Interleukin 1 β decreases prostacyclin synthase activity in rat mesangial cells via endogenous peroxynitrite formation

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We have reported that peroxynitrite (PON) selectively inactivated prostacyclin synthase (PGIS) by a mechanism of tyrosine nitration at the active site [Zou, Martin and Ullrich (1997) *Biol. Chem. Hoppe-Seyler* 378, 707–713]. We have now extended our studies on rat mesangial cells (RMC) and show that nitration can occur under the influence of cytokines. Pretreatment of RMC with interleukin 1 β (IL-1 β), which up-regulated cyclo-oxygenase 2 and inducible nitric oxide synthase (NOS-2), significantly attenuated the conversion of [¹⁴C]prostaglandin H₂ (PGH₂) into the stable prostacyclin (PGI₂) metabolite 6-oxo-prostaglandin F_{1 α} (6-oxo-PGF_{1 α}). The presence of superoxide dismutase (SOD, 100 units/ml) or the NOS synthase inhibitor *N*^ω-monomethyl-L-arginine (100 μ M) as well as cycloheximide (10 μ M) plus actinomycin (10 μ M) abolished IL-1 β -mediated down-regulation of 6-oxo-PGF_{1 α} from PGH₂. At the same time, 6-oxo-PGF_{1 α}

production from arachidonate (AA) increased at the expense of prostaglandin E₂ (PGE₂). Neither NO alone generated from different NO donors nor superoxide from xanthine/xanthine oxidase (1–100 m-units/ml) inhibited PGI₂ synthesis, either from PGH₂ or from AA. Bolus additions of chemically synthesized PON or the PON generator 3-morpholinomethyl-*N*-ethylcarbamide (SIN-1) exhibited a potent inhibition of 6-oxo-PGF_{1 α} release from both PGH₂ and AA. In addition, immunoprecipitation of nitrotyrosine-containing proteins from PON- and SIN-1-treated RMC yielded distinct nitrated PGIS bands but also from IL-1 β -pretreated cells alone, compared with a lack of nitrated PGIS in control cells. Taken together, our results strongly suggest that IL-1 β pretreatment of RMC via NOS-2 leads to the production of PON with the consequence of a partial nitration and inhibition of PGIS.

INTRODUCTION

Prostacyclin (PGI₂) is a potent vasodilator, as well as an anti-aggregatory, anti-adhesive and anti-proliferative mediator in the vascular system [1,2]. Its biosynthesis starts from released arachidonate (AA) and proceeds through the formation of 15-hydroxyprostaglandin-9,11-endoperoxide (PGH₂) which is finally rearranged to PGI₂ by a haem-thiolate-containing isomerase [3]. Prostacyclin synthase (PGIS) is highly expressed in the endothelium and releases PGI₂ to react with the corresponding receptor on smooth muscle cells, leading to vasorelaxation through stimulation of adenylate cyclase [1,2,4].

With regard to these physiological aspects it was of interest that we had previously reported a strong inhibitory action of peroxynitrite (PON) on PGIS activity [5]. PON is formed as a product of the almost diffusion-limited reaction of NO with the superoxide radical [6]. Indeed, both radicals generated together were also strongly inhibitory towards PGIS. Because NO is recognized as the endothelium-derived relaxing factor [7] and superoxide is up-regulated as a result of inflammatory or ischaemic conditions [8,9], inactivation of PGIS by PON could easily be envisaged under pathophysiological conditions. The underlying mechanism has now been identified as the nitration of a tyrosine residue at the active site of the enzyme under catalysis by its haem-thiolate prosthetic group [10]. However, because less than 100 nM PON as a bolus addition was required for 50% inhibition it was puzzling that this concentration could withstand the anti-oxidant potential of the cell as already demonstrated for cultured endothelial cells [10].

The kidney mesangium has been reported to be another source of PGI₂ [11,12], which together with NO seems to have an

important role in the autocrine and paracrine regulation of glomerular haemodynamics and functions as a negative feedback signal to attenuate inflammation-derived vasoconstriction caused by thromboxane A₂, PGF_{2 α} , PGH₂, platelet-activating factor or endothelins in glomerular arterioles and the mesangium [13]. Resting rat mesangial cells (RMC) do not synthesize inflammatory mediators constitutively, whereas under inflammatory conditions RMC are stimulated to produce interleukin 1 β (IL-1 β) and tumour necrosis factor, which with additional cytokines help to orchestrate the process of inflammation. RMC are also known to produce superoxide and other reactive oxygen species in response to several proinflammatory stimuli [14,15]. Thus, as a consequence of the high output of NO by inducible nitric oxide synthase (NOS-2) PON would probably be formed and could lead to the postulated inhibition of PGI₂ synthesis. As a consequence of increasing superoxide levels, not only would NO be scavenged but also the second potent vasodilator PGI₂ would be progressively down-regulated. Our results provide experimental evidence for such a model.

MATERIALS AND METHODS

Materials

Cell culture media were obtained from Gibco (Eggenstein, Germany). The recombinant human IL-1 β and the insulin-transferrin-selenite supplement were obtained from Boehringer Mannheim (Mannheim, Germany). [¹⁴C]AA (53 mCi/mmol) was purchased from Dupont (Dreieich, Germany). 3-Morpholinomethyl-*N*-ethylcarbamide (SIN-1), *N*^ω-monomethyl-L-ar-

Abbreviations used: AA, arachidonate; COX, cyclo-oxygenase; IL-1 β , interleukin 1 β ; 6-oxo-PGF_{1 α} , 6-oxo-prostaglandin F_{1 α} ; LDH, lactate dehydrogenase; L-NMMA, *N*^ω-monomethyl-L-arginine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NOS-2, inducible nitric oxide synthase; PG, prostaglandin; PGI₂, prostacyclin; PGIS, prostacyclin synthase; PON, peroxynitrite; RMC, rat mesangial cells; SIN-1, 3-morpholinomethyl-*N*-ethylcarbamide; SOD-PEG, superoxide dismutase-polyethylene glycol; SNAP, S-nitroso-*N*-acetyl-D,L-penicillamine; SP-NO, spermine NONOate.

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ginine (L-NMMA), *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), spermine NONOate (SP-NO), and diethylamide NONOate were from Cayman SPI (Massy, France). SOD-polyethylene glycol (SOD-PEG), mannitol, uric acid, tryptophan, haematin, ascorbic acid, α -tocopherol, glutathione, diethylenetriaminepenta-acetic acid, DMSO, EDTA, FeCl₃, Fe₂(SO₄)₃, tetranitromethane, hypoxanthine, xanthine oxidase, Trolox, cycloheximide, actinomycin D and H₂O₂ were from Sigma Biochemicals (Deisenhofen, Germany). Desferal was obtained from Ciba Pharmaceuticals (Wehr, Germany). Protein A-Sepharose CL-4B was from Pharmacia (Freiburg, Germany). A monoclonal antibody against 3-nitrotyrosine was obtained from Upstate Biotechnology (New York, NY, U.S.A.). The enhanced chemiluminescence (ECL) kit and nitrocellulose membranes (Hybond-C) were purchased from Amersham (Braunschweig, Germany). Organic solvents were obtained from Merck Darmstadt (Darmstadt, Germany).

Cell culture

RMC were used between passages 5 and 17 (with a constant split ratio of 1:3). Cells were maintained under growth conditions in RPMI 1640 supplemented with insulin (5 μ g/ml), transferrin (5 μ g/ml), sodium selenite (5 μ g/ml), L-glutamine (1 %, w/v), 1 % penicillin (100 i.u./ml) and streptomycin (100 μ g/ml) with 10 % (v/v) fetal calf serum. The confluent cells were transferred to the medium with 0.5 % (v/v) fetal calf serum to stop growth conditions 2 days before use. Confluent RMC were then incubated overnight with 1 nM IL-1 β in 3 ml of medium with or without SOD-PEG (100 units/ml), L-NMMA (100 μ M) or cycloheximide (10 μ M) in combination with actinomycin D (10 μ M). The supernatants were collected for nitrite measurements by the Griess reagent [16]. The cells were washed and supplemented with fresh medium devoid of serum 30 min before the experiment.

PON synthesis

PON was prepared by using a quenched-flow reaction as described by Reed et al. [17]. In brief, an aqueous solution of 0.6 M sodium nitrite was mixed rapidly with an equal volume of 0.7 M H₂O₂ containing 0.6 M HCl and immediately quenched with the same volume of 1.5 M NaOH. All solutions were kept on ice. The concentration of PON was determined spectrally in 0.7 M NaOH (ϵ_{302} 1670 M⁻¹·cm⁻¹).

Biosynthesis of [¹⁴C]PGH₂

Microsomes from sheep seminal vesicles were prepared by the method of Kulmacz and Lands [18]. [¹⁴C]PGH₂ was biosynthesized as described in [19].

Treatment of RMC with PON

With or without IL-1 β treatment, cells were incubated with 1.8 ml of PBS supplemented with 100 mM Hepes buffer, pH 7.4. The stock solution of PON was diluted in 0.1 M NaOH to give working solutions from which 0.2 ml portions were used to give the desired final concentrations. The reaction mixtures were mixed rapidly when PON was added. The pH of 7.4 was controlled after each addition.

Lactate dehydrogenase (LDH) assay

After the given times of incubation with indicated concentrations of PON, the supernatants were aspirated for LDH assays. Cells

were washed, dissolved in 0.2 % (v/v) Triton X-100, homogenized and sonicated. LDH was measured both in cells and in supernatants as described by Horder and Rej [20] in an Eppendorf ACP 5040 enzyme analyser (Hamburg, Germany). The percentage of LDH in the supernatant (S) was calculated as $100 \times \text{LDH}_s / (\text{LDH}_s + \text{LDH}_{\text{cells}})$.

Reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

Viability was also measured by the reduction of MTT, as an index of mitochondrial activity, by the method of Mosmann [21]. Confluent cells (10⁵) were treated with PON as described above. At appropriate intervals after treatment with PON, cells were incubated with 0.7 mg/ml MTT for 15 min. After removal of medium, the cells were lysed for 20 min in 200 μ l of propan-2-ol/formic acid (95:5, v/v). The reduced MTT was measured at 560 and 690 nm.

Measurement of PGI₂ synthase (PGIS) in RMC

At 10 min after pretreatment with PON, the cells were rinsed twice with 2 ml of PBS containing 1 mM Ca²⁺ and 1 mM Mg²⁺ (pH 7.4) and were supplemented with 10 μ M [¹⁴C]AA for 15 min or with 100 μ M [¹⁴C]PGH₂ for 3 min at 37 °C respectively. The reaction was stopped by acidification to pH 3.5 with 1 M HCl on ice. The supernatants were transferred to 4 vol. of ethyl acetate and the radioactive products were extracted into the organic solvent, which then was evaporated to dryness under N₂. The radioactive products were separated by TLC and quantified by autoradiography after detection with a PhosphorImager system (Image Quant; Molecular Dynamics). Thereafter, authentic prostanoids were stained with iodine vapour, and the radioactive zones were quantified. PGIS activity was monitored by the formation of [¹⁴C]6-oxo-PGF_{1 α} , the stable hydrolysis product of PGI₂. Values are given as counts by integrated absorbance (IA, or IOD).

Immunoprecipitation of nitrotyrosine-containing proteins

With or without IL-1 β treatment, cultured confluent RMC were washed and harvested, then homogenized in 50 mM Tris/HCl buffer, pH 8.0, containing 1 mM PMSF, 5 mM EDTA, 150 mM NaCl and 0.5 % (v/v) Nonidet P40. Solubilized extracts were sonicated for 10 s with a Branson Sonifier 250 (setting 5) at 50 % duty cycle, then centrifuged at 4 °C for 5 min to remove cellular debris. Protein concentrations were estimated by the method of Bradford [22].

Proteins (5 mg) were pre-cleared by 40 μ l of Protein A-Sepharose CL-4B and the supernatants were incubated (18 h, 4 °C) with 30 μ g of a monoclonal anti-nitrotyrosine antibody. Specific immune complexes were precipitated with 30 μ l of Protein A-Sepharose CL-4B and washed with 0.5 ml SNNT [0.5 % sucrose/1 % (v/v) NP40/0.5 M NaCl/50 mM Tris/HCl/5 mM EDTA (pH 7.4)]. The protein was precipitated by centrifugation (13000 g, 1 min) and resuspended in 40 μ l of Laemmli sample buffer/2-mercaptomethanol (9:1), heated to 95 °C for 5 min and kept on ice.

Western blots

Protein precipitates were separated by SDS/PAGE [7.5 % (w/v) gel] and blotted for 1 h with a constant current of 200 mA to a nitrocellulose membrane in a semi-dry blot procedure [48 mM Tris/39 mM glycine/20 % (v/v) methanol/0.037 % SDS]. Pro-

teins were revealed with 0.1 % Ponceau S in 5 % (v/v) acetic acid to check transfer efficiency. After destaining, the membrane was blocked with 5 % (w/v) milk powder in PBS/0.1 % (v/v) Tween 20 for 2 h and incubated with a polyclonal antibody directed against PGIS (2 µg/ml) overnight at 4 °C. After being washed several times with PBS/0.1 % (v/v) Tween 20, the membrane was further incubated with a goat anti-rabbit antibody at a dilution of 1:7500 for 45 min. Antibody binding was detected by the enhanced chemiluminescence technique, in accordance with the instructions of the supplier.

RESULTS AND DISCUSSION

In agreement with earlier results, primary confluent RMC (passages 5–17) contained PGIS activity as measured by the conversion of [14 C]PGH₂ to 6-oxo-PGF_{1α} [11]. However, with AA as a substrate only a very limited conversion to 6-oxo-PGF_{1α} or to other PGs took place owing to the low expression of cyclooxygenase 1 (COX-1) and COX-2 in unstimulated RMC [11,13] (Table 1).

In addition, nitrite formation as a parameter for NO production was below the detection limits in non-stimulated cells. Pretreatment of the cells with 1 nM IL-1β for 24 h greatly increased the expression of COX-2 [11], leading to enhanced synthesis of 6-oxo-PGF_{1α} from [14 C]AA as well as to a similar increase in PGE₂ but not in thromboxane B₂. These results, together with a high rate of nitrite formation, confirmed earlier results from us [11] and others [13,20–23] on the rapid expression of immediate-early gene products in RMC by IL-1β [11]. This was supported by the abolition of the induction effects in the presence of actinomycin D and cycloheximide.

Unexpected was a significant drop in 6-oxo-PGF_{1α} formation when [14 C]PGH₂ was incubated with IL-1β-pretreated cells compared with the primary cells. One reason might have been a blocked expression of PGIS under the influence of IL-1β. A Western blot, however, gave no indication that the amounts of enzyme in the controls and in IL-1β-treated RMC were different (results not shown). Under conditions of completely blocked protein synthesis in the presence of actinomycin D plus cycloheximide the inhibition of 6-oxo-PGF_{1α} was abolished in IL-1β-treated RMC. This result was paralleled by an inhibition of induction of NOS-2 under the influence of IL-1β, as evidenced by the marked drop in nitrite formation. Thus NO or a NO-derived species like PON could be the reason for the lowered 6-oxo-PGF_{1α} levels. Therefore the sensitivity of RMC against NO provided by various NO donors was tested in comparison with

Figure 1 Effect of NO-releasing compounds on [14 C]PGH₂-induced 6-oxo-PGF_{1α} release in confluent RMC

IL-1β-stimulated confluent RMC (5×10^6 cells) were first incubated with 1 mM NONOate and SP-NO for 30 min, or with SIN-1 and SNAP for 4 h, before the addition of 100 µM [14 C]PGH₂ in 2 ml of PBS/Hepes buffer. After 3 min the incubation was stopped by acidification (pH 3.5) with 1 M HCl on ice. PGs in the supernatants were extracted and analysed as described in the Materials and methods section. PGIS activity was monitored by the formation of 6-oxo-PGF_{1α}. The results (means ± S.E.M.) represent 12 samples from four different assays. Abbreviation: IOD (or IA), integrated absorbance.

SIN-1, which is known to release not only NO but also superoxide through an intrinsic auto-oxidation process [24] (Figure 1).

NO alone released by NONOate, SP-NO or SNAP caused no inhibition but rather a slight activation of 6-oxo-PGF_{1α} formation from [14 C]PGH₂, whereas SIN-1 led to a significant and concentration-dependent fall in 6-oxo-PGF_{1α} formation, in agreement with the supposed formation of PON.

Because a quantification of the release of PON from SIN-1 was difficult we tested the sensitivity of PGIS in RMC by bolus additions of chemically synthesized PON (Figure 2).

An inhibition appeared at 10 nM PON; an IC₅₀ of 60 nM was calculated. As controls a decomposed PON solution (24 h standing at 25 °C) and 0.1 M NaOH were used; no inhibition

Table 1 Effects of IL-1β on nitrite and PG production in confluent RMC

Confluent RMC (1.2×10^6 cells) were cultured in RPMI-1640 without or with the addition of IL-1β (1 nM) or IL-1β plus cycloheximide (CH) (10 µM) and actinomycin D (Act D) (10 µM) for 18 h. The concentration of nitrite in the culture medium was determined by the Griess test. After being washed with serum-free medium, the cells were stimulated either with 10 µM [14 C]AA for 15 min or 100 µM [14 C]PGH₂ for 3 min at 37 °C. The radioactive products were analysed and quantified as described in the Materials and methods section. The results (means ± S.D.) are percentages of each metabolite compared with total radioactivity and represent six samples from three different assays. *Significantly different from control ($P < 0.05$); †Significantly different from IL-1β alone ($P < 0.05$).

Addition	RMC stimulated with [14 C]AA			RMC incubated with [14 C]PGH ₂		Nitrite (nmol/ml)
	6-Oxo-PGF _{1α}	PGE ₂	Thromboxane B ₂	6-Oxo-PGF _{1α}	Thromboxane B ₂	
None (controls)	0.4 ± 0.1	0.6 ± 0.2	0.7 ± 0.2	30.5 ± 6.7	0.6 ± 0.4	0 ± 2
IL-1β	2.4 ± 0.6*	6.7 ± 1.7*	0.7 ± 0.4	14.8 ± 1.8*	0.7 ± 0.3	90.8 ± 7.7*
IL-1β + Act D + CH	0.4 ± 0.2†	0.5 ± 0.1†	0.6 ± 0.2	24.4 ± 3.6†	0.8 ± 0.4	0 ± 2†

Figure 2 Effects of PON and its decomposition products on PGH₂-induced 6-oxo-PGF_{1α} production in confluent RMC

IL-1 β -stimulated confluent RMC (5×10^6 cells) were treated with the indicated concentrations of PON. After 15 min 100 μ M [14 C]PGH₂ was added in 2 ml of PBS/Hepes buffer, incubated for 3 min and then analysed as described in the legend to Figure 1. The data (means $\pm$ S.E.M.) represent eight samples from four different experiments. Abbreviation: IOD, integrated absorbance.

Figure 3 Effects of PON on [14 C]AA-dependent prostanoid formation in IL-1 β -treated RMC

IL-1 β -pretreated RMC (5×10^6 cells) were incubated with 10 μ M [14 C]AA in 2 ml of PBS/Hepes buffer at 37 $^{\circ}$ C after pretreatment with the indicated concentrations of PON for 15 min and then analysed as described in the legend to Figure 1. The data (means $\pm$ S.E.M.) represent 10 samples from four different experiments. Abbreviation: IOD, integrated absorbance. 6-Keto-PGF_{1α} = 6-oxo-PGF_{1α}.

was found. It was also confirmed that no inhibition occurred with nitrite and nitrate (1 mM for 24 h) as the main degradation products of PON (results not shown).

Table 2 Effects of anti-oxidants on PON inhibition of PGIS activity in confluent RMC

IL-1 β -stimulated confluent RMC (5×10^6 cells) were first incubated with the agents indicated below before treatment with 1 μ M PON; 15 min after the PON treatment, 100 μ M [14 C]PGH₂ was added and incubated for a further 3 min. PGs were extracted and analysed as described in the Materials and methods section. The results are means $\pm$ S.E.M. for eight samples from four experiments. Abbreviations: DTPA, diethylenetriaminepenta-acetic acid; IA, integrated absorbance. *Significantly different from 1 μ M alone ($P < 0.05$); †Significantly different from non-treated cells ($P < 0.05$).

Treatment	6-oxo-PGF _{1α} (10^3 IA)
Control	1617 $\pm$ 463
+1 μ M PON	399 $\pm$ 167†
1 μ M PON + OH $^{\circ}$ scavengers	
Mannitol (1 mM)	429 $\pm$ 59†
Uric acid (1 mM)	385 $\pm$ 101†
DMSO (1 mM)	489 $\pm$ 89†
1 μ M PON + metal chelators	
DTPA (1 mM)	397 $\pm$ 78†
EDTA (1 mM)	413 $\pm$ 91†
Desferal (1 mg/ml)	496 $\pm$ 117†
1 μ M PON + anti-oxidants	
α -Tocopherol (1 mg/ml)	704 $\pm$ 278†
Trolox (10 mM)	672 $\pm$ 278†
Glutathione (10 mM)	1092 $\pm$ 213*†
Tyrosine (10 mM)	1474 $\pm$ 289*
Tryptophan (1 mM)	682 $\pm$ 261†
Ascorbate (1 mM)	623 $\pm$ 289†

When exogenous [14 C]AA was incubated with IL-1 β -pretreated RMC, the inhibition of 6-oxo-PGF_{1α} formation by PON proceeded with an IC₅₀ of 1 μ M (Figure 3).

Interestingly, the inhibition was paralleled by an increase in other prostanoids, preferentially PGE₂ and PGF_{2α}, suggesting that the remaining PGH₂ was directed to and used by other isomerases. The sum of all COX products was even slightly up-regulated (by 119.7 $\pm$ 6.7%; mean $\pm$ S.D) with a bolus addition of 1 μ M PON, which might have been due to an elevation of the peroxide tone [25].

Although this experiment indicated that PON did not cause damage to the IL-1 β -stimulated RMC, increasing PON concentrations were tested for tolerance by the resting cells. It was unexpected that even 200 μ M as a bolus did not influence the growth of primary RMC within 72 h. In addition, as determined by release of LDH and reduction of MTT, the PON-treated cells remained viable (results not shown). Even if the half-life of PON at pH 7.4 is in the order of 1 s [26] it is remarkable that this supposedly strong oxidant was not harmful, possibly owing to intrinsic cellular defence mechanisms. This raises the apparent discrepancy that in spite of this defence PGIS can be inactivated at PON concentrations 1/1000 of this. An explanation is given by the results in Table 2, in which anti-oxidants and known scavengers for PON were tested as potential blockers of PGIS inhibition by PON.

Chelating agents, $^{\circ}$ OH scavengers and anti-oxidants including known inhibitors for PON-mediated oxidations were employed. It was puzzling to find that none of the tested compounds had a potent inhibitory activity except that 10 mM concentrations of glutathione and tyrosine had some protective effects. These findings seemed incompatible with a direct action of PON as a strong one- or two-electron oxidant [26–28]. However, if we assume that haem catalysis occurs at the active site of PGIS [10] the nitration of a tyrosine residue might be three or four orders of magnitude faster than that of the uncatalysed reaction (M.

Figure 4 Immunoprecipitation with a monoclonal antibody against 3-nitrotyrosine and a Western blot with an antibody against PGIS

With or without IL-1 β pretreatment, confluent RMC were washed, trypsin-treated and homogenized in 50 mM Tris/HCl buffer, pH 8.0, containing 1 mM PMSF, 5 mM EDTA, 150 mM NaCl, 0.5% (v/v) Nonidet P40. Nitrotyrosine-positive proteins were immunoprecipitated by using a monoclonal antibody against 3-nitrotyrosine followed by Protein A–Sepharose CL-4B and were blotted as described in the Materials and methods section. Nitroated PGIS was identified by a polyclonal antibody against PGIS in the nitrotyrosine-positive immunoprecipitates after the indicated treatment. Lane a, purified bovine aortic PGIS treated with 1 μ M PON; lane b, purified bovine aortic PGIS treated with 100 μ M tetranitromethane; lane c, primary RMC (control); lane d, IL-1 β -stimulated RMC treated with 10 mM SIN-1 for 4 h; lane e, IL-1 β -stimulated RMC treated with 1 μ M PON; lane f, RMC stimulated with 1 nM IL-1 β for 18 h.

Mehl, A. Daiber, S. Herold, H. Shoun and V. Ullrich, unpublished work). In addition the commonly known oxidations and nitrations by PON require protonation to *cis*-peroxynitrous acid, its isomerization to the *trans*- form and a further conversion to the excited state [26]. This species reacts like an [HO $\cdot$ NO $_2$] complex and can be trapped by anti-oxidants [29].

It therefore was left to show that in RMC PGIS also underwent a tyrosine nitration and especially that the inhibition by endogenous PON was correlated with this covalent modification of an essential tyrosine residue. Immunoprecipitation with a monoclonal antibody against nitrotyrosine was performed to obtain only the nitrotyrosine-containing proteins. After detection of the immunoprecipitated protein with a polyclonal antibody against PGIS, a single band at 52 kDa was found (Figure 4). As a positive control, a sample of purified bovine PGIS treated with tetranitromethane, a known nitrating agent, was run in parallel.

Double staining with a monoclonal antibody against nitrotyrosine confirmed the nitration of PGIS (results not shown). Interestingly, a weaker band was also present in IL-1 β -pretreated cells that had not been exposed to PON. This nitrotyrosine-positive staining band in the IL-1 β -treated cells provided a direct proof for PGIS nitration and supported and explained the results of Table 1 in which such cells showed a partly blocked formation of 6-oxo-PGF $_{1\alpha}$.

In support of this hypothesis it had been reported that IL-1 β not only induced NO synthesis but also superoxide production [13], which then might cause an endogenous PON formation and PGIS nitration and inhibition. We therefore investigated the effects of a cell-permeable SOD (SOD-PEG) and of the NO synthase inhibitor L-NMMA on PG formation during incubation with IL-1 β .

Figure 5 Effects of SOD and L-NMMA on AA-stimulated PG production in IL-1 β -stimulated RMC

Confluent RMC (5×10^6 cells) were preincubated with 1 nM IL-1 β in the absence or presence of 100 units/ml SOD-PEG or 100 μ M L-NMMA in 3 ml of RPMI medium for 18 h. After being washed twice with PBS, the cells were added to 2 ml of PBS/Hepes buffer and incubated with 10 μ M [14 C]AA for 15 min. The reactions were stopped by acidification (pH 3.5) with 1 M HCl on ice. PGs in the supernatants were extracted and analysed as described in the Materials and methods section. The results (means $\pm$ S.E.M.) represent 10 samples from four different assays. $\square$, 6-Oxo-PGF $_{1\alpha}$; $\blacksquare$, PGE $_2$; $\blacksquare$, PGD $_2$. Abbreviation: IOD, integrated absorbance.

Interestingly, both SOD-PEG and L-NMMA significantly up-regulated PGI $_2$ production and concomitantly down-regulated PGE $_2$ when RMC were subsequently incubated with AA. PGD $_2$ decreased only to a smaller extent (Figure 5).

Such results were in line with the partial nitration of PGIS in IL-1 β -treated cells as observed in Figure 4 and provided additional support for the endogenous formation of PON by the combined action of NOS-2 activity with an as yet unidentified source of superoxide.

Thus for the first time we could demonstrate the nitration of PGIS as a pathophysiologically relevant regulation of enzyme activity. It is obvious that under conditions of increased superoxide production the release of PGI $_2$ from cytokine-stimulated RMC is further down-regulated. One could speculate that a critical situation would arise if the rates of NO and superoxide generation became even. Then neither NO nor PGI $_2$ would be available to compensate and counter-regulate the various inflammatory stimuli, which would take over and affect the glomerular filtration rate and could even provoke kidney failure.

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