Acetaminophen-sensitive prostaglandin production in rat cerebral endothelial cells

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Acetaminophen-sensitive prostaglandin production in rat cerebral endothelial cells. Am J Physiol Regul Integr Comp Physiol 288: R897–R902, 2005. First published December 23, 2004; doi:10.1152/ajpregu.00613.2004.—Acetaminophen is a widely used antipyretic and analgesic drug whose mechanism of action has recently been suggested to involve inhibitory effects on prostaglandin synthesis via a newly discovered cyclooxygenase variant (COX-3). Because COX-3 expression is high in cerebral endothelium, we investigated the effect of acetaminophen on the prostaglandin production of cultured rat cerebral endothelial cells (CECs). Acetaminophen dose-dependently inhibited both basal and LPS-induced PGE_2 production in CECs with IC_50 values of 15.5 and 6.9 μM, respectively. Acetaminophen also similarly inhibited the synthesis of 6-keto-PGF_1α and thromboxane B_2. LPS stimulation increased the expression of COX-2 but not COX-1 or COX-3. In addition, the selective COX-2 inhibitor NS398 (1 μM) was equally as effective as acetaminophen in blocking LPS-induced PGE_2 production. Acetaminophen did not influence the expression of the three COX isoforms and the inducible nitric oxide synthase. In LPS-stimulated isolated cerebral microvessels, acetaminophen also significantly inhibited PGE_2 production. Our results show that prostaglandin production in CECs during basal and stimulated conditions is very sensitive to inhibition by acetaminophen and suggest that acetaminophen acts against COX-2 and not COX-1 or COX-3. Furthermore, our findings support a critical role for cerebral endothelium in the therapeutic actions of acetaminophen in the central nervous system.

cyclooxygenase-3; enzyme-linked immunosorbent assay; lipopolysaccharide; NS398

ACETAMINOPHEN is a widely used antipyretic analgesic drug; however, its mechanism of action is still unknown. Although it has antipyretic and analgesic potential similar to that of non-steroidal anti-inflammatory drugs (NSAIDs), at therapeutic concentrations acetaminophen has been reported to have only a weak inhibitory effect on cyclooxygenase-1 (COX-1) and COX-2 and does not inhibit peripheral prostaglandin biosynthesis and inflammation (1). Three decades ago, it was suggested that acetaminophen might inhibit a COX in the brain and that tissue-specific COX isoforms may exist that are selectively inhibited by different NSAIDs (6). A new isoform of COX-1 has been recently identified in canine that is sensitive to acetaminophen and has been designated COX-3 (4). We described the existence of this new isoform in cultured rat brain cells and also in rat brain tissues (10, 11). COX-3 was particularly prevalent in cultured cerebrovascular endothelial cells (CECs) and in isolated capillaries (10, 11). Considerable evidence has indicated that transcriptional activation of COX and subsequent prostaglandin production in CECs is a crucial step in fever development (16). Prostaglandins produced and released by the cerebral endothelium also play an important role in the regulation of cerebrovascular tone and permeability (3). However, the relationship among COX isoforms, prostaglandin production, and acetaminophen has not been previously examined in CECs.

The purpose of this study was to examine the effects of acetaminophen on prostaglandin production in primary cultures of rat CECs under basal conditions and after bacterial lipopolysaccharide (LPS) challenge. LPS is a known potent stimulator of inflammatory genes and prostaglandin production in CECs both in vivo and in vitro, and it is used in in vivo experiments to induce fever in animals. We compared effectiveness of acetaminophen with that of NS398, which is a selective inhibitor of COX-2. In addition, we examined effects of acetaminophen in LPS-stimulated CECs on expression of COX-1, -2, and -3 and inducible nitric oxide synthase (iNOS) and the effect of acetaminophen on prostaglandin E_2 (PGE_2) production of LPS-stimulated isolated brain microvessels.

METHODS

Rat CEC culture. Wistar rats were obtained from Harlan (Indianapolis, IN). All animal experiments were approved by the Animal Care and Use Committee of Wake Forest University Health Sciences. Primary rat CECs were isolated and cultured as described previously (12). Brains were removed from 2-wk-old Wistar rats under deep halothane anesthesia and were collected in ice-cold phosphate-buffered saline. Larger surface vessels were removed from the brain with the use of fine forceps, and then the pial membranes were removed by rolling the hemispheres on dry chromatography paper (3MM; Whatman, Maidstone, UK). The white matter was removed, and cerebral cortices were finely minced with scalpels and then incubated in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO BRL, Grand Island, NY) containing collagenase (200 U/ml; Worthington, Lakewood, NJ) and DNase (50 U/ml; Sigma, St. Louis, MO) at 37°C for 2 h in a shaking water bath. After this incubation, the digest was thoroughly broken up by repeated aspiration through sterile Pasteur pipettes. The homogenate was centrifuged at 350 × g for 5 min. The supernatant was removed, and 20% bovine serum albumin (BSA, 2 ml/brain; Sigma) was added to the pellet. The digested brain tissue was completely redistributed in the BSA solution by repeated aspiration through sterile pipettes. The homogenate was centrifuged at 350 × g for 5 min. The supernatant was removed, and 20% bovine serum albumin (BSA, 2 ml/brain; Sigma) was added to the pellet. The digested brain tissue was completely redistributed in the BSA solution by repeated aspiration through sterile pipettes and then was centrifuged at 1,000 × g for 20 min. This centrifugation separates the brain microvessels from the digested neuronal elements, which appear on the top of the BSA solution as a myelin plug. The myelin plug and the BSA solution were discarded, and the pelleted microvessels were washed once in DMEM and then further digested with the above-mentioned enzymes for 1.5 h at 37°C. After the digestion, the cell suspensions were centrifuged at 500 × g for 5 min. The cells were carefully layered on a continuous 33% Percoll (Amersham, Uppsala, Sweden) gradient and centrifuged at
1,000 g for 10 min. The band of the endothelial cell clusters was aspirated and washed twice in DMEM. The cells were seeded onto collagen IV- and fibronectin-coated 35-mm dishes. Culture medium consisted of DMEM supplemented with 20% fetal bovine plasma-derived serum (Animal Technologies, Tyler, TX), 2 mM glutamine, 1 ng/ml basic fibroblast growth factor (Sigma), 50 μg/ml endothelial cell growth supplement (BD Biosciences, Bedford, MA), 100 μg/ml heparin, 5 μg/ml vitamin C, and antibiotics. Confluent cultures (4th-5th day in vitro) consisted of >95% of rat CECs, verified by positive immunohistochemistry for von Willebrand factor, and negative immunohistochemistry for glial fibrillary acidic protein and α-smooth muscle actin.

Isolation of cerebral microvessels. Male Wistar rats (body weight 250 ± 20 g) were killed by left ventricular transcardiac perfusion with chilled saline containing 1,000 U/l heparin under halothane (5% in oxygen) anesthesia. Brains were removed and collected in ice-cold phosphate-buffered saline. Larger surface vessels were removed from the brain with the use of fine forceps, and then the pial membranes were removed by rolling the hemispheres on dry chromatography paper (3M; Whatman). The white matter was removed, and cerebral cortices were finely minced with scalpels and then incubated in DMEM containing collagenase (200 U/ml) and DNase (30 U/ml) at 37°C for 2 h in a shaking water bath. After this incubation, the digest was thoroughly broken up by repeated aspiration through sterile Pasteur pipettes. The homogenate was centrifuged at 350 g for 5 min. The supernatant was removed, and 20% BSA (2 ml/brain) was added to the pellet. The digested brain tissue was completely redistributed in the BSA solution by repeated aspiration through sterile pipettes and then was centrifuged at 1,000 g for 20 min. This centrifugation separates the brain microvessels from the digested neuronal elements, which appear on the top of the BSA solution as a myelin plug. The myelin plug and the BSA solution were discarded, and the pelleted microvessels were washed twice with DMEM and then used for experiments. This preparation contains small pieces of cerebral microvessels and, after further processing, yields viable CECs that can be used for culturing (12).

Experimental protocol. Confluent cultures of CECs and freshly isolated cerebral microvessels were preincubated with different concentrations of acetaminophen (Sigma) or NS398 (Sigma) for 30 min, and then cells were washed twice with DMEM, and fresh culture medium was added. Cells were incubated for 6 h with the drugs with or without 100 ng/ml LPS (Escherichia coli O26:B6; Sigma). In experiments in which we measured the effect of acetaminophen on the basal PGE2 production of cultured CECs, the 6-h incubation was carried out in DMEM to eliminate the background prostaglandin activity of bovine serum in the culture medium.

After the incubation, media were collected and stored at −60°C until assayed for prostaglandins, and then the cells were washed twice with phosphate-buffered saline, and total RNA or proteins were isolated for RT-PCR or Western blot analysis. For each series of experiments, cells represented at least three independent cultures.

Prostaglandin measurements. PGE2, 6-keto-PGF1α, and thromboxane B2 (TXB2) concentrations in media were measured with specific ELISAs (Oxford Biomedical Research, Oxford, MI) according to the manufacturer’s instructions. The protein content was normalized to the amount of total cell protein quantified by the micro DC Protein assay (Bio-Rad, Hercules, CA).

Western blotting. Cultured cells were harvested by scraping in ice-cold NP-40 lysis buffer (1% Nonidet P-40, 135 mM NaCl, and 50 mM Tris, pH 8.0) supplemented with proteinase inhibitors (1 μg/ml aprotinin, 50 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin) and a phosphatase inhibitor cocktail (1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml benzamide, 1 mM sodium pyrophosphate, and 1 mM sodium fluoride). Lysates were centrifuged (10,000 g, 10 min, 4°C), and the supernatants were collected and stored at −60°C until assayed. Western blot analysis was performed as described previously (9). An equal amount of protein for each sample was separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride sheet (PolyScreen PVDF; Perkin Elmer Life Sciences, Boston, MA). Membranes were incubated in a blocking buffer (Tris-buffered saline, 0.1% Tween 20, and 5% skim milk powder) for 1 h at room temperature, and then blots were incubated with rabbit polyclonal anti-murine COX-1 (1:1,000), rabbit polyclonal anti-murine COX-2 (1:5,000; both from Cayman Chemical, Ann Arbor, MI), or monoclonal mouse anti-mouse iNOS (1:1,000; BD Biosciences) primary antibodies overnight at 4°C. The membranes were washed and incubated in Tris-buffered saline with 1% Tween 20 and then incubated for 1 h in the blocking buffer with rabbit-IgG (1:50,000; Jackson ImmunoResearch, West Grove, PA) or anti-mouse IgG (1:5,000; Jackson ImmunoResearch) conjugated to horseradish peroxidase. The final reaction products were visualized using enhanced chemiluminescence (SuperSignal West Pico; Pierce, Rockford, IL) and recorded on X-ray film.

Statistical analysis. Statistical analysis was performed with SigmaStat (SPSS). Data are presented as means ± SE. Differences between groups were assessed using one-way ANOVA followed by Tukey comparison tests. A value of P < 0.05 was considered to be statistically significant.

RESULTS

In cultured rat CECs, acetaminophen dose-dependently inhibited PGE2 production under basal conditions (Fig. 1A) and also after LPS application (Fig. 1B) with IC50 values of 15.5 and 6.9 μM, respectively. Acetaminophen also inhibited baseline and LPS-stimulated 6-keto-PGF1α and TXB2 production in cultured rat CECs (Fig. 2). The selective COX-2 inhibitor NS398 (1 μM) had an effect similar to that of acetaminophen
(100 μM) in inhibiting the production of PGE₂ (Fig. 1C), 6-keto-PGF₁α (Fig. 2A), and TXB₂ (Fig. 2B). LPS treatment did not further stimulate the PGE₂ production in freshly isolated cerebral microvessels (Fig. 3). Acetaminophen (100 μM) significantly inhibited the PGE₂ production of activated microvessels (Fig. 3).

Our RT-PCR analysis showed that 6-h incubation with LPS significantly stimulated COX-2 but not COX-1 and COX-3 mRNA expression in cultured rat CECs (Fig. 4A). Acetaminophen had no effect on expression of the COX isoforms (Fig. 4A). Our Western blot analysis showed the same results: 6-h incubation of cultured rat CECs with LPS significantly stimulated the expression of COX-2 but not COX-1 protein (Fig. 4B). LPS also stimulated the expression of iNOS protein in cultured rat CECs (Fig. 4B). Acetaminophen did not influence the expression of COX-1, COX-2, and iNOS proteins.

**DISCUSSION**

COX-3 has been suggested as a potential target for acetaminophen (4), and we previously demonstrated the highest COX-3 expression in CECs among other cultured brain cell types studied (11). We also reported that the expression pattern of COX-3 in the rat central nervous system primarily relates to the vascular density of a given region and that the microvessels showed the highest levels of COX-3 mRNA (10). Thus it was logical to examine the effect of acetaminophen on prostaglan-
Our present study demonstrates for the first time that cultured rat CECs have a highly acetaminophen-sensitive PGE2 synthesis; acetaminophen dose-dependently inhibited PGE2 production under basal conditions (Fig. 1A) and also after LPS application (Fig. 1B) in cultured rat CECs with IC50 values of 15.5 and 6.9 μM, respectively. These values are much lower than the therapeutic range of the plasma concentration of acetaminophen (66–132 μM) (26), suggesting that acetaminophen also may inhibit the prostaglandin production in the cerebral microvasculature in vivo. The slight difference in the IC50 between basal and LPS-stimulated conditions probably is due to minor differences in protocols. When we measured the PGE2 production under basal conditions, CECs were incubated in DMEM instead of the usual culture medium to eliminate the background prostaglandin level of the bovine serum. However, the bovine serum contains reduced glutathione, which is a normal constituent of the plasma, and has been shown to increase the effectiveness of acetaminophen in reducing prostaglandin production (18, 23). In addition to our results, acetaminophen-sensitive prostaglandin production has been shown previously in human umbilical vein endothelial cells (2, 17).

An important question is whether our results on cultured rat CECs can be transferred to the in vivo situation. Unlike the prostaglandin production of the brain tissue, which can be sampled in vivo by microdialysis, the prostaglandin production of the cerebral endothelium cannot be directly assessed in vivo. The best approach currently available to get information about the prostaglandin production of the CECs is via an ex vivo preparation, the isolated cerebral microvessels. We found a very high basal PGE2 production in our isolated cerebral microvessel preparations, and their PGE2 production was not further stimulated by LPS treatment (Fig. 3). The high basal PGE2 production in our microvessel preparation probably was the unavoidable consequence of the isolation procedure. Cerebral cortices were digested for 2 h at 37°C with collagenase, an enzyme derived from bacteria. Besides collagenase, the enzyme preparation contains several other bacterial enzymes and, likely, endotoxin and other bacteria-derived toxins. Thus it seems that the microvessels had already been stimulated during the isolation procedure, and this explains why they had very high basal PGE2 production and why LPS failed to further

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Fig. 3. Effects of ApAP on PGE2 production in isolated rat cerebral microvessels. Freshly isolated rat cerebral microvessels were preincubated for 5 min with or without ApAP (100 μM) and then incubated for 5 h in DMEM containing 5% fetal bovine serum with ApAP (100 μM) and/or LPS (100 ng/ml). After incubation, PGE2 was measured from the supernatants with ELISA. Values are means ± SE; n = 4. *P < 0.05.

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Fig. 4. Effects of ApAP on expression levels of cyclooxygenase (COX) isoforms and inducible nitric oxide synthase (iNOS) in rat CECs. Confluent rat CEC cultures were pretreated for 30 min with ApAP and then incubated for 6 h in culture medium with different concentrations of ApAP with or without LPS (100 ng/ml). After incubation, total RNA or proteins were isolated from the cells, and RT-PCR was performed to detect mRNAs of COX isoforms (A) or Western blotting was carried out to determine expression levels of COX-1, COX-2, and iNOS proteins (B). A: representative gel electrophoresis of RT-PCR products for COX-1, COX-2, and COX-3 in cultured rat CECs of 3 similar experiments (lower bands are β-actin). B: representative Western blots for COX-1, COX-2, and iNOS of 2 similar experiments. The faint nonspecific band above the band of COX-1 is likely to be the lymphocyte cytosolic protein 2, which has 81% epitope homology with the respective sequence of COX-1 that was used to produce the anti-murine COX antibody.
stimulate their PGE$_2$ production. Acetaminophen in the therapeutic range significantly inhibited the PGE$_2$ production in LPS-stimulated cerebral microvessels (Fig. 3). The effect of acetaminophen was slightly weaker on freshly isolated microvessels than on cultured CECs, which might be the consequence of the presence of other cellular elements (pericytes, astrocytic end feet, microglia) around the endothelial layer in the vascular wall that may not be as sensitive to acetaminophen as the endothelial cells.

Acetaminophen also inhibited baseline and LPS-stimulated 6-keto-PGF$_{1\alpha}$ (Fig. 2A) and TXB$_2$ (Fig. 2B) production in cultured rat CECs. However, the inhibition of TXB$_2$ production was not as complete as that of PGE$_2$ (Fig. 1C) and 6-keto-PGF$_{1\alpha}$ (Fig. 2A). We do not know why acetaminophen is less effective in inhibiting TXB$_2$ production, but similar to our results, acetaminophen was found to be twice as potent in inhibiting LPS-induced PGE$_2$ synthesis than TXB$_2$ production in blood (23).

The parallel measurements of PGE$_2$ (Fig. 1C), 6-keto-PGF$_{1\alpha}$, and TXB$_2$ (Fig. 2) suggest that the site of inhibition is proximal to PGE synthases. A possible target of acetaminophen could be phospholipase A$_2$, which is the rate-limiting enzyme of the eicosanoid biosynthesis, but several studies have clearly demonstrated that acetaminophen has no effect on phospholipase activity in different experimental preparations (2, 8, 15, 20, 25). Another target might be the multidrug resistance protein 4, a recently discovered prostaglandin efflux transporter that is expressed in CECs (unpublished observation), and its activity was inhibited by several NSAIDs (acetaminophen was not tested) (21).

A third likely target is one of the COX enzymes. Our present data indicate that the acetaminophen-sensitive COX isoform expressed in rat CECs may be the COX-2 or a COX-2 variant. CECs are known to constitutively express COX-2 (Fig. 4) (19); therefore, this isoform can play a role in the basal prostaglandin production in CECs. LPS stimulation increased the expression of COX-2 but not COX-1 or COX-3 (Fig. 4), and the LPS-induced prostaglandin production was as effectively blocked by acetaminophen as by NS398 (Fig. 1C). In support of our findings, Simmons et al. (24) reported an acetaminophen-sensitive COX isoform that was regarded as a COX-2 variant.

Acetaminophen is a very effective antipyretic drug, and a strong body of evidence supports the view that transcriptional activation of COX-2 in CECs is a crucial step in fever development (16). PGE$_2$ is the most abundant COX metabolite produced by CECs (12), as well as the main prostanooid implicated in fever generation (5). Experiments on COX-1 and -2 knockout mice clearly demonstrated the important role of the COX-2 but not of the COX-1 gene (which also encodes COX-3) in the fever response (14). These findings also suggest that the likely target of acetaminophen is the COX-2 in CECs.

Although COX-3 has been suggested as the acetaminophen-sensitive COX isoform (4), our present experiments do not support this hypothesis. Recently, we cloned and sequenced the rat COX-3 mRNA (GenBank accession no. AY523672), which suggests that unlike in canine, the rat COX-3 mRNA encodes a protein that has no homology with known COXs. Measurement of PGE$_2$ production in COS-7 cells transfected with rat COX-3 cDNA also revealed that this protein does not possess COX activity (25a). Warner et al. (28) demonstrated in a variety of rat tissues that the production of prostanooids is dependent on the two known isoforms of cyclooxygenase, COX-1 and COX-2, and that there is no evidence for the involvement of a particular acetaminophen-sensitive COX-3 isoform. Although we do not have any evidence supporting a relationship between the strong acetaminophen sensitivity of CECs and the high COX-3 expression in these cells, we do not exclude the possibility that there may be an as yet unknown interaction among acetaminophen, COX-3, and COX-2 activity. It is worthwhile to mention that Warner et al. (28) found the highest COX-3 expression in the lungs of rats, and interestingly, the lung was also the site at which acetaminophen produced its most potent inhibitory effects on prostanooid synthesis.

We could not detect an effect of acetaminophen on expression of the COX isoforms that we examined (Fig. 4), which is similar to findings of Sciulli et al. (23). We also could not detect an effect of acetaminophen on iNOS expression (Fig. 4). Ryu et al. (22) showed that acetaminophen suppressed LPS-induced iNOS expression in macrophages and suggested that acetaminophen may exert analgesic or anti-inflammatory effects by this mechanism given that NO has been shown to activate COXs and increases prostaglandin production (7). It should be noted that Ryu et al. (22) used very high concentrations of acetaminophen (2–10 mM) that were well beyond the range used clinically (26).

According to the generally accepted concept, acetaminophen acts centrally and does not exert its actions by inhibiting peripheral prostaglandin production (1). It has been suggested that acetaminophen reduces the oxidized form of the COX back to the catalytically inactive state, and consequently, low levels of oxidants potentiate this inhibition, whereas high levels decrease this effect (2, 18). Therefore, the weak anti-inflammatory activity of acetaminophen could be due to its poor ability to inhibit COX in inflamed tissue, where the peroxide level is high. In contrast, the unique, strictly controlled environment of the brain may favor the effect of acetaminophen (6). However, this view cannot explain why prostaglandin production in astrocytes, which possess the highest antioxidant levels in the brain, is not responsive to acetaminophen (13) or why microglia, which produce large amounts of reactive oxygen species, are sensitive to acetaminophen in blocking prostaglandin production even after LPS stimulation (8). Moreover, Swierkosz et al. (27) were not able to demonstrate in the mouse and rabbit that COX in the brain is more sensitive to acetaminophen than COX in the spleen. Although the molecular mechanism of acetaminophen has not been completely resolved, our findings provide the first evidence that the prostaglandin production in CECs is very sensitive to inhibition by acetaminophen, and this fact can explain the antipyretic action of this drug.

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