Generation of the Isoprostane 8-Epi-prostaglandin F$_{2\alpha}$ In Vitro and In Vivo via the Cyclooxygenases$^1$

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ABSTRACT

F$_2$-Isoprostanes are isomers of the prostaglandin PGF$_{2\alpha}$. At least one compound of this group, 8-epi-PGF$_{2\alpha}$, exhibits biological activity, and therefore special interest is focused on the mechanism of isoprostane formation: enzyme catalyzed or radical mediated. We analyzed the formation of isoprostanes in vitro and in vivo. In both systems, purified cyclooxygenase isoenzymes and cell models specific for the cyclooxygenase isoenzymes, 8-epi-PGF$_{2\alpha}$, formation could be totally suppressed by cyclooxygenase inhibitors. Indomethacin inhibited concentration-dependent 8-epi-PGF$_{2\alpha}$ formation in platelets stimulated with calcium ionophore, arachidonic acid or thrombin. Nordihydroguaiaretic acid, an antioxidant, blocked isoprostane formation with a similar IC$_{50}$ value as thromboxane B$_2$ synthesis, pointing toward cyclooxygenase as the primary target of inhibition. Based on the turnover number, cyclooxygenase-2 formed higher levels of 8-epi-PGF$_{2\alpha}$ than cyclooxygenase-1. Endogenous 8-epi-PGF$_{2\alpha}$ production in rat mesangial cells correlated well with the mRNA and protein expression of cyclooxygenase-2 during interleukin-1 induction. However, in contrast to human platelets, which produced different forms of isoprostanes, rat mesangial cells appeared to form only 8-epi-PGF$_{2\alpha}$. Further, this indicates that mesangial cells may represent a cellular origin for renal 8-epi-PGF$_{2\alpha}$ formation. Next, we analyzed the formation of isoprostanes in humans. A direct correlation was observed between indomethacin treatment and the decrease in 8-epi-PGF$_{2\alpha}$ and isoprostane levels, but compared with other prostanoids the inhibition was less pronounced. In summary, based on the in vitro studies, a clear cyclooxygenase-dependent formation of isoprostanes, especially 8-epi-PGF$_{2\alpha}$, was observed. However, in vivo additional formation via cyclooxygenase enzyme-independent mechanisms is likely.

Isoprostanes are recently discovered PG-like products formed from arachidonic acid (Morrow et al., 1990). The isoprostanes consist of stereoisomers and regioisomers of the common PGs and were first reported for PGF$_2\alpha$. Later, isoprostanes of the E$_2$ and D$_2$ series were discovered (Morrow et al., 1994). The best characterized isoprostane is 8-epi-PGF$_{2\alpha}$, which has been found to modulate platelet aggregation (Morrow et al., 1992a); later potent vasoconstrictor and bronchoconstriction were described in isolated lungs of rabbit (Banerjee et al., 1992) and rat (Kang et al., 1993). In rats, the intrarenal administration of 8-epi-PGF$_{2\alpha}$ exerted reduction in the glomerular filtration rate and renal plasma flow (Takahashi et al., 1992). Interestingly, these effects can be fully prevented by antagonists of the thromboxane receptor (Banerjee et al., 1992; Takahashi et al., 1992), indicating an interaction with this receptor subtype. Recently, a separate receptor for 8-epi-PGF$_{2\alpha}$ was postulated (Fukunaga et al., 1993).

In explaining the formation of these compounds in vivo, two different mechanisms are discussed. On the one hand, a free radical-catalyzed peroxidation of arachidonic acid with endocyclization, leading to a PGG$_2$-like compound, was suggested. Further reduction or rearrangement in the PGG$_2$-like compound results in F$_2\alpha$ or E$_2$/D$_2$-isoprostane formation (Morrow et al., 1990, 1994). Regarding this mechanism, it has been proposed that detection of isoprostanes might offer a quantitative index for the generation of free radicals and lipid oxidation in vivo (Nourooz Zadeh et al., 1995; Roberts and Morrow, 1994). In accordance with this assumption, elevated levels of isoprostanes have been detected in situations associated with increased free radical generation: during coronary reperfusion and in chronic cigarette smokers (Bachi et al., 1996; Morrow et al., 1995; Takahashi et al., 1992).

On the other hand, a COX-dependent isoprostane formation is discussed (Pratico et al., 1995; Pratico and Fitzgerald 1996). The COXs are rate-limiting enzymes in the arachidonic acid cascade, providing the endoperoxide PGH$_2$, which in turn is further metabolized by specific enzymes to PGs.

ABBREVIATIONS: PG, prostaglandin; COX, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drug; GC/MS/MS, gas chromatography-tandem mass spectrometry; RMC, rat mesangial cells; IL-1, interleukin-1β; TXB, thromboxane; HPS, hyperprostaglandin syndrome; RT, reverse transcription; PCR, polymerase chain reaction.
thromboxanes and prostacyclin. Several recent reports revealed the existence of two isoforms, COX-1 and COX-2 (for a review, see Otto and Smith, 1995). The former is thought to be constitutively expressed and involved in so-called housekeeping functions, such as homeostasis, organ function or hormone modulation, whereas the latter is highly inducible and implicated in the signal cross-talk during pathological situations, such as inflammation.

For further investigations into the mechanism of the formation of isoprostanes, especially 8-epi-PGF$_{2\alpha}$, we established a sensitive and specific assay based on GC/MS/MS for the detection of these compounds in diverse enzymatic and cellular systems as well as in humans and determined its formation under different conditions.

In all systems used, in vitro and in vivo, we observed a COX-dependent formation of 8-epi-PGF$_{2\alpha}$. The fact that isoprostane formation in human samples could not be fully prevented by COX inhibitors suggests that further mechanisms are involved in isoprostane formation in vivo.

Methods

Materials. All chemicals other than those listed below were purchased from Sigma Chemical (Deisenhofen, Germany). Arachidonic acid, COX-1 and COX-2 isoenzymes, purified from sheep tissues, as well as murine COX-2 antibodies were purchased from Cayman Chemicals (Paris, France). According to the supplier, the protein purity of COX-1 is $>95\%$, and that of COX-2 is $>70\%$. IL-18 and CGP 28238 were a kind gift of Dr. C. Vosbeck and Dr. I. Wiesenb (Ciba, Basel, CH). [3,3,4,4-2H$_4$]PGF$_2\alpha$, [3,3,4,4-2H$_4$]6-keto-PGF$_1\alpha$, [3,3,4,4-2H$_4$]TXB$_2$ and [3,3,4,4-2H$_4$]PGF$_2\alpha$, and their nondeuterated analogs were a kind gift from Dr. Udo Axen (Upjohn, Kalamao, MI). [18,18,19,19-18O$_2$]PGE$_2$ was a kind gift of Dr. C. Meese (Dr. Marga- rete Fischer-Bosch Institut, Stuttgart, Germany). 8-Epi-PGF$_{2\alpha}$ was purchased from Cayman Chemicals. [18O]$_2$-8-Epi-PGF$_{2\alpha}$ was prepared from 8-epi-PGF$_{2\alpha}$ and H$_2$18O as previously described (Leh- mann et al., 1992). Ethyl acetate, H$_2$18O (96.5\%) and chloroform were obtained from Promochem (Wesel, Germany). Butyrylcholinesterase and O-methylhydroxylamine hydrochloride were from Sigma. Pentafluorobenzyl bromide was from Lancaster (Mu¨ hlheim/Main, Germany). Water, methanol and formic acid were from Merck (Darms- tafluorobenzyl bromide was from Lancaster (Mu¨ hlheim/Main, Ger- many). Bis-(trimethylsilyl)trifluoroacetamide was from Macherey & Nagel (Du¨ ren, Germany). N,N-Diisopropylethylamine and 0.1 M hydroxylamine hydrochloride were from Aldrich (Milwaukee, Main, Germany). Ar, COX, peroxisomes, COX, and COX inhibitors was carried out on a (J & W) DB-1 (20 m, 0.25 mm i.d., 0.25-µm film thickness) capillary column (Analyst, Mu¨ hlheim, Germany) in a Finnigan MAT TQ700 GC/MS/MS equipped with a Varian 3400 gas chromatograph and a CTC A200S autosampler was used. Gas chromatography of prostaglandin deriv- atives was carried out on a (J & W) DB-1 (20 m, 0.25 mm i.d., 0.25-µm film thickness) capillary column (Analyst, Mu¨ hlheim, Germany) in
the splitless mode. GC/MS/MS parameters were exactly as described by Schweer et al. (1994). Products gained from zone 1 were analyzed for PGE\(_2\), TXB\(_2\), 6-keto-PGF\(_{1\alpha}\), PGF\(_{2\alpha}\), 8-epi-PGF\(_{2\alpha}\) and isoprostanes; products from zone 2 were analyzed for PGD\(_2\). For quantification of PGE\(_{2\alpha}\), 8-epi-PGF\(_{2\alpha}\) and isoprostanes, [P-3(CH\(_3\)]\(_3\)SiOH] daughter ions (m/z 299) were used.

**Statistical analysis.** Comparisons between groups were made by Student’s t test for paired or unpaired data as appropriate. Values of P < .05 were taken as significant.

### Results

**PGE and 8-epi-PGF\(_2\) formation by purified COX isoenzymes.** Opinions differ on whether isoprostane generation occurs via COX-dependent or -independent pathways. To study the possible formation via the enzyme COX, we first used purified COX-1 and COX-2 proteins. As a function of time, PGE\(_2\) and PGD\(_2\), the decay products of PGH\(_2\), reached a plateau after ~1000 sec in COX-2 enzyme samples incubated with arachidonic acid (fig. 1). Surprisingly, PGF\(_{2\alpha}\) and 8-epi-PGF\(_{2\alpha}\) formation did not follow with similar kinetics. These two products reached a maximum at ~150 sec, followed by a decrease to initial quantities after ~3500 sec. An almost identical kinetic behavior was observed in COX-1 assays (data not shown), albeit the product level was lower, with maximal values of 14 ng of PGF\(_{2\alpha}\) and 700 pg of 8-epi-PGF\(_{2\alpha}\) compared with 85 and 4 ng, respectively, for COX-2. For both enzymes, COX-1 and COX-2, a similar ratio of PGF\(_{2\alpha}\) to 8-epi-PGF\(_{2\alpha}\) (18:1) was found. No other isoprostanes except 8-epi-PGF\(_{2\alpha}\) were formed. Preincubation with diclofenac, a potent inhibitor for both isoenzymes, totally blocked the formation of PGs, including 8-epi-PGF\(_{2\alpha}\) (table 1).

**Prostanoid and isoprostane formation in human washed platelets and rat mesangial cells.** As a next approach to analyze isoprostane formation by the COX enzymes, we used cellular models with human washed platelets as COX-1 system and IL-1-stimulated rat mesangial cells as COX-2 system. Human washed platelets revealed time-dependent product formation after stimulation with arachidonic acid or calcium ionophore A23187. The synthesis of main metabolite TXB\(_2\) as well as isoprostanes and PGE\(_2\) showed kinetics of saturation, reaching maximum values after 5 min, followed by a moderate decrease in the following 5 min (data not shown). Values at maximal point are shown in table 2. Furthermore, the addition of a physiological stimulus, such as thrombin, caused a similar formation of 8-epi-PGF\(_{2\alpha}\). However, in contrast to purified COX enzymes, platelets produced an abundant fraction of isoprostanes (eluting profile shown in fig. 2). Interestingly, thrombin forced a much larger synthesis of isoprostanes than A23187 or arachidonic acid (table 2).

Next, we investigated the effects of the COX inhibitors indomethacin and diclofenac as well as the dual lipoxygenase/COX-inhibitor NDGA on prostanoid and isoprostane formation in platelets (fig. 3). Both, indomethacin and diclofenac almost completely suppressed the formation of TXB\(_2\), PGE\(_2\) and 8-epi-PGF\(_{2\alpha}\), whereas less inhibition was observed within the isoprostane fraction. In comparison to that, NDGA was a more effective inhibitor of the isoprostanes than of the prostanoids and 8-epi-PGF\(_{2\alpha}\). Because NDGA is an antioxidant, we determined the IC\(_{50}\) values for isoprostane inhibition in comparison with indomethacin, thereby discriminating the enzyme inhibitory potency from antioxidant potency. For the inhibition of TXB\(_2\) and isoprostane formation by indomethacin, we obtained a half-maximal inhibition of 2.8 and 500 nM, respectively, and the IC\(_{50}\) values of NDGA were 5 \mu M for TXB\(_2\) and 10 \mu M for isoprostanes. The similarity between TXB\(_2\) and isoprostane suppression by NDGA points toward COX activity as the primary target of inhibitor action.

Similar results in product formation and inhibition were obtained by using the calcium ionophor A 23187 or thrombin to stimulate the metabolism of arachidonic acid endogenously (data not shown).

Incubation of rat mesangial cells with IL-1 resulted in a time-dependent increase in prostanoid formation (fig. 4) with 6-keto-PGF\(_{1\alpha}\) as main metabolite. Also, 8-epi-PGF\(_{2\alpha}\) release increased and reached 15% of PGE\(_{2\alpha}\) formation.

To assess isoprostane formation dependent on exogenous substrate, cells were treated with IL-1 for 20 hr after stimulation with 3 \mu M arachidonic acid (fig. 5). A similar kinetic of time-dependent product formation was observed for all prostanoids, including 8-epi-PGF\(_{2\alpha}\). Compared with PGE\(_{2\alpha}\), 12% 8-epi-PGF\(_{2\alpha}\) was formed. As illustrated in figure 6, the COX inhibitors indomethacin and diclofenac completely blocked endogenous PG formation, but 8-epi-PGF\(_{2\alpha}\) formation was blocked slightly less efficiently. 8-Epi-PGF\(_{2\alpha}\) ap-

### Table 1

**Inhibition of product formation from purified COX-1 and COX-2 enzymes by diclofenac**

COX isoenzymes were preincubated with 1 \mu M diclofenac and reaction was started by the addition of 25 \mu M arachidonic acid. In controls, the inhibitor diclofenac was omitted. The experiment was terminated after 150 sec by acidification and extraction of the lipid phase. Values represent percent inhibition compared to control and are reported as mean \pm S.E.M. of four experiments.

<table>
<thead>
<tr>
<th></th>
<th>PGE(_2)</th>
<th>PGI(_2)</th>
<th>PGF(_{2\alpha})</th>
<th>8-Epi-PGF(_{2\alpha})</th>
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<tbody>
<tr>
<td>COX-1</td>
<td>99.75 \pm 0.05</td>
<td>99.5 \pm 0.5</td>
<td>98.1 \pm 0.4</td>
<td>98.5 \pm 0.9</td>
</tr>
<tr>
<td>COX-2</td>
<td>98.8 \pm 0.04</td>
<td>99.6 \pm 0.1</td>
<td>99.5 \pm 0.15</td>
<td>99.3 \pm 0.29</td>
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Fig. 1. Time-dependent formation of PGs by COX-2. Purified COX-2 (40 units) was incubated with 25 \mu M arachidonic acid for the indicated time points. Synthesis of PGs was stopped by extraction of the lipid phase, and PGs were determined by GC/MS/MS. Inset, formation of 8-epi-PGF\(_{2\alpha}\). The figure is representative of three independent experiments. ○, PGD\(_2\); ●, PGE\(_2\); ▽, PGF\(_{2\alpha}\); ▼, 8-epi-PGF\(_{2\alpha}\).
peared to be the only isoprostane formed by mesangial cells (data not shown).

To evaluate whether IL-1-induced production of these compounds was dependent on de novo synthesis of COX-2, we investigated its expression. Western blot (fig. 7A) and RT-PCR analysis (fig. 7B) of cell lysates confirmed the induction of transcription and translation of COX-2 after IL-1 treatment. An important finding was that dexamethasone, which is known to suppress de novo synthesis of COX-2, inhibited the formation of the PGs as well as 8-epi-PGF$_{2\alpha}$ at similar extent (fig. 6). Confirmation that product formation was dependent on COX-2 activity was given by the complete suppression of PG and 8-epi-PGF$_{2\alpha}$ formation by CGP 28238, a selective COX-2 inhibitor (Klein et al., 1994, 1996) (table 3). In all cell experiments, 8-epi-PGF$_{2\alpha}$ was the only isoprostane formed (data not shown).

**Influence of the COX-inhibitor indomethacin on in vivo prostanoid and isoprostane formation.** We investi-
gated the effect of the COX inhibition on human prostanoid and isoprostane formation in two different collectives. In group 1, healthy volunteers were treated with indomethacin for 2 days, and urine samples were collected and analyzed for COX products. After indomethacin withdrawal for 72 hr, urinary prostanoid levels were analyzed again and taken as control values. Comparison between the urine samples taken before and after indomethacin withdrawal showed that indomethacin significantly suppressed the synthesis of TXB2 and of its metabolization products 2,3-dinor-TXB2 and 11-dehydro-TXB2 (fig. 8A). Although lowered, prostanoid and 8-epi-PGF2α formation were significantly different, but isoprostane formation was not (P ≠ 0.09).

Consequently, a second group with HPS (Seyberth et al., 1985) was selected. HPS is a complex congenital tubular disorder associated with excessive renal and systemic PGE2 biosynthesis (Seyberth et al., 1997). In this collective, long-term treatment with indomethacin improves the prognosis of HPS by suppressing PGE-M and PGE2 levels. To investigate the inhibitory effect of indomethacin on prostanoid and isoprostane formation, long-term medication with indomethacin was interrupted for 72 hr, and urinary excretion rates of prostanoids and isoprostanes were determined before and at the end of inhibitor withdrawal, according to the study protocol. During this time interval, PGE2 and indomethacin concentrations were monitored to control PG biosynthesis. In contrast to the results gained in the control group, we ob-
8-epi-PGF2\(_a\) was not consistent for all metabolites obtained within the isoprostane fraction. The comparably small amounts of 8-epi-PGF2\(_a\) in the assays of the purified COX proteins suggest the synthesis as a by-product during catalysis, as earlier demonstrated for several products of [\(^{14}\)C]arachidonic acid-stimulated microsomes from sheep seminal vesicels (Hecker et al., 1997). A plausible explanation would be that radicals formed during the metabolism of PGG2\(_a\) to PGH2\(_a\) (Kuehl et al., 1977) are responsible for the formation of 8-epi-PGF2\(_a\). Accordingly, NDGA, which is known to act as an antioxidant, exerts similar inhibitory potency on isoprostane formation compared with inhibition on TXB2 formation, indicating the involvement of COX activity. However, assuming this mechanism, other isoprostanes should be expected, because a free radical-catalyzed mechanism is unlikely to result in only one specific product. Therefore, the observation of 8-epi-PGF2\(_a\) as the only isoprostane formed by isolated proteins and rat mesangial cells points against the radical theory and to a more complex mechanism, including enzyme-specific activity. In this context, the decreasing levels of PGF2\(_a\) and 8-epi-PGF2\(_a\) after several minutes to basal levels in the protein assays could be a sign of radical cooxidation of these compounds, as has been reported for different xenobiotics as well as for PGs (Eling et al., 1990).
In the cell system, this cooxidation may be blocked by reducing agents, such as proteins or glutathione. In human urinary samples, the generation of 8-epi-PGF$_2$$\alpha$ is not as obvious as in the protein or cell experiments. First, in contrast to the enzymatic formation described, isoprostanes appear as a large fraction in the GC/MS/MS-chromatogram (data not shown), indicating a great variety of compounds. Second, 8-epi-PGF$_2$$\alpha$ levels are in a similar range as PGE$_2$ or PGF$_2$$\alpha$. Moreover, isoprostanes are the most abundant metabolites detected. This is in clear contrast to the synthesizing ability of the COX proteins and of the used cell systems.

In the control group, indomethacin suppressed mainly the thromboxane metabolites but also significantly 8-epi-PGF$_2$$\alpha$ formation. This is consistent with the higher susceptibility of the systemic prostanooid synthesis toward COX inhibition. In agreement, similar decrease in urinary excretion of 11-dehydro-TXB$_2$ by aspirin in healthy volunteers was reported (Wang et al., 1995). However, although a suppression of serum levels of 8-epi-PGF$_2$$\alpha$ and TXB$_2$ was detectable, no decrease in 8-epi-PGF$_2$$\alpha$ levels was observed. The authors concluded that there was COX-independent 8-epi-PGF$_2$$\alpha$ formation in the kidney. However, in this study, other PGs were not determined; therefore, it is unknown whether COX activity was totally abolished. We observed in the HPS group with long-term indomethacin treatment that all urinary prostanooid products were significantly suppressed, albeit by quite different extents: the inhibitory potency of indomethacin on isoprostanes and 8-epi-PGF$_2$$\alpha$ is reduced in respect to regularly formed prostanooids. These facts point toward the evidence of further pathways in addition to an enzymatic generation.

In conclusion, we assume that COXs are able to synthesize 8-epi-PGF$_2$$\alpha$ but that in vivo, further, most likely radical-based, mechanisms do exist. However, the combined action of these mechanisms may exclude isoprostanes and especially 8-epi-PGF$_2$$\alpha$ as an exclusive parameter for oxidative injuries. Therefore, it might be necessary to determine under such conditions in addition to 8-epi-PGF$_2$$\alpha$ the classical PGs to exclude any contribution by the COX pathway.

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**References**


