylcholine

were not affected significantly by in-vitro COX blockade [18,19]. In contrast, NOS inhibition induced a significant reduction of these vascular responses, the effect of which was aggravated by simultaneous administration of indomethacin or diclofenac. These results indicate that in the absence of NO, the compensatory release of a vasodilator prostanoid may significantly contribute to the mediation of endothelium-dependent relaxations in the coronary artery [18,19]. Indeed, in isolated perfused hearts, increased release of PGI_2 and PGE_2 has been demonstrated after NOS blockade and indomethacin aggravated the effect of NOS inhibition on the coronary circulation [3–5].

The mechanism of the adaptation of the cerebral circulation to NO deficiency is far less understood although eNOS knockout mice show normal blood flow not only in the heart [20] but also in the brain [21], indicating the presence of effective compensatory mechanisms. Vasodilator prostanoids, released constitutively from brain microvessels, are apparently involved in the maintenance of the resting cerebral blood flow in some species including humans and rats [22] and enhancement of their influence may serve as a reserve regulatory mechanism in NO deficiency. In this study, we observed a significant increase in the levels of PGI_2 and PGE_2 in the CSF after NOS blockade, which indicates the activation of a prostanoid-mediated compensatory mechanism in NO deficiency. Surprisingly, however, the CBF-decreasing effect of indomethacin failed to increase in the absence of NO. This apparent discrepancy can be explained by a dual action of COX blockade. It has been shown recently in cultured porcine cerebrovascular endothelial cells that NOS blockade enhances the release of thromboxane A_2 [23] and previous studies indicated that thromboxane receptors mediate the cerebral vasoconstriction induced by inhibition of NO synthesis [24,25]. Therefore, in the absence of NO, both the vasodilator (PGI_2 and PGE_2) and vasoconstrictor (thromboxane A_2) prostanoid pathways seem to be activated and therefore the CBF effect of COX blockade remains unaltered. This, however, does not mean that the adverse cerebrovascular effects of COX inhibitors are not enhanced in NO deficiency. Our observations show that the resting CBF is decreased by *22%* and COX blockade induces a further *26%* reduction in the absence of NO. As the oxygen extraction fraction of the resting brain tissue is relatively high ($>40\%$), COX inhibition in NO-deficient pathophysiological states may lead to neuronal hypoxia with its harmful functional consequences.

Conclusion

We conclude that although cerebral release of vasodilator prostanoids is enhanced after the inhibition of NOS, they play an equally potent role in the maintenance of the resting CBF under physiological conditions and in NO deficiency. As both NOS and COX blockade result in significant CBF reduction and their cerebrovascular effects are additive, COX inhibitors may induce cerebral ischemia in pathological states associated with diminished NO availability.

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