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Reduction of urinary 8-epi-prostaglandin $F_{2\alpha}$ during cyclo-oxygenase inhibition in rats but not in man

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1 8-epi-prostaglandin (PG) $F_{2\alpha}$, a major F_2 isoprostane, is produced *in vivo* by free radical-dependent peroxidation of lipid-esterified arachidonic acid. Both cyclo-oxygenase isoforms (COX-1 and COX-2) may also form free 8-epi-$PGF_{2\alpha}$ as a minor product. It has been recently seen in human volunteers that the overall basal formation of 8-epi-$PGF_{2\alpha}$ *in vivo* is mostly COX-independent and urinary 8-epi-$PGF_{2\alpha}$ is therefore an accurate marker of 'basal' oxidative stress *in vivo*.

2 To test the validity of this marker in the rat, we evaluated *in vivo* the effect of COX inhibition on the formation of 8-epi-$PGF_{2\alpha}$ vs prostanoids. Two structurally unrelated COX inhibitors (naproxen: 30 mg kg^{-1} day^{-1}; indomethacin: 4 mg kg^{-1} day^{-1}) were given i.p. to rats kept in metabolic cages. *In vivo* formation of 8-epi-$PGF_{2\alpha}$ was assessed by measuring its urinary excretion. Prostanoid biosynthesis was assessed by measuring urinary excretion of major metabolites of thromboxane (TX) and prostacyclin (2,3-dinor-TXB_1 and 2,3-dinor-6-keto-$PGF_{1\alpha}$). All compounds were selectively measured by immunopurification/gas chromatography-mass spectrometry.

3 Naproxen reduced urinary excretion of 2,3-dinor-TXB_1 and 2,3-dinor-6-keto-$PGF_{1\alpha}$ but, unexpectedly, also that of 8-epi-$PGF_{2\alpha}$ (82, 49 and 52% inhibition, respectively). Indomethacin had a similar effect (77, 69 and 55% inhibition). Esterified 8-epi-$PGF_{2\alpha}$ in liver and plasma remained unchanged after indomethacin.

4 These findings prompted us to re-assess the contribution of COX activity to the systemic production of 8-epi-$PGF_{2\alpha}$ in man. We gave naproxen (1 g day^{-1}) to healthy subjects (four nonsmokers and four smokers). Urinary 8-epi-$PGF_{2\alpha}$ remained unchanged in the two groups (9.63 ± 0.99 before vs 10.24 ± 1.01 after and 20.14 ± 3.00 vs 19.03 ± 2.45 ng h^{-1} 1.73 m^{-2}), whereas there was a marked reduction of major urinary metabolites of thromboxane and prostacyclin (about 90% for both 11-dehydro-TXB_2 and 2,3-dinor-TXB_2; $>50\%$ for 2,3-dinor-6-keto-$PGF_{1\alpha}$).

5 To investigate whether rat COX-1 produces 8-epi-$PGF_{2\alpha}$ more efficiently than human COX-1, we measured the *ex vivo* formation of 8-epi-$PGF_{2\alpha}$ and TXB_2 simultaneously in whole clotting blood. Serum levels of 8-epi-$PGF_{2\alpha}$ and TXB_2 were similar in rats and man.

6 We conclude that a significant amount of COX-dependent 8-epi-$PGF_{2\alpha}$ is present in rat but not in human urine under normal conditions. This implies that urinary 8-epi-$PGF_{2\alpha}$ cannot be used as an index of near-basal oxidant stress in rats. On the other hand, our data further confirm the validity of this marker in man.

Keywords: 8-epi-$PGF_{2\alpha}$; F_2-isoprostanes; thromboxane; prostacyclin; cyclo-oxygenase; indomethacin; naproxen; oxidative stress

Introduction

F_2-isoprostanes, produced by free-radical mediated peroxidation of arachidonic acid, have recently been found to be accurate markers of oxidant stress *in vivo* (Morrow & Roberts, 1991; 1996). 8-epi-Prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$) is the most widely studied F_2-isoprostane, since it is a major product *in vivo* (Morrow *et al*., 1994) and has potent biological activities (Kang *et al*., 1993; Morrow & Roberts, 1996). The validity of 8-epi-$PGF_{2\alpha}$ as a marker of oxidant stress has been recently re-examined since, in contrast to other F_2-isoprostanes, which can only be produced nonenzymatically, this isomer can also be formed by cyclo-oxygenase (COX) as a minor product (Hecker *et al*., 1987). Both the constitutive and the inducible COX isoforms (COX-1 and COX-2) can form 8-epi-$PGF_{2\alpha}$. In fact, platelet COX-1 (Praticò *et al*., 1995) and monocyte COX-2 (Praticò & FitzGerald, 1996; Patrignani *et al*., 1996) produce small amounts of 8-epi-$PGF_{2\alpha}$ concomitantly with prostanoid biosynthesis.

The *in vivo* contribution of COX-derived 8-epi-$PGF_{2\alpha}$ to its basal overall production, evaluated from its urinary excretion, appears to be insignificant in man (Wang *et al*., 1995; Delanty *et al*., 1996). These findings reinforce the concept that basal 8-epi-$PGF_{2\alpha}$ is mostly formed *in vivo* through a nonenzymatic free radical-mediated mechanism, and in particular that selective measurement of this isoprostane in urine permits accurate monitoring of oxidant stress in man. To verify whether this holds true for the rat too, we administered two structurally unrelated COX inhibitors (indomethacin and naproxen) to normal rats and evaluated the systemic formation of 8-epi-$PGF_{2\alpha}$ compared with two COX products, thromboxane and prostacyclin. Since COX inhibition was accompanied by significant decreases of both urinary 8-epi-$PGF_{2\alpha}$ and prostanoids, we reconsidered the origin of urinary 8-epi-$PGF_{2\alpha}$ in healthy volunteers.

Methods

Animals

Male Sprague-Dawley CD COBS rats (300–350 g body weight; Charles River, Calco, Italy) were used. Procedures involving animals and their care were conducted in accordance with the Institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 Febbraio 1992) and international laws and policies (EEC Council Directives 86/609, OJ L 358, 1, Dec. 12, 1987; NIH Guide for the Care and

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Use for Laboratory Animals, NIH Publication No. 85-23, 1985).

Rat urine

Urine was collected from rats kept in metabolic cages, with free access to food and water. Collections were made before (−24 to 0 h) and after (0 to 24 h) treatment with vehicle or COX-inhibitors (at time 0 and 12 h, 15 mg kg^{-1} naproxen sodium or 2 mg kg^{-1} indomethacin). Samples were stored at −20°C until analysed.

Rat plasma

Blood, taken from anaesthesized rats by intracardiac puncture, was collected into heparin-treated syringes and spiked with butylated hydroxytoluene (BHT, final concentration 50 μg ml^{-1}). An aliquot of plasma, obtained by centrifugation, was immediately immunoextracted for free 8-epi-$PGF_{2\alpha}$ and analysed as below. Another aliquot was stored at −20°C for one week, then assayed for total (free plus esterified) 8-epi-$PGF_{2\alpha}$ after hydrolysis (1 ml plasma plus 1 ml KOH 1 M, 30 min at 37°C).

Rat liver

Livers of rats treated with indomethacin or vehicle were rapidly frozen in liquid nitrogen and stored at −70°C until analysed. Samples were processed as described by Morrow & Roberts (1994), with modifications. Briefly, livers were homogenized in ice-cold Folch solution containing 0.005% BHT (10 ml g^{-1} of tissue). After extraction of lipids, [$^2H_4$]-8-epi-$PGF_{2\alpha}$ was added and the samples were hydrolysed at 37°C for 30 min with a 1 : 1 mixture of methanol (0.005% BHT) and aqueous KOH (15%). The samples were then acidified to pH 3 and purified on C18 solid phase extraction (SPE) columns. The final eluate was evaporated to dryness, redissolved in phosphate buffer (0.05 M, pH 7.4) and immunopurified as below.

Human volunteers

Healthy volunteers (four nonsmokers and four smokers, one female and three males per group; age: 29±6 and 35±3 y, respectively) were recruited at our Institute. Subjects had not taken drugs or vitamin supplements in the preceding two weeks. Their informed consent was obtained and they were given two oral doses of naproxen sodium (550 mg) at meals, with a 12 h interval (20 h 00 min on day 1 and 08 h 00 min on day 2). Urine was collected for 6 h, from 10 h 00 min to 16 h 00 min on day 1 and on day 2. Samples were frozen and stored at −20°C until analysed.

Rat and human serum

Blood was collected without anticoagulant by intracardiac puncture from control rats ($n=3$) or from the antecubital vein from healthy drug-free nonsmokers (age 32±7 y, $n=3$). Each sample was distributed into glass test tubes, with or without indomethacin (10 μg ml^{-1}, final concentration). Blood was left to clot at 37°C for 1 h and serum was prepared as described by Alessandrini *et al.* (1985). An aliquot of each sample was immediately assayed for 8-epi-$PGF_{2\alpha}$ as described below; another aliquot was stored at −20°C until used for thromboxane B_2 (TXB_2) analysis (extraction on C18 SPE columns).

Immunoaffinity extractions

The immunoaffinity extraction procedures for 8-epi-$PGF_{2\alpha}$, 2,3-dinor-6-keto-$PGF_{1\alpha}$, 11-dehydro-TXB_2, 2,3-dinor-TXB_2 and 2,3-dinor-TXB_1 have been described in detail elsewhere (Chiabrando *et al.*, 1989; 1993; 1994; Bachi *et al.*, 1996b). Briefly, biological specimens (urine, plasma, serum, tissue extract) were diluted to 20 ml with phosphate buffer (0.05 M, pH 7.4) containing deuterated analogues of the analytes as internal standards. Samples were filtered and percolated through an immunoaffinity column prepared with the appropriate immobilized antibody. The column was washed with water and eluted with acetone-water (95 : 5, v/v).

Gas chromatography-mass spectrometry

Samples from immunoaffinity or SPE extractions were dried and derivatized to pentafluorobenzyl (PFB) ester, trimethylsilyl ether (TMS) for 8-epi-$PGF_{2\alpha}$ or to PFB, TMS, O-methyloxime for 2,3-dinor-6-keto-$PGF_{1\alpha}$, 11-dehydro-TXB_2, 2,3-dinor-TXB_2, 2,3-dinor-TXB_1 and TXB_2, as described previously (Chiabrando *et al.*, 1989; 1993; 1994; Bachi *et al.*, 1996b). Samples were analysed by gas chromatography/negative-ion chemical ionization mass spectrometry in the selected ion recording mode, monitoring the carboxylate anions (M-PFB) as described.

Drugs

Naproxen sodium and indomethacin (as meglumine salt) were used.

Statistics

Results are expressed as mean±s.e.mean. Statistical significance was analysed by ANOVA followed by Tukey's test or by paired or unpaired Student's *t* test, as appropriate. Differences were considered significant when $P<0.05$.

Results

In vivo *formation of 8-epi-$PGF_{2\alpha}$ and prostanoids during COX inhibition in rats*

To assess inhibition of COX activity *in vivo*, we measured urinary excretion of major enzymatic metabolites of thromboxane (2,3-dinor-TXB_1) and prostacyclin (2,3-dinor-6-keto-$PGF_{1\alpha}$) in this species (Sun & Taylor, 1978; Chiabrando *et al.*, 1994).

Under basal conditions, mean urinary excretion of 2,3-dinor-TXB_1 and 2,3-dinor-6-keto-$PGF_{1\alpha}$ was 23.61±1.35 and 14.51±1.62 ng 24 h^{-1} ($n=15$), respectively. After naproxen, urinary excretion of 2,3-dinor-TXB_1 and 2,3-dinor-6-keto-$PGF_{1\alpha}$ was reduced to 18.4±1.1% and 50.7±3.0% of the pretreatment values ($P<0.001$ vs vehicle; $n=5$) (Figure 1). Indomethacin reduced the two metabolites to 22.7±3.8% and 31.4±2.8% of their basal levels ($P<0.001$ *vs* vehicle, $n=5$). The vehicle did not affect urinary excretion of 2,3-dinor-TXB_1 (89.8±7.7%), and increased that of 2,3-dinor-6-keto-$PGF_{1\alpha}$ (161±20%, $P<0.05$ vs basal values, $n=5$). Basal urinary excretion of 8-epi-$PGF_{2\alpha}$ was 4.34±0.48 ng 24 h^{-1} ($n=15$). Both naproxen and indomethacin significantly ($P<0.005$) reduced levels of 8-epi-$PGF_{2\alpha}$ to 48.0±5.9% and 45.5±5.3% of their pretreatment values, while vehicle had no effect (97.8±12.2%).

Urinary 8-epi-$PGF_{2\alpha}$ (y; ng 24 h^{-1}) before and during COX inhibition weakly but significantly correlated ($y=0.064x+1.726$; $r=0.51$; $n=30$; $P<0.005$) with the sum of 2,3-dinor-TXB_1 and 2,3-dinor-6-keto-$PGF_{1\alpha}$ (x; ng 24 h^{-1}). Similar figures were found when the correlation of 8-epi-$PGF_{2\alpha}$ with each COX metabolite was considered separately (data not shown).

In vivo *formation of 8-epi-$PGF_{2\alpha}$ and prostanoids during COX inhibition in man*

To assess COX activity *in vivo* in healthy volunteers before and during administration of naproxen, we measured urinary excretion of the major enzymatic metabolites of thromboxane

(2,3-dinor-TXB_2 and 11-dehydro-TXB_2) and prostacyclin (2,3-dinor-6-keto-$PGF_{1\alpha}$) reflecting systemic biosynthesis of these prostanoids (FitzGerald *et al*., 1983; Catella & FitzGerald, 1987). In parallel, urinary excretion of 8-epi-$PGF_{2\alpha}$ was measured. Results, expressed as ng h^{-1} 1.73 m^{-2}, are shown in Figure 2. Both thromboxane metabolites and 8-epi $PGF_{2\alpha}$ were higher in smokers than nonsmokers, as found by Delanty *et al*. (1996). Naproxen caused a marked inhibition of systemic COX activity, as indicated by the reduced excretion of COX-derived metabolites, while urinary 8-epi-$PGF_{2\alpha}$ remained unchanged (Figure 2). No correlation was found between urinary 8-epi-$PGF_{2\alpha}$ and total urinary prostanoids before and during COX inhibition in smokers and nonsmokers ($r=0.32$; $n=16$; $P=0.22$).

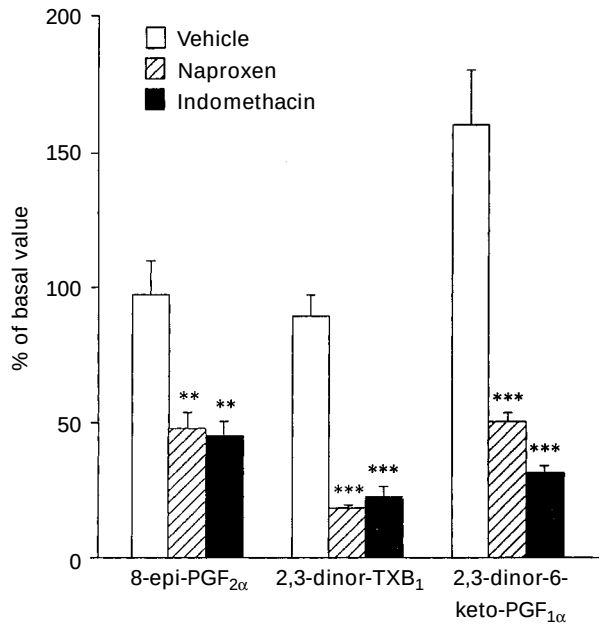

Figure 1 Urinary excretion of 8-epi-$PGF_{2\alpha}$, 2,3-dinor-TXB_1 and 2,3-dinor-6-keto-$PGF_{1\alpha}$ in rats after administration of vehicle, naproxen or indomethacin. Data are expressed as means ± s.e.mean ($n=5$). Individual percentages refer to the corresponding basal value. $^{**}P<0.005$; $^{***}P<0.001$ vs vehicle.

Lipid-esterified 8-epi-$PGF_{2\alpha}$ in rats given indomethacin

To investigate whether, in our setting, reduction of urinary 8-epi-$PGF_{2\alpha}$ by COX inhibitors was caused by a reduction of its nonenzymatic free radical-mediated formation, we measured the levels of 8-epi-$PGF_{2\alpha}$ esterified to lipids. Livers of rats treated with indomethacin had levels of esterified 8-epi-$PGF_{2\alpha}$ similar to those of controls (590 ± 52 and 619 ± 34 pg g^{-1}; $n=5$, $P=0.65$). In plasma, total (esterified plus free) 8-epi-$PGF_{2\alpha}$ was 443 ± 25 and 478 ± 30 pg ml^{-1} ($n=5$, $P=0.4$), while the corresponding plasma levels of free 8-epi-$PGF_{2\alpha}$ were 6.1 ± 0.62 and 14.6 ± 2.5 pg ml^{-1} ($n=5$, $P<0.01$).

COX-dependent ex vivo *formation of 8-epi-$PGF_{2\alpha}$ and prostanoids in rat and man*

To explore whether rat and human COX-1 enzyme differs in the production of 8-epi-$PGF_{2\alpha}$ and prostanoids, we measured the amounts of 8-epi-$PGF_{2\alpha}$ and TXB_2 produced during spontaneous whole blood clotting *ex vivo* in the two species. TXB_2 is in fact the major COX-derived product in control serum, mostly reflecting platelet COX activity (Alessandrini *et al*., 1985), in turn related to the constitutive isoform of the enzyme, COX-1. In rats, serum levels of TXB_2 were 147 ± 19 ng ml^{-1}, while 8-epi-$PGF_{2\alpha}$ was three orders of magnitude less (251 ± 8 pg ml^{-1}). Indomethacin added to blood similarly reduced TXB_2 and 8-epi-$PGF_{2\alpha}$ (50 ± 7% and 44 ± 7%). Levels of these two compounds with and without indomethacin ($n=4$) strongly correlated in each animal ($r=0.98\pm0.003$, all $P<0.01$), confirming their common origin in this preparation (Wang *et al*., 1995).

Levels of TXB_2 and 8-epi-$PGF_{2\alpha}$ in serum from three healthy nonsmoking drug-free volunteers were, respectively, 303 ± 93 ng ml^{-1} and 113 ± 26 pg ml^{-1}. Inhibition by indomethacin was 89 ± 5% for TXB_2 and 86 ± 6% for 8-epi-$PGF_{2\alpha}$. As with rat serum, levels of TXB_2 and 8-epi-$PGF_{2\alpha}$ with and without indomethacin ($n=4-5$) were highly correlated in all subjects ($r=0.98\pm0.01$, all $P<0.01$).

Discussion

F_2-isoprostanes are a complex family of isomeric compounds formed by nonenzymatic free radical-mediated peroxidation of arachidonic acid, reflecting lipid peroxidation *in vivo* in a wide range of conditions, both in man and experimental animals

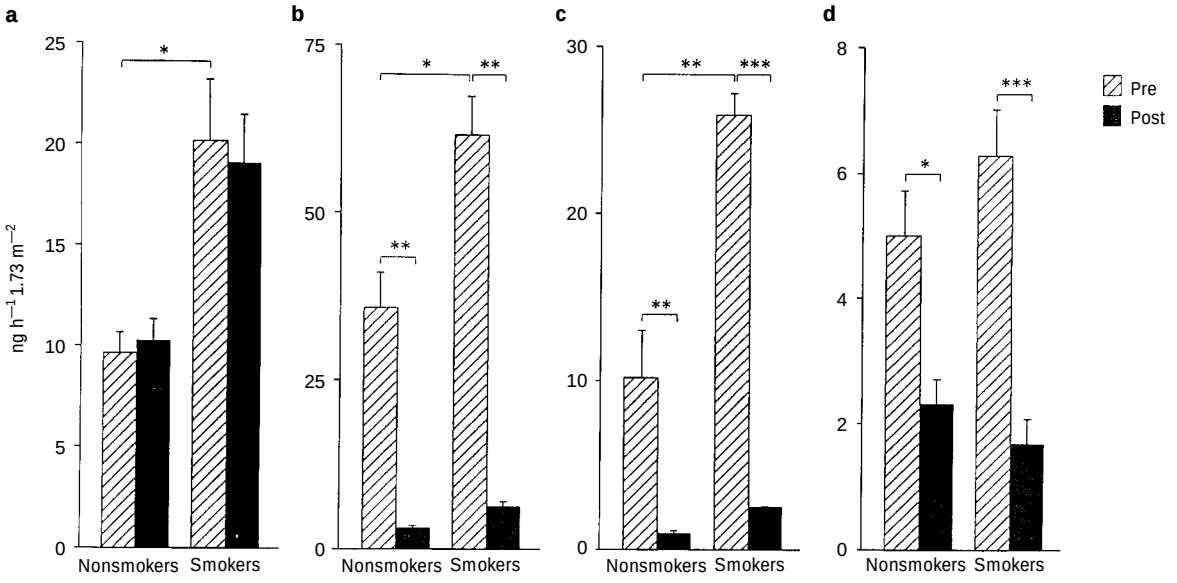

Figure 2 Urinary excretion of (a) 8-epi-$PGF_{2\alpha}$, (b) 11-dehydro-TXB_2, (c) 2,3-dinor-TXB_2 and (d) 2,3-dinor-6-keto-$PGF_{1\alpha}$ in nonsmoking or smoking healthy volunteers before and after administration of naproxen. Data are expressed as ng h^{-1} 1.73 m^{-2} ± s.e.mean ($n=4$). $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

(Morrow & Roberts, 1996). F_2-isoprostanes are therefore currently used as indices of oxidant stress *in vivo* (Morrow & Roberts, 1991).

8-epi-$PGF_{2\alpha}$ is the best known and most widely studied F_2-isoprostane isomer since it is a major product *in vivo* (Morrow *et al*., 1994) and has potent biological activities possibly contributing to oxidant damage (Kang *et al*., 1993). Selective measurement of 8-epi-$PGF_{2\alpha}$ in urine is an attractive noninvasive tool for time-integrated measurement of its formation *in vivo* (Delanty *et al*., 1996). Although the origin of urinary 8-epi-$PGF_{2\alpha}$ has not been specifically investigated, ample evidence suggests that it comes mostly from free 8-epi-$PGF_{2\alpha}$ filtered from the circulation. In fact, conditions believed to be associated with increased oxidant stress in target organs other than kidney (e.g., chronic cigarette smoking, paracetamol or paraquat intoxication, alcohol induced liver disease or coronary reperfusion with thrombolytic drugs) were all accompanied by increased urinary excretion of 8-epi-$PGF_{2\alpha}$ (Delanty *et al*., 1996). The twofold increase in F_2-isoprostane formation detected in smokers by measuring the free compounds in plasma and their metabolites in urine (Morrow *et al*., 1995) corresponds to an equal increase in urinary excretion of 8-epi-$PGF_{2\alpha}$ (Catella *et al*., 1995; Wang *et al*., 1995; Bachi *et al*., 1996b) again suggesting a common origin of urinary and circulating F_2-isoprostanes. Our present data agree with this hypothesis, since in the rat a reduction of urinary 8-epi-$PGF_{2\alpha}$ was indeed accompanied by a comparable drop in circulating free 8-epi-$PGF_{2\alpha}$.

In contrast to other isoprostanes which can only come from nonenzymatic peroxidation of free or esterified arachidonic acid, 8-epi-$PGF_{2\alpha}$ can also be formed as a minor product during biotransformation of free arachidonic acid to PGH_2 by COX (Hecker *et al*., 1987). 8-Epi-$PGF_{2\alpha}$ was recently shown to be formed enzymatically by COX-1 in a number of preparations such as sheep microsomal and purified enzyme, human isolated platelets, human serum, or by COX-2 in LPS-treated human monocytes (Hecker *et al*., 1987; Praticò *et al*., 1995; Praticò & FitzGerald, 1996; Patrignani *et al*., 1996). Therefore, as opposed to lipid-esterified 8-epi-$PGF_{2\alpha}$ which can only be formed nonenzymatically (Morrow *et al*., 1992), free 8-epi-$PGF_{2\alpha}$ in biological fluids may not exclusively reflect free radical-mediated lipid peroxidation.

Despite these possible drawbacks, urinary excretion of 8-epi-$PGF_{2\alpha}$ has been recently validated as a marker of oxidant stress in man. In fact, different COX inhibitors did not reduce urinary excretion of 8-epi-$PGF_{2\alpha}$, while significantly inhibiting COX activity *in vivo* (Catella *et al*., 1995; Wang *et al*., 1995; Reilly *et al*., 1996). Moreover, urinary 8-epi-$PGF_{2\alpha}$ was not modified during selective inhibition of platelet COX activity *in vivo* with chronic low-dose aspirin (Catella *et al*., 1995). These results convincingly validated the use of urinary 8-epi-$PGF_{2\alpha}$ as a marker of oxidant stress in man, both basally and under circumstances of platelet COX activation, such as in chronic smokers (Reilly *et al*., 1996).

In the rat, although *in vivo* formation of F_2-isoprostanes is not reduced by high-dose COX inhibitors (Morrow *et al*., 1990; Awad *et al*., 1993), no data are available specifically regarding 8-epi-$PGF_{2\alpha}$. Since we intended to use this isomer as a marker of oxidant stress in rats, we checked whether it was mostly COX-independent in this species too. To establish a clear relationship between COX activity and formation of 8-epi-$PGF_{2\alpha}$ *in vivo*, we simultaneously and highly selectively measured major prostanoid metabolites and 8-epi-$PGF_{2\alpha}$ in urine. Unexpectedly, we found that two structurally unrelated COX inhibitors significantly reduced 8-epi-$PGF_{2\alpha}$. Inhibition was less marked for 8-epi-$PGF_{2\alpha}$ than prostanoids, but its amplitude was consistent with the presence of a significant amount of the isoprostane derived from COX activity in rat urine. We did find evidence of a weak but significant correlation between urinary 8-epi-$PGF_{2\alpha}$ and COX-derived prostanoid metabolites in rats ($P<0.005$) but not in man ($P=0.22$), suggesting that these compounds have a partially common origin only in the former species. We have recently collected further indirect evidence supporting this hypothesis: (a) in diabetic rats, an excess of urinary COX products is accompanied by increased urinary 8-epi-$PGF_{2\alpha}$ which can be reduced by indomethacin (Bachi *et al*., 1997); (b) rats given lipopolysaccharide (LPS) have abnormally high urinary excretion of prostanoids and 8-epi-$PGF_{2\alpha}$ (Bachi *et al*., 1996a).

To exclude the possibility that the reduction of urinary 8-epi-$PGF_{2\alpha}$ was due to some unforeseen effect of the COX inhibitors on the formation or release of free radical-derived 8-epi-$PGF_{2\alpha}$ rather than its COX-dependent production, we measured tissue and plasma levels of esterified 8-epi-$PGF_{2\alpha}$, which cannot be formed by COX. Since indomethacin did not cause any reduction, it seems reasonable to conclude that urinary 8-epi-$PGF_{2\alpha}$ derives in part from free 8-epi-$PGF_{2\alpha}$ synthesized by COX. It seems unlikely that the COX inhibitors have reduced urinary 8-epi-$PGF_{2\alpha}$ through other mechanisms, such as an antioxidant action, because this would have probably become apparent in man in this and other studies. Also, in rats urinary 8-epi-$PGF_{2\alpha}$ appears to be poorly sensitive to antioxidants, since it was not reduced by high-dose chronic probucol (1% in the diet for one month) (Chiabrando *et al*., unpublished observations).

We then decided to explore whether, at least in man, we could confirm that urinary 8-epi-$PGF_{2\alpha}$ is mostly COX-independent. In addition to nonsmokers, we also examined chronic smokers. Cigarette smoking on the one hand causes chronic oxidant stress which is reflected by higher formation of F_2-isoprostanes (Morrow *et al*., 1995) and on the other hand induces platelet activation with elevated formation of thromboxane (Nowak *et al*., 1987). In full agreement with previous observations (Wang *et al*., 1995; Reilly *et al*., 1996), we found that, in the presence of a marked reduction of urinary prostanoids, urinary 8-epi-$PGF_{2\alpha}$ remained unchanged both in nonsmokers and smokers. The latter, as expected, had higher levels of 8-epi-$PGF_{2\alpha}$ and higher levels of thromboxane metabolites. These data, obtained with a different analytical method, confirm the validity of urinary 8-epi-$PGF_{2\alpha}$ as a COX-independent marker of oxidant stress in man at least under basal conditions and under circumstances of moderate COX activation.

To explain the different effects on urinary 8-epi-$PGF_{2\alpha}$ during comparable reductions of COX activity in the two species, we checked for any major difference between human and rat COX enzymes as to the fractional conversion of arachidonic acid to PGH_2 and 8-epi-$PGF_{2\alpha}$. Focusing on the constitutive isoform COX-1, which probably contributes mostly to the physiological biosynthesis of prostanoids (Feng *et al*., 1993; Wu, 1996), we assumed that, if rat COX-1 had a high capacity for producting 8-epi-$PGF_{2\alpha}$ under basal conditions, the fraction of urinary 8-epi-$PGF_{2\alpha}$ derived from COX would be appreciable.

Since human platelets have been fully characterized as regards COX-1-dependent biosynthesis of 8-epi-$PGF_{2\alpha}$ *vs* TXB_2 (Wang *et al*., 1995; Praticò *et al*., 1995) we measured both compounds in rat and human serum. Our results with human serum are identical to those obtained by others, confirming that human platelet COX-1 produces 8-epi-$PGF_{2\alpha}$ and TXB_2 with a molar ratio of about 1 : 1000. Rat platelet COX-1 formed the two compounds in similar amounts, indicating that in this intact cell system, without any exogenous substrate added, the relative production of 8-epi-$PGF_{2\alpha}$ and TXB_2 by COX-1 is similar in the two species. These results suggest that the original assumption is unlikely. However, the fact that the catalytic activity of COX-1 *ex vivo* is similar in man and rats does not exclude the possibility that other factors *in vivo*, such as different substrate availability in the two species, might lead to different amounts of COX by-products.

We conclude that in the rat a substantial amount of basal urinary 8-epi-$PGF_{2\alpha}$ derives from COX activity or is reduced by COX inhibitors. Therefore, urinary excretion of 8-epi-$PGF_{2\alpha}$ cannot be used as an accurate marker of oxidant stress in the rat, at least under near-basal conditions, and other selected COX-independent F_2-isoprostane isomers should be investigated as better candidates (Delanty *et al*., 1996). On the

other hand, our data further confirm the validity of urinary 8-epi-$PGF_{2\alpha}$ as an accurate, noninvasive marker of oxidant stress in man.

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References

ALESSANDRINI, P., AVOGARO, P., BITTOLO BON, G., PATRIGNANI, P. & PATRONO, C. (1985). Physiologic variables affecting thromboxane B2 production in human whole blood. *Thrombosis Res.*, **37,** 1–8.

AWAD, J.A., MORROW, J.D., TAKAHASHI, K. & ROBERTS II, L.J. (1993). Identification of non-cyclooxygenase-derived prostanoid (F2-isoprostane) metabolites in human urine and plasma. *J. Biol. Chem.*, **268,** 4161–4169.

BACHI, A., VILLA, P., BRAMBILLA, R., BARBIERE, M., FANELLI, R. & CHIABRANDO, C. (1996a). *In vivo* formation of 8-epi-$PGF_{2\alpha}$ in the rat after lipopolysaccharide (LPS). *Prostaglandins, Leukot. Essent. Fatty Acids*, **55,** 102.

BACHI, A., ZUCCATO, E., BARALDI, M., FANELLI, R. & CHIABRANDO, C. (1996b). Measurement of urinary 8-epi-prostaglandin $F_{2\alpha}$, a novel index of lipid peroxidation *in vivo*, by immunoaffinity extraction/gas chromatography-mass spectrometry. Basal levels in smokers and nonsmokers. *Free Radic. Biol. Med.*, **20,** 619–624.

BACHI, A., BIANCHI, R., BRAMBILLA, R., MILANO, W., FANELLI, R. & CHIABRANDO, C. (1997). *In vivo* formation of 8-epi-PGF2a *vs* prostanoids in STZ diabetic rats. *Diabetes*, **46**(suppl.1), 0912 (abstract).

CATELLA, F. & FITZGERALD, G.A. (1987). Paired analysis of urinary thromboxane B_2 metabolites in humans. *Thrombosis Res.*, **47,** 647–656.

CATELLA, F., REILLY, M.P., DELANTY, N., LAWSON, J.A., MORAN, N., MEAGHER, E. & FITZGERALD, G.A. (1995). Physiological formation of 8-epi-$PGF_{2\alpha}$ *in vivo* is not affected by cyclooxygenase inhibition. *Adv. Prostaglandin Thromboxane Leukot. Res.*, **23,** 233–236.

CHIABRANDO, C., CORADA, M., BACHI, A. & FANELLI, R. (1994). Urinary excretion of 2,3-dinor-thromboxane B_1, a major metabolite of thromboxane B_2 in the rat. *Prostaglandins*, **47,** 409–422.

CHIABRANDO, C., PINCIROLI, V., CAMPOLEONI, A., BENIGNI, A., PICCINELLI, A. & FANELLI, R. (1989). Quantitative profiling of 6-keto-prostaglandin $F_{1\alpha}$, 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$, thromboxane B_2 and 2,3-dinor-thromboxane B_2 in human and rat urine by immunoaffinity extraction with gas chromatography-mass spectrometry. *J. Chromatogr.*, **495,** 1–11.

CHIABRANDO, C., RIVOLTELLA, L., ALBERTI, E., BONOLLO, M., DJURUP, R. & FANELLI, R. (1993). Urinary excretion and origin of 11-dehydro-2,3-dinor-thromboxane B_2 in man. *Prostaglandins*, **45,** 401–411.

DELANTY, N., REILLY, M., PRATICO, D., FITZGERALD, D.J., LAWSON, J.A. & FITZGERALD, G.A. (1996). 8-Epi-$PGF_{2\alpha}$ specific analysis of an isoeicosanoid as an index of oxidant stress *in vivo*. *Br. J. Clin. Pharmacol.*, **42,** 15–19.

FENG, L., SUN, W., XIA, Y., TANG, W.W., CHANMUGAM, P., SOYOOLA, E., WILSON, C.B. & HWANG, D. (1993). Cloning two isoforms of rat cyclooxygenase: differential regulation of their expression. *Arch. Biochem. Biophys.*, **307,** 361–368.

FITZGERALD, G.A., PEDERSEN, A.K. & PATRONO, C. (1983). Analysis of prostacyclin and thromboxane biosynthesis in cardiovascular disease. *Circulation*, **67,** 1174–1177.

HECKER, M., ULLRICH, V., FISCHER, C. & MEESE, C.O. (1987). Identification of novel arachidonic acid metabolites formed by prostaglandin H synthase. *Eur. J. Biochem.*, **169,** 113–123.

KANG, K.H., MORROW, J.D., ROBERTS II, L.J., NEWMAN, J.H. & BANERJEE, M. (1993). Airway and vascular effects of 8-epi-prostaglandin $F_{2\alpha}$ in isolated perfused rat lung. *J. Appl. Physiol.*, **74,** 460–465.

MORROW, J.D., AWAD, J.A., BOSS, H.J., BLAIR, I.A. & ROBERTS II, L.J. (1992). Non-cyclooxygenase-derived prostanoids (F_2-isoprostanes) are formed *in situ* on phospholipids. *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 10721–10725.

MORROW, J.D., FREI, B., LONGMIRE, A.W., GAZIANO, J.M., LYNCH, S.M., SHYR, Y., STRAUSS, W.E., OATES, J.A. & ROBERTS II, L.J. (1995). Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers. Smoking as a cause of oxidative damage. *N. Engl. J. Med.*, **332,** 1198–1203.

MORROW, J.D., HILL, K.E., BURK, R.F., NAMMOUR, T.M., BADR, K.F. & ROBERTS II, L.J. (1990). A series of prostaglandin F2-like compounds are produced *in vivo* in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 9383–9387.

MORROW, J.D., MINTON, T.A., BADR, K.F. & ROBERTS II, L.J. (1994). Evidence that the F2-isoprostane, 8-epi-prostaglandin $F_{2\alpha}$ is formed *in vivo*. *Biochim. Biophys. Acta*, **1210,** 244–248.

MORROW, J.D. & ROBERTS II, L.J. (1991). Quantification of noncyclooxygenase derived prostanoids as a marker of oxidative stress. *Free Radic. Biol. Med.*, **10,** 195–200.

MORROW, J.D. & ROBERTS II, L.J. (1994). Mass spectrometry of prostanoids: F2-isoprostanes produced by non-cyclooxygenase free radical-catalyzed mechanism. *Methods Enzymol.*, **233,** 163–174.

MORROW, J.D. & ROBERTS II, L.J. (1996). The Isoprostanes. Current knowledge and directions for future research. *Biochem. Pharmacol.*, **51,** 1–9.

NOWAK, J., MURRAY, J.J., OATES, J.A. & FITZGERALD, G.A. (1987). Biochemical evidence of a chronic abnormality in platelet and vascular function in healthy individuals who smoke cigarettes. *Circulation*, **76,** 6–14.

PATRIGNANI, P., SANTINI, G., PANARA, M.R., SCIULLI, M.G., GRECO, A., ROTONDO, M.T., DI GIAMBERARDINO, M., MACLOUF, J., CIABATTONI, G. & PATRONO, C. (1996). Induction of prostaglandin endoperoxide synthase-2 in human monocytes associated with cyclo-oxygenase-dependent F_2-isoprostane formation. *Br. J. Pharmacol.*, **118,** 1285–1293.

PRATICO', D. & FITZGERALD, G.A. (1996). Generation of 8-epiprostaglandin $F_{2\alpha}$ by human monocytes. *J. Biol. Chem.*, **271,** 8919–8924.

PRATICO, D., LAWSON, J.A. & FITZGERALD, G.A. (1995). Cyclooxygenase-dependent formation of the isoprostane, 8-epi-prostaglandin $F_{2\alpha}$. *J. Biol. Chem.*, **270,** 9800–9808.

REILLY, M., DELANTY, N., LAWSON, J.A. & FITZGERALD, G.A. (1996). Modulation of oxidant stress *in vivo* in chronic cigarette smokers. *Circulation*, **94,** 19–25.

SUN, F.F. & TAYLOR, B.M. (1978). Metabolism of prostacyclin in rat. *Biochemistry*, **17,** 4096–4101.

WANG, Z., CIABATTONI, G., CREMINON, C., LAWSON, J.D., FITZGERALD, G.A., PATRONO C. & MACLOUF, J. (1995). Immunological characterization of urinary 8-epi-prostaglandin $F_{2\alpha}$ excretion in man. *J. Pharmacol. Exp. Ther.*, **275,** 94–100.

WU, K.K. (1996). Cyclooxygenase 2 induction: molecular mechanism and pathophysiologic roles. *J. Lab. Clin. Med.*, **128,** 242–245.

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