Cyclooxygenases 1, 2, and 3 and the Production of Prostaglandin I2: Investigating the Activities of Acetaminophen and Cyclooxygenase-2-Selective Inhibitors in Rat Tissues

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ABSTRACT

It has been suggested recently that cyclooxygenase-3, formed as a splice variant of cyclooxygenase-1, is the enzymatic target for acetaminophen. To investigate the relative roles of the putative three cyclooxygenase isoforms in different target tissues, we compared the inhibitory effects of acetaminophen, a cyclooxygenase-2-selective inhibitor; rofecoxib, a nonsteroid anti-inflammatory drug; naproxen; and a cyclooxygenase-1-selective inhibitor, SC560 [5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole]. Prostanoid production by aorta, heart, lung, and whole blood was inhibited by all drugs tested with the order of potency SC560 > naproxen > acetaminophen > rofecoxib. In brain and cerebellum, no differences among drug potencies were found. Reverse transcription-polymerase chain reaction analysis of aorta, brain, cerebellum, heart, and lung showed general expression of cyclooxygenase-1 and cyclooxygenase-3 mRNA and particular expression of cyclooxygenase-2 mRNA in brain and cerebellum. Western blotting demonstrated general expression of cyclooxygenase-1 in test tissues and cyclooxygenase-2 within the brain and cerebellum. Western blotting using a commercially available antibody raised against canine cyclooxygenase-3 failed to detect any immunoreactive proteins. In conclusion, our studies indicate that cyclooxygenase-1 and cyclooxygenase-2 are the functional forms of the enzyme present in the rat tissues tested and that acetaminophen is not a selective inhibitor of “cyclooxygenase” activities in the central nervous system. This is consistent with the apparent impossibility for the expression of cyclooxygenase active protein from cyclooxygenase-3 mRNA in the rat. Also, our experiments show that the ability of rofecoxib to depress the circulating levels of prostaglandin I2 is more readily associated with its ability to reduce production from the lung, heart, or brain than from arterial vessels.

Since the early 1990s, it has been appreciated that there are two cyclooxygenase enzymes, cyclooxygenase-1 and cyclooxygenase-2, responsible for the production of prostaglandin H2, the first step in prostanoid biosynthesis. In simple terms, it seemed that cyclooxygenase-1 was responsible for the physiological production of prostanoids and that cyclooxygenase-2 was responsible for the elevated production of prostanoids that occurred in sites of disease and inflammation (Mitchell et al., 1993; Dubois et al., 1998; Vane et al., 1998; Mitchell and Warner, 1999; Smith et al., 2000; Fitzgerald and Patrono, 2001; Warner and Mitchell, 2004). These two cyclooxygenase proteins are products of two distinct genes for cyclooxygenase-1 and cyclooxygenase-2 located, respectively, on chromosomes 9 and 1. Although the genes for cyclooxygenase-1 and cyclooxygenase-2 are very different, the proteins share approximately 60% homology at the amino acid level; both catalyze from arachidonic acid the formation of prostaglandin G2 followed by prostaglandin H2 via a peroxidase function and have similar molecular weights and protein lengths. It has been suggested recently that there is an additional cyclooxygenase enzyme, formed as a splice variant of cyclooxygenase-1, named in the initial report of this enzyme as cyclooxygenase-3 (Chandrasekharan et al., 2002). Cyclooxygenase-3 is a product of the cyclooxygenase-1 gene identical to cyclooxygenase-1 but with the retention of intron

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; SC560, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole; RT-PCR, reverse transcription-polymerase chain reaction; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; ANOVA, analysis of variance.
1 in its mRNA. The difference at the protein level between cyclooxygenase-3 and cyclooxygenase-1 is the insertion of 30 to 34 aa, depending on the mammalian species, into the hydrophobic signal peptide. In cyclooxygenase-3, this signal peptide is not cleaved, and the protein is glycosylated and displays cyclooxygenase activity.

The initial report of cyclooxygenase-3 showed that, in comparative assays using canine cyclooxygenase-3, murine cyclooxygenase-1, and murine cyclooxygenase-2 expressed by transfected insect cells, cyclooxygenase-3 was selectively inhibited by analgesic/antipyretic drugs such as acetaminophen, phenacetin, antipyrine, and dipyrone. Inhibition of cyclooxygenase-3, it was suggested, could represent a primary central mechanism by which these drugs decrease pain and possibly fever. This makes an attractive hypothesis. Acetaminophen has an idiosyncratic profile: it can be shown to inhibit the activity of both cyclooxygenase-1 and cyclooxygenase-2 in isolated tissues and cells and also reduce prostanoid production in vivo (Prescott, 2000; Warner and Mitchell, 2002). However, acetaminophen seems to act only at certain sites in vivo; it is anti-pyretic and analgesic (suggesting inhibition of cyclooxygenase-2 within the central nervous system), poorly effective on platelets (weak inhibition of peripheral cyclooxygenase-1), and shows no anti-inflammatory activity (weak inhibition of peripheral cyclooxygenase-2). Interestingly, acetaminophen also reduces the circulating levels of prostaglandin I$_2$, suggesting inhibition of cyclooxygenase within blood vessels/endothelial cells (Green et al., 1989). This ability of acetaminophen mirrors that of selective inhibitors of cyclooxygenase-2, which also reduce the circulating levels of prostaglandin I$_2$ while leaving platelet thromboxane A$_2$ production unaffected (McCadam et al., 1999; Fitzgerald and Patrono, 2001). This has led to suggestions that cyclooxygenase-2 may underlie the production of prostaglandin I$_2$ within blood vessels. However, no direct evidence for cyclooxygenase-3 in “fresh” human blood vessels has yet been described. Indeed, human fresh internal mammary artery and saphenous vein contain only cyclooxygenase-1 protein with undetectable levels of cyclooxygenase-2 or cyclooxygenase-3 (unpublished observations). Thus, the primary central mechanism by which these drugs decrease pain and possibly fever makes an attractive hypothesis.

Materials and Methods

Materials. All compounds used were obtained from Sigma Chemical (Poole, Dorset, UK) unless otherwise stated. Rofecoxib and SC560 were gifts from Boehringer Ingelheim GmbH (Ingelheim, Germany). For the radioimmunoassays, antisera to 6-keto-prostaglandin F$_{1\alpha}$ and thromboxane B$_2$ were obtained from Sigma Chemical; [H]6-keto-prostaglandin F$_{1\alpha}$ and [H]thromboxane B$_2$ were purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK).

Collection of Rat Tissues. Male Wistar rats were obtained from Tuck (Rayleigh, Essex, UK) and kept according to the guidelines set by the Home Office Code of Practice for the Housing and Care of Animals used in Scientific Procedures (1989). Rats (250–280 g) were injected with a lethal dose of pentobarbitone sodium (Sagatal, 120 mg kg$^{-1}$, intraperitoneal). Upon occurrence of deep anesthesia, a laparotomy was performed, and blood from the abdominal aorta was collected into 30 U ml$^{-1}$ heparin using a plastic syringe connected to an 18-gauge needle. Animals were then killed by thoracotomy. Tissues were rapidly removed, cleaned of connective tissue, and finely chopped using a scalpel blade.

Assays of Cyclooxygenase Activity. For whole blood assays, blood was incubated (37°C) with test agents (naproxen, acetaminophen, rofecoxib, or SC560, all 10$^{-6}$ to 10$^{-3}$ M) or vehicle [0.1% (v/v) dimethyl sulfoxide] for 60 min followed by the addition of the calcium ionophore A23187 (50 μM). After a further 30-min incubation, the plates were centrifuged (1500 g, 4°C, 5 min), and the plasma was removed and immediately frozen. Concentrations of thromboxane B$_2$ (as a measure of thromboxane A$_2$ formation and cyclooxygenase activity) in samples were then determined by radioimmunoassay.

For the assay of cyclooxygenase activities in tissue samples, finely chopped tissues were placed into 100-μl incubates of rat plasma. Test compounds (as above) were then added, and incubations continued for 60 min. Plasma (50 μl) was then removed from the incubates (basal) and frozen; this was followed by the addition to the incubates of the calcium ionophore A23187 (50 μM). Thirty minutes later, the incubates were centrifuged (1500g, 4°C, 5 min), the plasma was removed (stimulated) and frozen, and the wet weight of the tissues in each incubate was determined. The frozen plasma samples from the tissue incubates were subsequently assayed for their contents of 6-keto-prostaglandin F$_{1\alpha}$ as a marker of the production of prostaglandin I$_2$.

RT-PCR. Total RNA was extracted from the tissues by homogenization and converted to cDNA by standard methods. Polymerase chain reaction was performed with a Technc Techgene thermocycler (Techne, Cambridge, UK). Initial denaturation was done at 94°C for 2 min and followed by 28 to 40 cycles of amplification. Each cycle consisted of 35 s of denaturation at 94°C, 35 s of annealing at 58°C (for COX-1 and COX-2) or 53°C (for COX-3), and 45 s for enzymatic primer extension at 72°C. After the final cycle, the temperature was held at 72°C for 10 min to allow reannealing of amplified products. Polymerase chain reaction products were then size-fractionated through a 2% agarose gel, and the bands were visualized with ethidium bromide. The sequences for primers for COX-1 and COX-2 were selected according to the published sequences in GenBank and were as follows: COX-1 (160 base pairs) sense, 5’-TAAGTACCAG-GTCCTGGATG-3’ and antisense, 5’-GATCTGAGAACGTCATA-CA-3’; COX-2 (242 base pairs) sense, 5’-TCCAATCGCTGTA-3’ and antisense, 5’-TCCCCAAAAGATAGCATCTGG-3’ (Bishop-Bailey et al., 1997). The sequences for primers for COX-3 (497 base pairs) were sense, 5’-TGGACAAGCTAGGATCGTTACTG-3’ and antisense, 5’-AGAGGGCCAGAATCGACGGTAT-3’ (Ki et al., 2003). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH), a constitutively expressed gene, was chosen as a control. The sequence for G3PDH (452 base pairs) was sense, 5’-ACCACGTCATGGCCATTGAC-3’ and antisense, 5’-TCCACACCTGGTCTGTA-3’.

Western Blot Analysis. Rat tissues, or centrifuged blood cells, were homogenized in 6 volumes of ice-cold protein radioimmunopre-
Results

Cyclooxygenase Activity. Whole blood. Under control conditions, the production of thromboxane B2 by whole blood was 84 ± 11 ng ml⁻¹ (n = 4). This production was inhibited by test agents with the order of potency SC560 > naproxen > acetaminophen ≥ rofecoxib (Fig. 1; Table 1) (ANOVA, p < 0.0001; post test for linear trend, p < 0.0001).

Aorta, heart, and lung. The productions of 6-keto-prostaglandin F1α by aortic, heart, and lung tissue in response to A23187 (stimulated minus basal) were 380 ± 43 pg mg⁻¹ (n = 15), 68 ± 16 pg mg⁻¹ (n = 15), and 103 ± 22 pg mg⁻¹ (n = 15), respectively. As for thromboxane B2 production in whole blood, prostanoid production in these tissues was inhibited by test agents in the order of potency SC560 > naproxen ≥ acetaminophen > rofecoxib (Fig. 2; Table 1) (aorta: ANOVA and post test for linear trend, both p < 0.0001; lung: ANOVA, p < 0.01 and post test for linear trend, p < 0.002; and heart: ANOVA and post test for linear trend, both p < 0.0001).

Brain and cerebellum. The productions of 6-keto-prostaglandin F1α by brain and cerebellum in response to A23187 (stimulated minus basal) were 16 ± 2 pg mg⁻¹ (n = 21) and 37 ± 5 pg mg⁻¹ (n = 21), respectively. In contrast to the other tissues studied, no differences in potencies were found between the test agents (Fig. 3; Table 1) (brain: ANOVA and post test for linear trend, both N.S.; cerebellum: ANOVA and post test for linear trend, both N.S.).

Relative tissue sensitivities. Neither naproxen nor acetaminophen showed any difference in potencies as inhibitors of prostanoid production between test tissues (ANOVA and post test for linear trend, both N.S.). Rofecoxib was markedly more active as an inhibitor of prostaglandin I2 production in brain and cerebellum than in heart and lung and was more active in these latter tissues than in the whole blood or aorta (ANOVA, p < 0.03; post test for linear trend, p < 0.0008). SC560 showed activity in the blood > heart > aorta > lung > brain > cerebellum (ANOVA and post test for linear trend, both p < 0.0001) (Table 1 for all).

### Table 1

Potencies of compounds as inhibitors in the different assay systems

<table>
<thead>
<tr>
<th></th>
<th>Blood</th>
<th>Aorta</th>
<th>Heart</th>
<th>Lung</th>
<th>Brain</th>
<th>Cerebellum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naproxen</td>
<td>-5.7 ± 0.1</td>
<td>-5.0 ± 0.1</td>
<td>-5.5 ± 0.1</td>
<td>-5.4 ± 0.3</td>
<td>-4.6 ± 0.1</td>
<td>-4.5 ± 0.7</td>
</tr>
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<td></td>
<td>2.0</td>
<td>9.3</td>
<td>3.0</td>
<td>4.4</td>
<td>23</td>
<td>33</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>-3.7 ± 0.1</td>
<td>-4.0 ± 0.5</td>
<td>-4.0 ± 0.2</td>
<td>-4.5 ± 0.2</td>
<td>-4.2 ± 0.2</td>
<td>-4.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>204</td>
<td>95</td>
<td>104</td>
<td>35</td>
<td>61</td>
<td>47</td>
</tr>
<tr>
<td>Rofecoxib</td>
<td>-3.3 ± 0.2</td>
<td>-3.1 ± 0.7</td>
<td>-4.5 ± 0.1</td>
<td>-4.6 ± 0.3</td>
<td>-5.8 ± 1.2</td>
<td>-5.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>522</td>
<td>887</td>
<td>29</td>
<td>28</td>
<td>1.5</td>
<td>4.4</td>
</tr>
<tr>
<td>SC560</td>
<td>-1</td>
<td>-6.9 ± 0.2</td>
<td>-7.8 ± 0.6</td>
<td>-5.8 ± 0.3</td>
<td>-5.2 ± 0.4</td>
<td>-4.5 ± 0.3</td>
</tr>
</tbody>
</table>

Data Analysis. Data are expressed as mean ± S.E.M. of separate determinations as specified in individual figure legends. Unless otherwise stated, statistical analyses were performed by applying a one-way analysis of variance (ANOVA) followed by a Tukey-Kramer post test. A p value smaller than 0.05 indicated a statistical difference. All graphs and analyses were determined by Prism 4.00 (GraphPad Software Inc., San Diego, CA.).
Expression of Cyclooxygenase Isoforms. With the exception of blood, RT-PCR analysis demonstrated expression of mRNA for COX-1 and COX-3 in all tissues studied (Fig. 4). COX-2 expression seemed to be limited to the brain and cerebellum. Blood samples showed no detectable mRNA for any of the COX isoforms or G3PDH.

Western blot analysis of cell extracts demonstrated that cyclooxygenase-1 was expressed in all tissues, whereas cyclooxygenase-2 was only found to be expressed in brain and cerebellum (Fig. 5). Commercially available anti-canine cyclooxygenase-3 antibody did not demonstrate expression of cyclooxygenase-3 in any of the tissues examined (data not shown).

Discussion

Here we show that, in a variety of rat tissues, the production of prostanoids is dependent upon the two known isoforms of cyclooxygenase, cyclooxygenase-1 and cyclooxygenase-2, with evidence for neither an involvement of cyclooxygenase-3 nor the presence of a particular, acetaminophen-sensitive cyclooxygenase isoform.

We chose tissues on two bases: first, tissues that have been previously used to characterize the selectivity of NSAIDs;
The effects upon prostaglandin I2 production of the aorta was also characterized as being largely dependent upon cyclooxygenase-1, with rofecoxib producing only a weak effect. This does not explain the blood vessels as cyclooxygenase-1-driven, and Western blot analysis suggests that it is rather the presence of a mixture of cyclooxygenase-1 and cyclooxygenase-2 that is congruent with the drug potencies we found. This is consistent with the apparent impossibility for the expression of a functional cyclooxygenase protein from cyclooxygenase-3 mRNA in the rat. In addition, our experiments show that the ability of cyclooxygenase-2-selective inhibitors to depress the circulating levels of prostaglandin I2 is more readily associated with their ability to reduce production from the lung than from arterial vessels.

References


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