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Generation of 8-epi-prostaglandin $F_{2\alpha}$ in isolated rat kidney glomeruli by a radical-independent mechanism

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1 Isoprostanes comprise a group of free radical-catalyzed products of arachidonic acid. However, there is recent evidence pointing towards an enzyme-dependent formation of isoprostanes.

2 With the use of isolated rat glomeruli we addressed the mechanisms of isoprostane generation. Synthesis of prostanoids and isoprostanes, including 8-epi-$PGF_{2\alpha}$, was studied under conditions favouring radical formation.

3 Cultured glomeruli formed different prostanoids including 8-epi-$PGF_{2\alpha}$. Upon LPS challenge cyclo-oxygenase (COX)-2 expression was enhanced, and this was paralleled by a 2–9-fold increase in prostanoid formation, including isoprostanes. Addition of COX-isoform unselective inhibitors (diclofenac, indomethacin) or a selective inhibitor (NS-398) suppressed the synthesis of prostanoids, 8-epi-$PGF_{2\alpha}$ and total isoprostane fraction; however, inhibition of the latter was less pronounced.

4 Antioxidants such as butylated hydroxytoluene (BHT), nordihydroguaiaretic acid (NDGA), or dimethylurea exhibited an only minimal inhibitory effect on 8-epi-$PGF_{2\alpha}$ synthesis. Moreover, ROS-generating drugs (menadione, methylviologen) or NADPH-driven radical formation were unable to cause the generation of significant amounts of 8-epi-$PGF_{2\alpha}$ by rat glomeruli. In contrast, the total isoprostane fraction could be increased by menadione addition.

5 These data provide further evidence for a radical-independent, but COX-dependent formation of 8-epi-$PGF_{2\alpha}$ in renal tissue. Regarding the other isoprostanes, both radicals and COX enzymes contribute to their formation. Based on our data we assume that elevated release of vasoactive 8-epi-$PGF_{2\alpha}$ has to be expected under conditions when the prostanoid system in the kidney is stimulated, e.g. under inflammatory conditions. Regarding renal oxidative injuries, the usefulness of 8-epi-$PGF_{2\alpha}$ as a representative marker molecule of oxidative stress has to be questioned.

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Abbreviations: AMT, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine; BHT, butylated hydroxytoluene; COX, cyclo-oxygenase; 4,5-DHB, 4,5-dihydroxy-1,3-benzene disulphonic acid; GC/MS/MS, gas chromatography-tandem mass spectrometry; IL-1, interleukin-1β; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NDGA, nordihydroguaiaretic acid; NSAIDs, non-steroidal anti-inflammatory drugs; SOD, superoxide dismutase

Introduction

Free radicals have been implicated in the pathophysiology of a wide variety of diseases and also of ageing (Beckman & Ames, 1998; Floyd, 1999; Singal *et al*., 1998; Smith *et al*., 2000). However, definitive evidence for their causative aetiological role is often missing due to limited methods to assess oxidative stress *in vivo*. Isoprostanes comprise a group of free radical-catalyzed products of arachidonic acid (for review see Lawson *et al*., 1999; Morrow & Roberts, 1996). In contrast to the classical cyclo-oxygenase (COX)-dependent formed prostaglandins, isoprostanes result from interaction of arachidonic acid with different reactive oxygen species. Radicals such as HO^-, O_2^-, and ROO^- result as by-products of enzymatic reactions or are formed specifically by immune-competent cells. Therefore, the detection of isoprostanes in different fluids and tissues *in vivo* was assumed to be a representative marker of lipid peroxidation or of oxidative injury (for review Morrow, 2000; Roberts & Morrow, 1994). However, these assumptions have recently been questioned due to the discovery of the cyclo-oxygenase-dependent formation of isoprostanes by several groups (Jourdan *et al*., 1997; Klein *et al*., 1997; Pratico *et al*., 1995; Pratico & Fitzgerald, 1996). For both the constitutive COX-1 as well as the inducible isoform COX-2, an involvement in the generation of isoprostanes has been demonstrated.

Currently, the best characterized isoprostane is 8-epi-$PGF_{2\alpha}$ (also called 15-F_{2t}-IsoP (Taber *et al*., 1997) or $iPF_{2\alpha}$-III (Rokach *et al*., 1997)), and most of the biological action of 8-epi-$PGF_{2\alpha}$ appears to be due to its interaction with the thromboxane receptor (Kinsella *et al*., 1997; Mohler *et al*., 1996) although a specific receptor for 8-epi-$PGF_{2\alpha}$ has been postulated (Fukunaga *et al*., 1997). For example, 8-epi-$PGF_{2\alpha}$ acts as a potent vasoconstrictor in the pulmonary vascular bed (Banerjee *et al*., 1992; John & Valentin, 1997). Besides its bronchoconstrictory characteristics a second major target of 8-epi-$PGF_{2\alpha}$ action is the modulation of renal functions. Intrarenal infusion of 8-epi-$PGF_{2\alpha}$ causes an

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abundant reduction in glomerular filtration rate and blood flow, thereby acting clearly as a glomerular vasoconstrictor (Takahashi *et al*., 1992).

Initial work on renal prostanoid synthesis identified the cortex region of the kidney as one major source for thromboxane and PGI_2, in addition to PGE_2, $PGF_{2\alpha}$ and PGD_2. The medullary region is more restricted to generating significant amounts of PGE_2. Elevated levels of the constrictive-acting prostanoids may account for the imbalance in glomerular blood flow and filtration rate observed in the different forms of glomerulonephritides (Lianos *et al*., 1983; Patrono *et al*., 1985; Stahl *et al*., 1987). Therefore, it was of interest to investigate the ability and capacity of isolated glomeruli with respect to isoprostane generation, and moreover, to reveal the precise–cyclo-oxygenase-dependent or -independent–mechanism. Recently, we could demonstrate that cultured rat mesangial cells predominantly produce 8-epi-$PGF_{2\alpha}$ *via* the cyclo-oxygenase pathway, however, without formation of other isoprostanes in parallel (Klein *et al*., 1997). With the use of isolated rat glomeruli we addressed the mechanisms of isoprostane generation under proinflammatory and pro-oxidative conditions.

Methods

Preparation of isolated rat glomeruli

Kidneys were removed from sacrificed rats (Spraque-Dawley, 200–250 g, of either sex). The cortex region was separated from medulla and finely minced in 10 ml of prewarmed RPMI 1640 supplemented with 10% FCS. The glomeruli were separated from other cortical tissues by passing the minced tissue consecutively through sieves of different sizes (106, 150 and 63 μm). The glomeruli were obtained from the 63 μm sieve and purified again from contaminating tubular fragments by 150 μm sieving. The resulting glomerular fraction was checked microscopically for purity and centrifuged 20 min at 100 $\times g$ to remove single cells or cell fragments. The resulting pellet was resuspended in phosphate buffered saline or medium and glomeruli were cultured in tissue plates.

Measurement of cyclo-oxygenase activity by product analysis

Metabolism of exogenously added arachidonic acid: freshly prepared glomeruli were preincubated with inhibitors or vehicle for 15 min, and different concentrations of arachidonic acid were added for 15 min at room temperature. The reaction was stopped by acidification with formic acid to pH 2.5. The formed prostaglandins and isoprostanes were extracted by three volumes ethyl acetate and analysed by GC/MS/MS.

Metabolism of arachidonic acid from endogenous sources: isolated glomeruli were held overnight in RPMI 1640, 0.5% FCS, supplemented with 1% L-glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin at 37°C in a humid atmosphere with 5% CO_2. The glomeruli were stimulated with 1 nM interleukin-1β (IL-1) and 1 μg ml^{-1} LPS for 24 h to initiate COX-2 induction. Inhibitors were coincubated and supernatants were collected after 24 h and stored at −80°C until analysis.

Western blot analysis and reverse transcription–polymerase chain reaction (RT–PCR)

Western blot analysis of COX-2, iNOS and prostacyclin synthase were performed as we recently reported (Klein *et al*., 1994). Briefly, isolated glomeruli were pelleted following IL-1, or LPS stimulation or control and lysed in PBS/0.1N NaOH. Protein content was determined by using bicinchoninic acid (Smith *et al*., 1985) (BCA assay, Pierce, Rockford, U.S.A.), and equal amounts of lysates were separated by 10% SDS–PAGE. The proteins were transferred onto a nitrocellulose membrane (Amersham, Braunschweig, Germany) and visualized with a 5% Ponceau S solution. After destaining, the membrane was blocked with a solution of 5% milk powder in PBS and incubated with the respective antibodies (cyclo-oxygenase-2, 1 : 100; iNOS, 1 : 100; prostacyclin synthase 1 : 100). The characterization of specific antibodies directed against prostacyclin synthase was described recently (Klein *et al*., 1998). For visualization a peroxidase-labelled goat-anti rabbit antibody (1 : 7500, Dianova, Hamburg, Germany) was added followed by enhanced chemiluminescence detection method (Amersham, Braunschweig, Germany) after several rounds of intensive washing. RT–PCR analysis was performed with primer constructs as described by us elsewhere (Nüsing *et al*., 1996).

Sample preparation and GC/MS/MS-analysis for prostanoids and isoprostanes

Samples were prepared as described recently (Schweer *et al*., 1994) with minor modifications (Klein *et al*., 1997) and analysed with a Finnigan MAT TSQ700 GC/MS/MS equipped with a Varian 3400 gas chromatograph and a CTC A200S autosampler. Total fraction of isoprostanes was analysed after purification of the samples by thin-layer chromatography. To determine concentrations of 8-epi-$PGF_{2\alpha}$, following thin layer chromatography samples were further purified by high-performance liquid chromatography. The amount of isoprostanes was calculated by subtraction of the amount of $PGF_{2\alpha}$ from the entire amount of F-isoprostanes.

Detection of chemiluminescence as a parameter of O_2^- and radical generation and the antioxidative potency of compounds

Measurement of chemiluminescence was carried out in a single photomultiplier tube (Lumat, Berthold) using lucigenin (250 μM) as a detector for radical formation. In the case of cell free generated O_2^-, xanthine (1–10 μM) was dissolved in Krebs buffer enriched with 20 mM HEPES. This solution was transferred to the scintillation tube, and the reaction was initiated by the addition of xanthine oxidase (2 mU). Immediately lucigenin was injected and radical formation was monitored at intervals of 10 s. The capacity of isolated glomeruli to generate reactive oxygen species was determined using the same buffer system with the addition of NADPH (500 μM).

Statistical analysis

Comparisons between groups were made by the Student's *t*-test for paired or unpaired data as appropriate. *P* values of less than 0.05 were regarded as significant.

Materials

All chemicals beside the ones mentioned below were purchased from Sigma (Deisenhofen, Germany). Arachidonic acid, polyclonal antibodies directed against COX-2 and inducible nitric oxide synthase (iNOS) were purchased from Cayman Chemicals (Paris, France). [3,3,4,4-$^2H_4$]-PGE_2, [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 non-deuterated analogues were a kind gift from Dr Udo Axen (Upjohn, Kalamazoo, MI, U.S.A.). [18,18,19,19-$^2H_4$]-PGD_2 was a kind gift of Dr C. Meese (Dr Margarete Fischer- Bosch Institute, Stuttgart, Germany). [$^{18}O_2$]-8-epi-$PGF_{2\alpha}$ was prepared from 8-epi-$PGF_{2\alpha}$ and H_2 ^{18}O as described (Schweer *et al*., 1994). Ethyl acetate, H_2 ^{18}O (96.5%) and chloroform were obtained from Promochem (Wesel, Germany), pentafluorobenzyl bromide from Lancaster (Mühlheim/Main, Germany), water, methanol, and formic acid from Merck (Darmstadt, Germany), hexane and sodium acetate from Riedel-de Haen (Seelze, Germany). Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Macherey and Nagel (Düren, Germany) and N,N-diisopropylethylamine from Pierce (Oud Beijerland, The Netherlands). Silica gel thin layer chromatography (TLC) plates (LK6D, 5×20 cm) were from Whatman (Maidstone, U.K.). Helium (99.996%), methane (99.995%) and argon (99.998%) were from Messer Griesheim (Herborn, Germany).

Results

Demonstration of COX-1, COX-2, prostacyclin and thromboxane synthase and iNOS expression in isolated rat glomeruli

To gain insight into prostanoid formation by isolated rat glomeruli we analysed the expression of the corresponding enzymes by means of RT–PCR and Western blot analysis. Figure 1 demonstrates a basal mRNA expression for the enzymes COX-1, thromboxane synthase, prostacyclin synthase and also COX-2. Under these conditions, the mRNA for inducible nitric oxide synthase was not detectable. This enzyme and its product, known to be undetectable under basal conditions, served as an internal control for bacterial contamination (which causes not only the induction of iNOS expression but also of COX-2) and in stimulated cells as a control for cytokine efficacy.

Following IL-1/LPS challenge of glomeruli for 24 h, significant upregulation of COX-2 and iNOS message was observed; however, the specific mRNAs for prostacyclin and thromboxane synthase were barely affected compared to untreated samples (Figure 1). In agreement with this observation, the constitutive expression of prostacyclin synthase protein and the induced expression of COX-2 and iNOS were detected by Western blot (Figure 2). The elevated protein expression of iNOS and COX-2 was sensitive to dexamethasone treatment, whereas prostacyclin synthase expression remained unaffected.

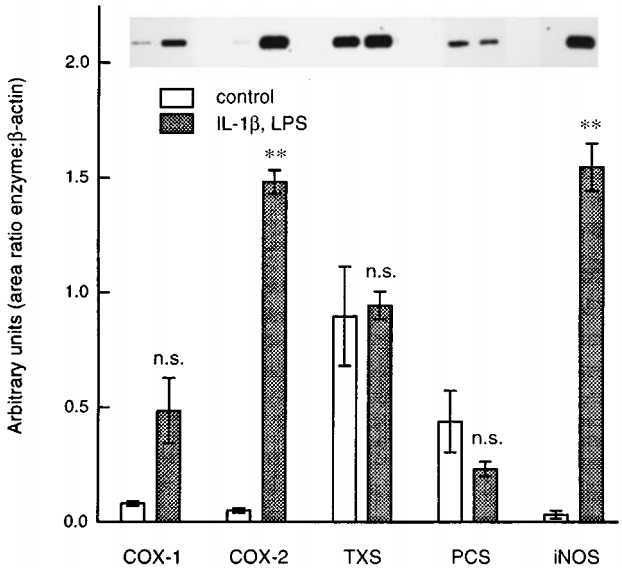

Figure 1 mRNA expression of COX-1/2, thromboxane synthase, prostacyclin synthase and iNOS in IL-1/LPS-stimulated rat glomeruli. Glomeruli were incubated with IL-1/LPS or vehicle for 20 h and analysed for the expression of the specific mRNAs by means of RT–PCR. The densitometric evaluated values of 3–5 different mRNA analysis were normalised *versus* β-actin and expressed as arbitrary units. Inset, a representative of the amplified cDNA fragments is depicted. TXS, thromboxane synthase; PCS, prostacyclin synthase.

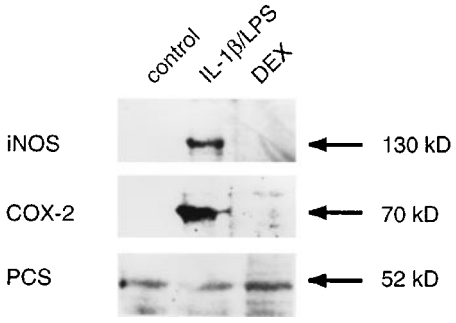

Figure 2 Western blot analysis of iNOS, COX-2 and prostacyclin synthase in IL-1/LPS-treated glomeruli. Following treatment (20 h) with vehicle, IL-1/LPS or IL-1/LPS in addition to 2 μM dexamethasone, glomerular proteins were separated by SDS–PAGE; iNOS, COX-2 and prostacyclin synthase (PCS) were detected by specific polyclonal antibodies.

Glomerular prostaglandin and isoprostane formation from endogenous esterified and exogenously added arachidonic acid

Following IL-1/LPS treatment of enriched glomerular fractions we observed an increase in the release of prostaglandins and isoprostanes into the glomerular supernatant. The main metabolites were PGE_2 and $PGF_{2\alpha}$, PGD_2, TXB_2, isoprostanes, 8-epi $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ were formed in much lower amounts (Table 1). A similar pattern of prostanoid synthesis, but with lower amounts was

observed in untreated glomeruli, suggesting a pivotal role of COX-2 in increased prostanoid formation in cytokine-treated glomeruli. Accordingly, preincubation with the glucocorticoid dexamethasone (1 μM) decreased metabolite formation to control levels (Table 1). Noteworthy, synthesis of 8-epi-$PGF_{2\alpha}$ was completely suppressed, but the isoprostane fraction was not significantly reduced.

Stimulation of glomeruli with 30 μM arachidonic acid caused the synthesis of large amounts of prostanoids (Table 2). However, a modified product pattern was observed with the preferential formation of PGD_2 and isoprostanes. Linearity of metabolite production was observed in the range of 5–50 μM of added arachidonic acid (Figure 3). Application of low concentration of diclofenac caused 59–84% inhibition of the classical prostanoids, including 8-epi-$PGF_{2\alpha}$. In comparison, suppression of the total isoprostane fraction was less pronounced.

Role of ROS-based formation of isoprostanes

In the next approach our aim was to analyse isoprostane generation independent from cyclo-oxygenase and dependent on the presence of radicals, such as O_2^-. The reaction of O_2^- with lucigenin, which results in the emittance of chemiluminescences was chosen to detect O_2^-. Authenticity of O_2^- radicals was demonstrated by the complete inhibition with added superoxide dismutase (SOD, 10 U ml^{-1}).

Figure 4 summarizes NADPH-driven radical formation from isolated glomeruli in the presence of different inhibitors. The three antioxidants tested–4,5-dihydroxy 1,3-benzene disulphonic acid, NDGA, as well as superoxide dismutase (SOD)–abrogated NADPH induced photoemission, whereas the hydroxyl radical scavenger DMSO was without effect. The non-steroidal compounds indomethacin, diclofenac and the COX-2-selective inhibitor NS-398 exhibited no antioxidative effect at 10 μM in our assay system.

To evaluate the contribution of cyclo-oxygenase inhibitors or antioxidants to the metabolic pathways leading to prostaglandins or isoprostanes, glomeruli were treated with a number of these compounds in the presence of IL-1/LPS. As evidenced in Table 3, the use of the antioxidant BHT or the cell permeable superoxide dismutase analogue (PEG–SOD) resulted in a minimal decrease of prostanoid and isoprostane production (6–23%). Compounds such as dimethylurea and NDGA showed some inhibition of isoprostane formation (up to 26%); however, synthesis of PGE_2 and TXB_2 was similarily or even more affected (up to 55%). Coincubation with 1 μM AMT, a potent inhibitor of nitric oxide synthase (Boer *et al*., 2000), caused 92±6% inhibition of NO formation determined as nitrite/nitrate, but only marginally blocked synthesis of PGE_2, TXB_2 8-epi $PGF_{2\alpha}$, and isoprostanes. This rules out the possibility that nitric oxide or associated radicals such as peroxynitrite are involved in prostanoid or isoprostane formation.

In striking contrast to the observation above were the results obtained with COX inhibitors. At concentrations where these inhibitors exhibited no radical scavenging effect, diclofenac and indomethacin significantly blocked isoprostane and 8-epi $PGF_{2\alpha}$ formation in addition to PGE_2 and TXB_2 formation. At a concentration as low as 100 nM diclofenac was still effective in lowering PGE_2 by 89%, and 8-epi $PGF_{2\alpha}$ by 64%. A modest inhibitory effect of 31% was obtained within the isoprostane fraction. Similar results were obtained

Table 1 Endogenous prostaglandin and isoprostane production from isolated rat glomeruli

	Control	*LPS/IL-1*	*LPS/IL-1 + Dex*
	(ng μg^{-1} protein)		
PGE_2	34.2±10.7	275±64	21.7±3.1**
TxB_2	8.4±1.3	26.9±5.3	9.8±1.3**
$PGF_{2\alpha}$	38.3±5.5	95±14.9	35.6±7.4**
PGD_2	22.6±3.6	70.2±15.3	21.17±3.6**
6-keto $PGF_{1\alpha}$	4.0±0.5	6.3±0.6	3.5±1.1**
8-epi $PGF_{2\alpha}$	2.1±0.3	5.5±0.8	2.5±0.67**
F-isoprostanes	11.2±1.5	18.9±2.3	16.1±2.36

Glomeruli were treated either with vehicle, LPS/IL-1 or LPS/IL-1 with the addition of 1 μM dexamethasone for 24 h. Prostanoids and isoprostanes were detected by GC/MS/MS in the supernatant and expressed as ng per μg protein. Values are given as means±s.e.mean of 10–24 separate experiments. Asterisk indicates significance of LPS/IL-1+Dex to LPS/IL-1.

Table 2 Formation of prostaglandins and isoprostanes from isolated rat glomeruli following stimulation with arachidonic acid

	30 μM arachidonic acid	*AA + 1 μM diclofenac*	*% Inhibition*
	(ng μg^{-1} protein)		
PGE_2	11.3±2.6	4.3±2.0	62
TxB_2	4.9±1.0	0.8±0.18	84
$PGF_{2\alpha}$	41.4±19.2	17.0±8.6	59
PGD_2	90.5±21	35.8±7.2	61
6-keto $PGF_{1\alpha}$	1.6±0.32	0.46±0.1	72
8-epi $PGF_{2\alpha}$	2.8±1.1	0.57±0.22	80
F-isoprostanes	54.5±19	24.9±6.1	55

Following preincubation of glomeruli with diclofenac (30 min), arachidonic acid was added for 15 min at RT and eicosanoids were determined as described. Values are expressed as ng per μg protein mean±s.e.mean of 6–12 experiments.

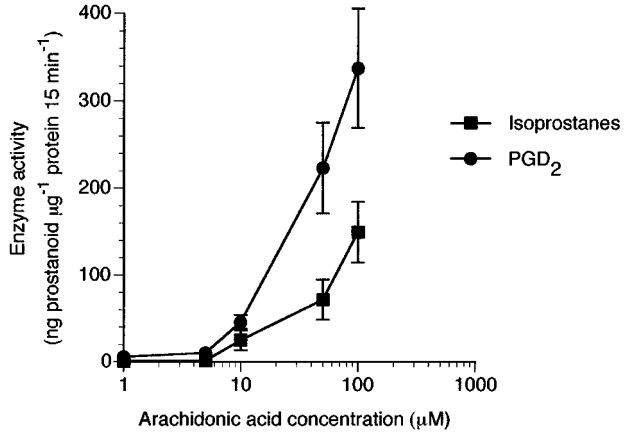

Figure 3 Arachidonic acid concentration dependency of prostanoid enzyme activity. Glomeruli were cultured and incubated with different concentrations of arachidonic acid for 15 min. Thereafter enzyme reaction was stopped by acidification and extraction with three volumes ethyl acetate. Prostanoids were analysed by GC/MS/MS. Data represents mean±s.e.mean of five experiments.

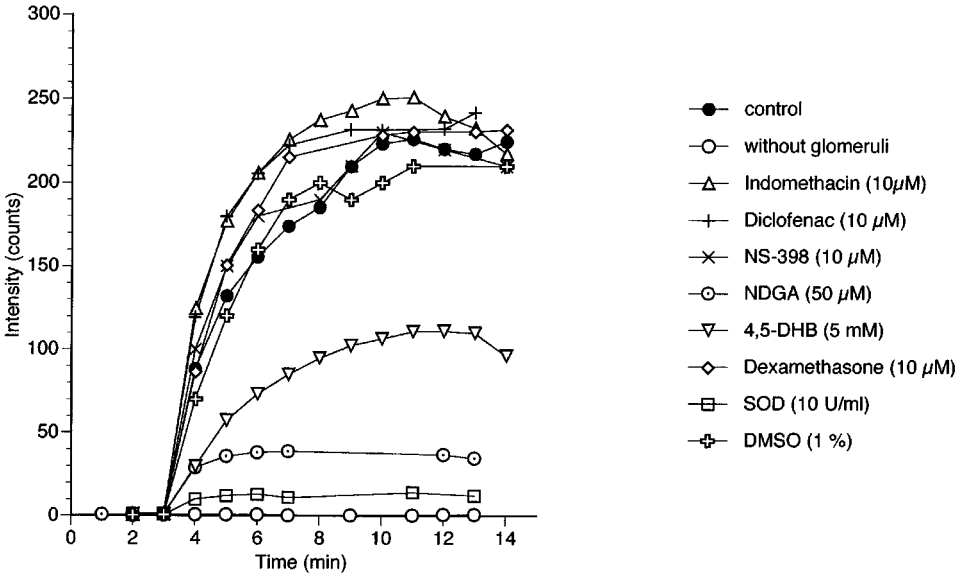

Figure 4 Antioxidative properties of various compounds. The plot shows original time course and inhibitory action of NSAIDs, dexamethasone and selected antioxidants on NADPH (500 μM)-driven superoxide generation from isolated glomeruli. Lucigenin-mediated chemiluminescence was taken as a measure for superoxide production. A representative of 5–7 experiments is shown.

Table 3 Inhibitory efficacy of antioxidants and NSAIDs on eicosanoid synthesis in stimulated glomeruli

	PGE_2	*TxB_2*	*8-epi $PGF_{2\alpha}$*	*Isoprostanes*
	% inhibition (compared to control stimulated glomeruli)			
BHT 30 μM	18.4±6.7	13.6±8.1	18.2±9.4	6.7±3.7
PEG-SOD 100 u ml^{-1}	23.1±11.2	4.8±3.8	21.3±8	9.3±4.3
NDGA 50 μM	55.4±12.7	45.1±16.5	26.2±14.9	19.6±14
Dimethylurea 100 μM	32.6±11.1	24±13.5	19.6±1.6	18.2±2.2
AMT 1 μM	12.7±8.1	11.0±8.8	21.0±1.5	19.2±9.5
Diclofenac 10 μM	95.3±7.4	82.8±5.8	78.5±5	62.7±6.8
Diclofenac 1 μM	97.0±0.7	85.4±4	67.1±8.3	46.0±24.2
Diclofenac 100 nM	88.6±6.8	68.0±9.8	63.6±1.2	30.6±17.3
Indomethacin 100 nM	79.5±3.2	67.5±3.5	64.2±8.1	34.0±17.5
NS-398 10 μM	89.1±5.2	79.3±6.1	71.8±4.8	58.3±12.1

Glomeruli were incubated overnight with LPS/IL-1, and the depicted concentrations of diverse antioxidants and NSAIDs. Values are given in per cent inhibition±s.e.mean of 4–8 samples.

with the structurally dissimilar NSAID indomethacin and the COX-2 inhibitor NS-398.

Prooxidative conditions impair the inhibitory potency of diclofenac and increase isoprostane formation

In another approach we investigated the long term effect of radicals on isoprostane formation. For this purpose isolated rat glomeruli were incubated in the presence of NADPH, a cofactor of NADPH oxidase which causes the formation of O_2^-. To ensure continuous presence of O_2^- radicals NADPH was added at several time points. Although generation of radicals was demonstrated in NADPH-treated glomeruli (Figure 5), we observed only a slight increase in isoprostane, 8-epi $PGF_{2\alpha}$, and PGE_2 synthesis, following 24 h LPS/IL-1 treatment (Table 4). Regarding the inhibitory potency of diclofenac (1 μM) we observed a significantly decreased suppression of isoprostane synthesis in NADPH-treated glomeruli compared with untreated glomeruli. Suppression of the prostanoids such as PGE_2 and TXB_2 but also 8-epi $PGF_{2\alpha}$ remained unaffected.

In a similar approach we used the redox cycler paraquat (methylviologen) as well as menadion (a vitamin K analogue) as O_2^--generating compounds in the presence of cytokine-stimulated glomeruli. In the case of menadione we observed a significant stimulation of isoprostane formation compared to PGE_2, TXB_2 and 8-epi $PGF_{2\alpha}$. The increase of isoprostanes mediated by paraquat, however, was only significant with respect to 8-epi $PGF_{2\alpha}$ (Table 4).

Discussion

The kidney cortex represents an important source of renal prostanoids, and therefore isolated glomeruli have been chosen as a complex system to study the mechanisms of isoprostanes and 8-epi $PGF_{2\alpha}$ formation. The abundant generation of the prostanoids PGE_2, $PGF_{2\alpha}$, and thromboxane from stimulated glomeruli was in line with the literature (Dunn & Hood, 1977). The detection of large amounts of isoprostanes, however, contrasted with our findings in cultivated rat mesangial cells as previously reported (Klein

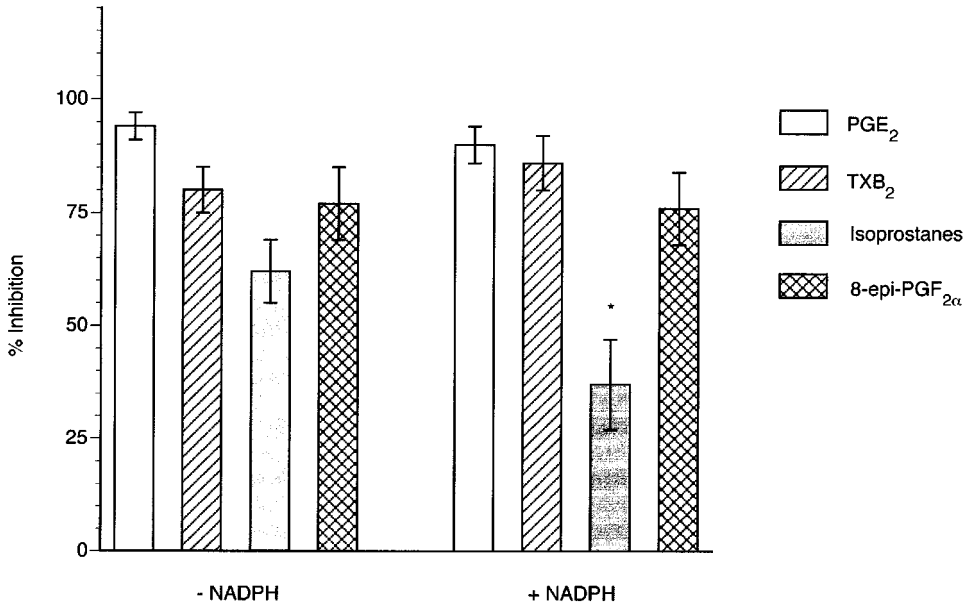

Figure 5 Inhibitory action of diclofenac on NADPH-treated glomeruli. NADPH (500 μM) was given at the time points 0, 6 and 10 h to LPS/IL-1-stimulated glomeruli. Release of prostanoids and isoprostanes in the supernatant was determined at time point 20 h. The efficacy of diclofenac (1 μM) to lower eicosanoids is expressed as per cent inhibition compared to vehicle treated samples $\pm$ s.e.mean of 4–6 experiments. Asterisk indicates significance between NADPH-treated and -untreated glomeruli.

Table 4 Effect of redox cycler and NADPH-driven radical formation on prostanoid and isoprostanes synthesis

	PGE_2	*TxB_2*	*8-epi $PGF_{2\alpha}$*	*Isoprostanes*
	x-fold induction (compared to control stimulated glomeruli)			
Menadion 50 μM	0.7$\pm$0.16**	1.02$\pm$0.15**	0.78$\pm$0.13**	2.64$\pm$0.59
Methylviologen 1 mM	1.32$\pm$0.38	1.31$\pm$0.11	0.82$\pm$0.08**	1.53$\pm$0.16
NADPH 500 μM	1.21$\pm$0.24	0.93$\pm$0.26	1.31$\pm$0.19	1.41$\pm$0.19

Isoloated rat glomeruli were treated with the compounds indicated in the presence of LPS/IL-1. Means $\pm$ s.e.mean of 7.8–8 independent experiments were expressed as x-fold increase compared to LPS/IL-1 controls. Asterisk indicates significance with respect to isoprostanes.

et al., 1997). In this study mainly prostacyclin (44 ng μg^{-1} protein/24 h) and minor amounts of 8-epi $PGF_{2\alpha}$ (0.2 ng μg^{-1} protein/24 h) were detected following IL-1 treatment. Other isoprostanes were undetectable. These observations indicate the involvement of different cell types within the glomerulus, responsible for the isoprostane and 8-epi $PGF_{2\alpha}$ synthesis.

Rat glomeruli express large amounts of cyclo-oxygenase-2 mRNA and protein upon IL-1/LPS stimulation with the consequence of elevated arachidonic acid metabolism. Prostacyclin synthase, thromboxane synthase, and COX-1 expression remained unchanged under proinflammatory conditions, confirming COX-2 as a rate-limiting step for enhanced arachidonic acid metabolism. The ability of dexamethasone to inhibit *de novo* protein synthesis of COX-2 supports this notion.

As expected, the COX-dependent prostanoid production (PGE_2, TXB_2) was completely suppressed by the use of diclofenac, indomethacin and NS-398. But, more importantly formation of 8-epi $PGF_{2\alpha}$ exhibited a similar sensitivity toward the various NSAIDs used. The hypothesis of a predominantly enzyme-dependent 8-epi $PGF_{2\alpha}$ synthesis in rat glomeruli was further supported by the observation that neither antioxidants protected from LPS-induced glomerular 8-epi $PGF_{2\alpha}$ formation nor the pro-oxidative environment due to menadione or paraquat presence increased the 8-epi-$PGF_{2\alpha}$ formation. Unlike monocytes (Pratico & Fitzgerald, 1996), where both a COX-dependent as well as a free radical-catalyzed formation of 8-epi $PGF_{2\alpha}$ generation have been reported, we provide evidence that in isolated rat glomeruli 8-epi $PGF_{2\alpha}$ formation is largely COX-dependent. The possibility of inhibition due to antioxidative properties of the NSAID was excluded. Similar to the effect on COX-2, treatment of glomeruli with LPS results in an immediate induction of nitric oxide synthase. Pulmonary arteries in organ culture produce 8-epi $PGF_{2\alpha}$ upon cytokine challenge, and this formation could be blocked by indomethacin, but also by the NO synthase inhibitor L-NAME (Jourdan *et al*., 1997). Under our experimental conditions the potent NO synthase inhibitor AMT did not show a large effect on isoprostane or prostanoid formation in rat glomeruli.

Comparing the mechanisms of isoprostane formation to those of 8-epi $PGF_{2\alpha}$ it is clear that dexamethasone and NSAIDs were less effective and only partially inhibited the total fraction of isoprostanes. This is consistent with the observations of others (summarized in Morrow & Roberts, 1997) and indicates that at least a part of the isoprostane fraction is formed in a radical-dependent manner. However,

in isolated rat glomeruli NSAIDs were still more effective inhibitors of isoprostane formation than several antioxidative agents. One conceivable explanation may be found in the chemistry of COX catalysis. The conversion of arachidonic acid to prostaglandin endoperoxide is accompanied by O_2^- production from the peroxidase subunit (Kuehl *et al*., 1977). Therefore, tissues with high COX activity such as the kidney may be involved in isoprostane generation due to common arachidonic acid turnover. The presence of oxygen radicals from additional sources, e.g. activation of NADPH oxidase, may contribute in part to further isoprostane formation.

Thus, from the results of the current study we postulate a COX-dependent 8-epi-$PGF_{2\alpha}$ formation and a combined COX- and radical-dependent formation of the other isoprostanes in rat glomeruli. Therefore, under pathological conditions related to an increase in COX activity, an increase in isoprostane and specifically in 8-epi-$PGF_{2\alpha}$ formation can be anticipated. Thus in the context of renal oxidative injuries, the reliability of isoprostanes or 8-epi-$PGF_{2\alpha}$ as a suitable molecular marker for processes involving intrarenal peroxidative damage has to be questioned. At least determination of the classical prostanoids in addition to isoprostanes and 8-epi-$PGF_{2\alpha}$ is necessary to exclude a contribution from an activated COX pathway.

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