Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 340 (2006) 318–325
BBRC
www.elsevier.com/locate/ybbrc

Autocatalytic tyrosine nitration of prostaglandin endoperoxide synthase-2 in LPS-stimulated RAW 264.7 macrophages

Stefan Schildknecht [a,1], Kathrin Heinz [a,1], Andreas Daiber [b], Jürg Hamacher [c], Cengiz Kavaklí [d], Volker Ullrich [a], Markus Bachschmid [a,*]

[a] Department of Biology, University of Konstanz, D-78457 Konstanz, Germany
[b] Department of Cardiology, University Hospital Mainz, D-55101 Mainz, Germany
[c] Department of Internal Medicine, University Hospital Homburg, D-66421 Homburg, Germany
[d] Faculty of Pharmacy, University of Ankara, 06100 Sihhiye, Ankara, Turkey

Received 23 November 2005
Available online 12 December 2005

Abstract

In the literature, biological tyrosine nitrations have been reported to depend not only on peroxynitrite but also on nitrite/hydrogen peroxide linked to catalysis by myeloperoxidase. In endotoxin-stimulated RAW 264.7 macrophages, we have detected a major nitrotyrosine positive protein band around 72 kDa and identified it as prostaglandin endoperoxide synthase-2 (PGHS-2). Isolated PGHS-2 in absence of its substrate arachidonate was not only tyrosine-nitrated with peroxynitrite, but al\so with nitrite/hydrogen peroxide in complete absence of myeloperoxidase. Our data favor an autocatalytic activation of nitrite by PGHS-2 with a subsequent nitration of the essential tyrosine residue in the cyclooxygenase domain. Under inflammatory conditions, nitrite formed via NO-synthase-2 may therefore act as an endogenous regulator for PGHS-2 in stimulated macrophages. Nitration of PGHS-2 by the autocatalytic activation of nitrite further depends on the intracellular concentration of arachidonate since arachidonate reacted competitively with nitrite and could prevent PGHS-2 from nitration when excessively present.

Keywords: Prostaglandin endoperoxide synthase; Nitrite; Nitration; Macrophages; Prostaglandins; Tyrosyl radical; Nitric oxide synthase

Activated macrophages release large quantities of nitric oxide ($^{\cdot}NO$) and superoxide ($^{\cdot}O_2^-$) [1,2]. Next to their role in the innate immune system [3] these radicals may also interact with the prostanoid signaling pathway on different levels. Such interactions range from the regulation of mRNA and protein expression [4,5] to posttranslational modifications of the enzymes involved in the prostanoid biosynthetic pathway [6].

The key enzymatic step in prostanoid biosynthesis is catalyzed by prostaglandin endoperoxide H_2 synthase (PGHS). While the constitutively expressed PGHS-1 maintains housekeeping functions at various sites of the body, PGHS-2 is usually induced in response to pathophysiological stimuli like endotoxin (lipopolysaccharide, LPS), cytokines or mechanical stress [7,8].

Both isoenzymes of PGHS possess two distinct but interconnected catalytic sites, the cyclooxygenase domain located in a hydrophobic channel near the core of the enzyme and a peroxidase domain located close to the surface [9]. Both domains share a single heme prosthetic group which initially reacts with a peroxide like H_2O_2 or peroxynitrite to yield a ferryl species (Fe^{4+}=O) and a tyrosyl radical (Tyr^{385} in PGHS-1; Tyr^{371} in PGHS-2) [10–14]. This initiation and maintenance of the PGHS catalytic cycle by peroxides is summarized by the term ''peroxide tone'' [15]. The tyrosyl radical then initiates the cyclooxygenase reaction by abstracting a hydrogen atom at C13 of

* Corresponding author. Fax: +49 7531 884084 .
E-mail address: Markus.Bachschmid@t-online.de (M. Bachschmid).
[1] The first two authors contributed equally to this work.

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.12.009

arachidonic acid. The resulting arachidonate radical subsequently reacts with a first molecule of O_2 to form a C9–C11 endoperoxide bond and with a second molecule of O_2 to form the 15-hydroperoxy-endoperoxide (PGG_2) which is then reduced by the peroxidase activity to finally yield PGH_2. PGH_2 can then be further converted by downstream synthases to PGE_2, PGD_2, prostacyclin (PGI_2), $PGF_{2\alpha}$ or thromboxane A_2 (TxA_2).

Nitric oxide ($^{\bullet}NO$) activates guanylyl cyclase but also represents the precursor for a variety of reactive nitrogen species [16,17]. It can react with $^{\bullet}O_2^-$ in a nearly diffusion-limited reaction to form peroxynitrite ($ONOO^-$). Its acid form (ONOOH) can easily form the highly reactive oxidants $^{\bullet}NO_2$ and $^{\bullet}OH$. In absence of other paramagnetic species or when present excessively, $^{\bullet}NO$ can also undergo autoxidation with O_2 yielding nitrite (NO_2^-) as the stable end product [18]. In healthy subjects, plasma NO_2^- levels can be detected between 0.5 and 3 μM whereas in septic patients plasma levels of more than 50 μM were observed which originate mainly as a consequence of NO-synthase-2 (NOS-2) induction in various cell types [19–21].

Several reports have indicated an increase of nitrated tyrosine residues (3-NT) in cells, tissues, and organs exposed to severe stress conditions [22–24] but examples of physiological or pathophysiological roles of Tyr-nitration are scarce and difficult to approach experimentally. For prostacyclin (PGI_2)-synthase nitration and inhibition by peroxynitrite has been described in association with endotoxemia, ischemia–reperfusion, atherosclerosis, and diabetes [25–28]. Another reported example for a regulatory role of Tyr-nitration is manganese superoxide dismutase (Mn-SOD) [29,30]. Doubts had been raised in the literature about the ability of the physiologically low levels of peroxynitrite in the low micromolar range to perform Tyr-nitrations [31–34] but such criticisms do not consider the role of metal centers during catalysis which certainly applies for PGI_2-synthase and other heme proteins and possibly also for Mn-SOD [35–37].

An alternative pathway leading to Tyr-nitration can be observed with nitrite in the presence of peroxidatic and pseudo-peroxidatic activities as exemplified by myeloperoxidase (MPO) [38,39] or myoglobin [40–42]. MPO has been associated with host defence mechanisms of activated granulocytes. It uses H_2O_2 to oxidize its primary substrate chloride to generate the highly reactive hypochlorous acid but can alternatively also oxidize NO_2^- to yield the $^{\bullet}NO_2$ radical [43,44]. Recently, the MPO-mechanism was proposed for Tyr-nitration of unidentified proteins in stimulated macrophages [31,32]. The presence of MPO, nitrite, and H_2O_2 in these cells and an inhibition of the nitration process by catalase were taken as an argument that this nitration proceeded through an activation of nitrite by MPO and endogenous peroxides. In contrast, Galinanes and Matata [45] demonstrated that the MPO mechanism does not account for Tyr-nitration in human leukocytes. Thus, the issue of nitrite as a physiological nitrating agent is still open for speculation.

During our work on PGI_2-synthase nitration, we noticed a dominating Tyr-nitrated band at about 72 kDa in LPS-stimulated RAW 264.7 macrophages. We herein identify this protein as prostaglandin endoperoxide synthase-2 (PGHS-2) and provide evidence that its nitration in vitro originates mainly from an autocatalytic activation of nitrite by the peroxide-activated form of PGHS-2. Thus, at least for stimulated macrophages the nitrite/peroxide-driven nitration pathway seems to depend mostly on the heme-containing active site of PGHS-2 and not on a catalytic action of MPO.

Materials and methods

Materials. Arachidonic acid (AA), purified PGHS-2, NDGA, and spermine-NONOate were obtained from Cayman Chemicals (Ann Arbor, MI, USA). SIN-1, MPO, and catalase was purchased from Calbiochem–Novabiochem (Schwalbach, Germany). Allopurinol, gliotoxin, protein A– and G–Sepharose, DMP, pronase, and LPS (*Salmonella minnesota*) was purchased from Sigma (St. Louis, MO, USA). AMT was a product of Alexis Biochemicals (San Diego, USA). Anti-PGHS-2 and anti-NOS-2 monoclonal antibodies were from Transduction Laboratories (Lexington, KY, USA) and the monoclonal and polyclonal antibodies for 3-nitrotyrosine were from HBT (Uden, The Netherlands) and Upstate Biotechnology (Lake Placid, USA). The secondary antibody GAM-POX (goat anti-mouse, HRP-labeled) was obtained from Pierce Biotechnology (Rockford, IL, USA). Cell culture medium DMEM and the additions FCS, penicillin, streptomycin, and amphotericin B were supplied by Biochrom AG (Berlin, Germany). Thin-layer chromatography plates were purchased from Merck (Darmstadt, Germany) and [^{14}C]arachidonic acid was from American Radiolabeled Chemicals (St. Louis, MO, USA).

Western blots. Sonicated samples were mixed with Laemmli loading buffer and boiled for 10 min at 95 °C. Proteins were separated by 8% SDS–PAGE and transferred onto nitrocellulose membranes (Hybond-C extra, Amersham, GB) by the semi-dry procedure. Equal protein loading and transfer were controlled by Ponceau S staining. Blots were blocked for 2 h with 5% milk powder and probed with the primary antibodies overnight. The secondary GAM-POX antibody was incubated for 45 min at room temperature, staining visualized by the enhanced chemiluminescence technique (Uptima; Interchim, France), and exposure to X-ray hyperfilm (Fuji, Tokyo, Japan).

Immunoprecipitation. To prepare covalent protein G/antibody complexes, 40 μg of PGHS-2 antibody was added to 1 ml of protein G–Sepharose beads and incubated at room temperature for 2 h. The beads were washed with phosphate buffer (0.2 M NaH_2PO_4, pH 7.5), 6 mg/ml DMP (approximately 40 mM) was added, and the mixture was incubated for 2 h at room temperature. Beads were washed with 0.2 M ethanolamine (pH 8.2) and incubated in this buffer for further 2 h. Finally they were washed with PBS, before being resuspended in PBS/0.05% Na-azide for further use. For immunoprecipitation, whole cell lysates were incubated with 50 μl of protein A–Sepharose beads in a 1.5 ml tube for 1 h. Beads and membrane fraction were removed by centrifugation for 25 min at 12,000*g*. The supernatant was incubated with 100 μl of antibody/ protein G complex at 4 °C overnight. Beads were sedimented and washed with PBST. PBST was removed, samples were mixed with 40 μl Laemmli buffer containing 5% 2-mercaptoethanol and boiled for 5 min. One hundred microliters of the antibody/protein G complex was run as a control. Samples were allowed to cool to room temperature and subjected to SDS–PAGE and Western blot as described.

PGHS activity assay. PGHS activity was determined by conversion of ^{14}C-labeled-arachidonic acid. Cells were washed twice, collected in cold PBS, and centrifuged at 1000*g* for 3 min. The pellet was dissolved in lysis buffer (20 mM Hepes, 1% Triton X-100, 1% aprotinin, and 10% glycerol, pH 7.5) for 30 min. Nitration was induced by adding the

peroxynitrite-generating compound SIN-1 or nitrite in combination with H_2O_2 at various concentrations for 15 min at 37 °C. Following centrifugation at 12,000*g* for 1 min, the supernatant was incubated with the reaction buffer (80 mM Tris–HCl, 0.8 mM phenol, 10 μg/ml hematin, and 17.2 μM ^{14}C-AA, pH 8.0) for 2 min. The reaction was terminated by the addition of ethyl acetate/2 M citric acid (30:1). The organic phase was spotted by glass capillaries onto silica TLC plates (Silica 60, Merck, Darmstadt, Germany) and subjected to chromatography. The solvent consisted of ethyl acetate:2,2,4-trimethylpentane:acetic acid:-water (110:50:20:100). Plates were dried and exposed to a phosphorimager screen overnight. For reading the screen, a phosphorimager system from Molecular Dynamics, USA, was used. Quantification was performed by the detection of total prostanoid formation utilizing "ImageQuant" software. Background values were subtracted by running a separate lane loaded with reaction buffer only.

HPLC analysis of protein-bound 3-NT. For nitration purified PGHS-2 was incubated as descibed above. Inhibitors (aspirin 200 μM; diclofenac 10, 100 μM; NDGA 10 μM; KCN 1 mM; phenol 50, 200 μM) were added before initiating the nitration and incubated for 1 h. The reaction was terminated by adding digestion solution (phosphate buffer containing 1 mM calcium, 5% acetonitrile, and 2 mg/ml pronase). Cells (10^6 cells/ml) were incubated with LPS (10 μg/ml, *Salmonella minnesota*) at 37 °C under 5% CO_2. After 24 h, cells were harvested in PBS and lysed. After pronase digestion (24 h, 37 °C), samples were evaporated to dryness and resuspended in HPLC buffer. The nitrite content of the cell culture medium was routinely determined, to exclude any influence of the PGHS inhibitors on the induction of NOS-2. Samples were analyzed by using an HPLC system (Jasco) equipped with a ternary low-pressure gradient system and a variable-wavelenght UV detector. Separation was achieved by a Nucleosil column (125/4.6; 100-3-C_{18} Macherey-Nagel, Düren, Germany), isocratically eluted with potassium phosphate buffer (50 mM, pH 7.4) containing 3% acetonitrile at a flow rate of 0.8 ml/min. 3-Nitrotyrosine was identified by spiking the samples with authentic nitrotyrosine and by reduction of nitrotyrosine with sodium dithionite. Concentrations were calculated by integration of the peak area and comparison with a standard curve of authentic 3-nitrotyrosine. In addition, HPLC-electrochemical detection (ECD) analysis was used with an ESA (Bedford, MA, USA) Coulochem II detector. 3-Nitrotyrosine was separated on a C_{18} Nucleosil-100-5 250 × 4.6 reversed-phase column from Macherey-Nagel (Düren, Germany) by isocratic elution with 10% (v/v) methanol in phosphate buffer, pH 6.0, at a flow rate of 0.8 ml/min and detected at a potential of +825 mV.

GC–MS. Purified PGHS-2 was incubated with 10 μM nitrite and 20 μM H_2O_2 for 10 min at 37 °C. Samples were prepared as described recently [46,47] with minor modifications. Briefly, sample extracts were spiked with deuterated internal standards. Prostaglandins and isoprostanes were extracted by 3 volumes of ethyl acetate and the solvent was removed. The methoxime was obtained through reaction with an *O*-methylhydroxylamine hydrochloride-acetate buffer. After acidification to pH 3.5, prostanoid derivatives were extracted and the pentafluorobenzylesters were formed. Samples were purified by TLC and two broad zones with R_v 0.03–0.39 and 0.4–0.8 were eluted. After evaporation of the organic layers, trimethylsilyl ethers were prepared by reaction with bis(trimethylsilyl) trifluoroacetamide and thereafter subjected to GC/MS/MS analysis. A Finnigan MAT TSQ700 GC/MS/MS equipped with a Varian 3400 gas chromatograph and a CTC A200S autosampler was employed. Gas chromatography of prostanoid derivatives was carried out on a J&W DB-1 (20 m, 0.25 mm i.d., 0.25 μm film thickness) capillary column (Analyt, Mühlheim, Germany) in the splitless mode. GC/MS/MS parameters were as described in [46]. Products gained from zone 1 were analyzed for PGE_2, TxB_2, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, and isoprostanes, products from zone 2 for PGD_2. For the quantification of $PGF_{2\alpha}$, and isoprostanes, [P-3 $(CH_3)_3SiOH$]-daughter ions ($m/z = 299$) were used.

Cell culture. The mouse RAW 264.7 macrophage cell line (ATCC—American Type Culture Collection, Manassas, VA, USA) was cultured in DMEM containing 10% FCS, 4 mM glutamine, 1 mM sodium pyruvate, 1.5% sodium bicarbonate, 100 U/ml penicillin/streptomycin, and 0.002% amphotericin B. Confluent cells were challenged with LPS (10 μg/ml; *Salmonella minnesota*). Cells were centrifuged (5 min, 200*g*, 20 °C) and the pellet was frozen in liquid nitrogen.

Nitrite determination. Nitrite as the stable autoxidation product of ·NO was measured by the Griess method. Thirty microliters of 12.5 mM sulfanilamide and 30 μl of 6 N HCl were mixed with 300 μl of cell culture supernatant and incubated for 5 min at room temperature. Absorbance was measured before and after the addition of 25 μl of 12.5 mM NEDA (*N*-(1-naphthyl)ethylenediamide) at 560 nm using a microplate reader. Nitrite concentrations were calculated from a $NaNO_2$ standard curve in the range of 0.5–50 μM. Nitrate was quantified by the nitrate reductase-catalyzed conversion of nitrate to nitrite prior to the Griess reaction [48].

Statistics. Where indicated, data were expressed as means ± SD and were compared by one-way analysis of variance (ANOVA) using $^*P \leqslant 0.05$ as the significance level.

Results

Exposure of RAW 264.7 macrophages to 10 μg/ml lipopolysaccharide (LPS) resulted in a time-dependent induction of PGHS-2 (Fig. 1A). When such blots were stripped and incubated with an anti-3-nitrotyrosine (3-NT) antibody, a main positive band at around 72 kDa was observed, suggesting the nitration of PGHS-2 after 10 h with a maximum at 16 h. The increase of 3-NT between 10 and 16 h was further confirmed by GC–MS analysis of a total hydrolysate of RAW 264.7 macrophages (not shown). In order to verify a specific nitration of PGHS-2,

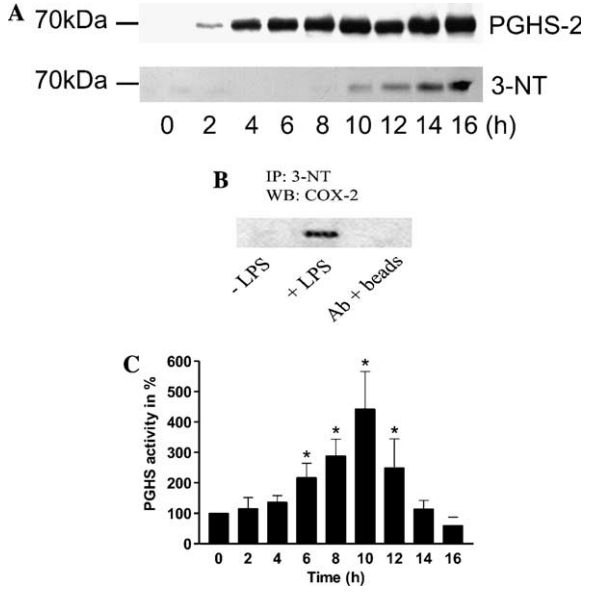

Fig. 1. (A) Nitration of PGHS-2 in RAW 264.7 macrophages. RAW 264.7 macrophages were exposed to LPS (10 μg/ml) for the time periods indicated. After staining of PGHS-2, the membrane was stripped and probed for 3-NT. PGHS-2 protein was under detection limit at $t = 0$ and gradually increased until a maximum was reached after 8–10 h. The same band was stained positive for 3-NT after 10 h with a gradual increase up to 16 h. (B) Homogenates of LPS-exposed RAW 264.7 macrophages (24 h) were immunoprecipitated with an anti-3-NT antibody, staining against PGHS-2 indicated nitration of PGHS-2. (C) Total PGHS activity of RAW 264.7 homogenates incubated with LPS for various time intervals. A maximum was reached after 10 h followed by a decline between 12 and 16 h. Values are means ± SD ($n = 4$). $^*P < 0.05$ vs. $t = 0$.

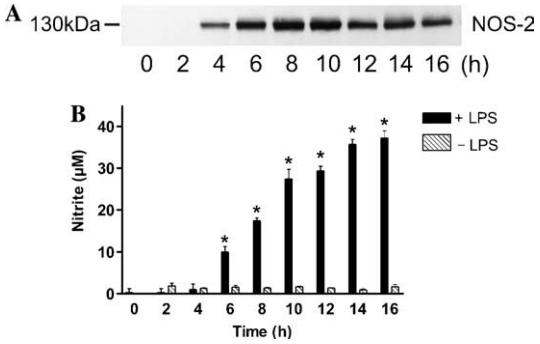

Fig. 2. NOS-2 dependent nitrite formation in RAW 264.7 macrophages. (A) NOS-2 protein expression of LPS (10 μg/ml) exposed RAW 264.7 macrophages was first visualized after 4 h with an increase up to 10 h. From 12 to 16 h, a decline in protein expression was detected. (B) Nitrite formation in cell culture supernatants was observed as indicator for total NOS activity. In the absence of LPS nearly no generation of nitrite was detected. The presence of LPS caused significant upregulation after 6 h. From 10 to 16 h de novo synthesis of nitrite declined. Values are means ± SD ($n = 4$). *$P < 0.05$ vs. $t = 0$.

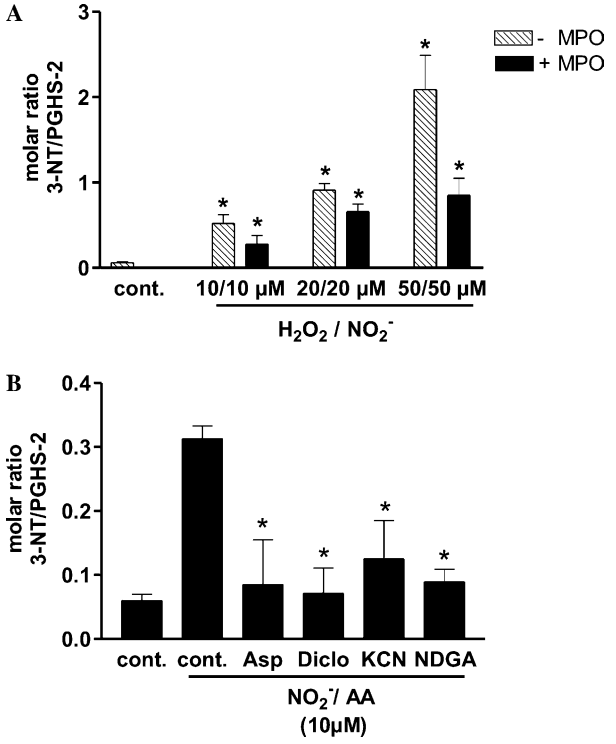

Fig. 3. Involvement of peroxides in PGHS-2 nitration by NO_2^-. (A) Purified PGHS-2 enzyme was incubated with increasing concentrations of NO_2^-/H_2O_2. Nitrotyrosine formation increased in a concentration-dependent manner. The presence of myeloperoxidase (MPO, 1 μM, black bars) even reduced PGHS-2 nitration by NO_2^-/H_2O_2. (B) Purified PGHS-2 was incubated with 20 μM NO_2^-, 20 μM AA, and different inhibitors. Inhibition of the cyclooxygenase activity of PGHS-2 by aspirin (Asp, 500 μM) or diclofenac (Diclo, 10 μM) as well as inhibition of the catalytic heme center by cyanide (KCN, 1 mM) or inhibition of the peroxidase activity by NDGA (10 μM) resulted in a significant inhibition of PGHS-2 nitration. Values are means ± SD ($n = 5$). *$P < 0.05$ vs. cont.

immunoprecipitation with an anti-3-NT antibody followed by Western blotting and incubation with an anti-PGHS-2 antibody confirmed the observed nitrated band as PGHS-2 (Fig. 1B). The reverse experiment failed because of insufficient precipitation with the available PGHS-2 antibody. Total PGHS activity of cell homogenates was measured by the conversion of ^{14}C-labeled arachidonate (Fig. 1C). PGHS activity increased with time until a maximum at 10 h was reached. From 12 to 16 h, in spite of high PGHS-2 protein expression, PGHS activity rapidly declined.

The presence of 3-NT in PGHS-2 implies the activity of NO-synthases in stimulated RAW 264.7 macrophages. Inducible NOS-2 was not detectable without stimulation but was clearly upregulated between 4 and 10 h followed by a decline between 12 and 16 h (Fig. 2A). Nitrite accumulation as indicator for total NOS activity paralleled NOS-2 protein expression with a significant increase between 6 and 10 h followed by a reduced increment from 12 to 16 h (Fig. 2B). Thus, nitrite release closely followed NOS-2 expression. Without LPS, no significant basal release of ·NO was detected which indicates NOS-2 as the major source of NO in LPS-treated RAW 264.7 macrophages.

Since PGHS-2-nitration was initiated when maximal NOS-2 activity was already transgressed, it seems rather plausible that the stable end product nitrite, but not the short-lived ·NO or peroxynitrite, mainly accounts for the observed inhibition of the enzyme. To exclude a possible involvement of MPO present in the cells, these conclusions were further substantiated by experiments with isolated PGHS-2.

Fig. 3A demonstrates the nitration of isolated PGHS-2 by NO_2^-/H_2O_2 in the absence or presence of MPO. Surprisingly, without MPO 3-NT was massively formed in PGHS-2 up to a molar ratio of 2, whereas in the presence of MPO 3-NT-formation was only about half yielding a molar ratio of about 1:1. These observations support a peroxidatic activation of nitrite by the intrinsic peroxidase activity of PGHS-2. Not only H_2O_2 but also the primary PGHS-2 product 15-hydroperoxy-prostaglandin endoperoxide G_2 (PGG_2) formed by the cyclooxygenase activity of the PGHS enzyme is supposed to provide the peroxide tone. Blocking the cyclooxygenase function by aspirin or diclofenac and also a 1 mM concentration of the heme-ligand KCN as well as the phenolic antioxidant NDGA caused an inhibition of PGHS-2-nitration (Fig. 3B). This underlines the role of the heme active site of PGHS-2 for the activation of nitrite to result in Tyr-nitration. To better compare the efficacy of nitrite as a Tyr-nitrating agent for PGHS-2 against ·NO and peroxynitrite, the activity of the purified enzyme was determined after an incubation with NO_2^-, the ·NO-releasing compound spermine-NONOate or the peroxynitrite-generating compound SIN-1 for 15 min (Fig. 4). Both NO_2^- and SIN-1 demonstrated a dose-dependent inhibition whereas incubation with spermine-NONOate resulted only in a moderate inhibition at high concentrations. Since autoxidation of ·NO could occur under the conditions employed, inhibi-

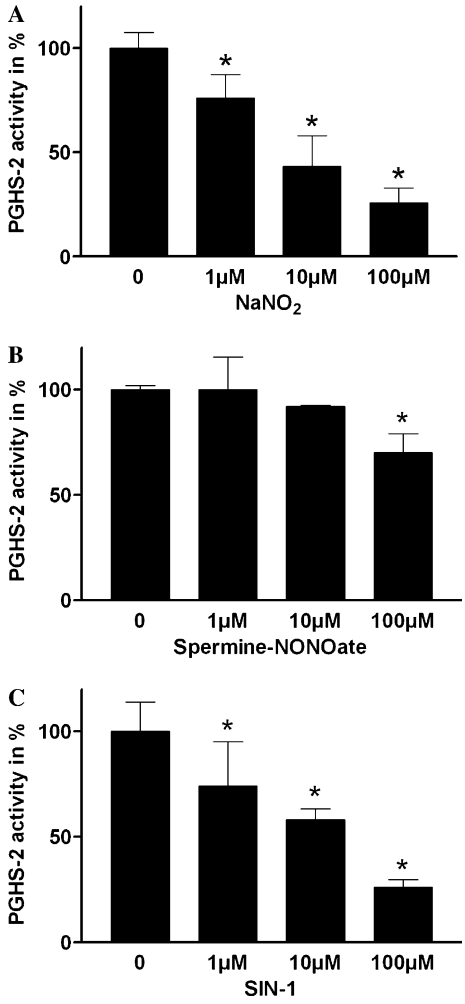

Fig. 4. Inhibition of PGHS-2 activitiy by $ONOO^-$ and NO_2^-. Purified PGHS-2 was preincubated with NO_2^-, ·NO-releasing spermine-NONOate or peroxynitrite-generating SIN-1 in the concentrations indicated for 10 min. ^{14}C-AA (17.2 μM) was then added, the reaction was terminated after 2 min. Total prostaglandin formation was evaluated for PGHS activity detection. NO_2^- (A) and SIN-1 (C) resulted in a dose-dependent inhibition of PGHS-2 whereas ·NO (B) caused only a weakly reduced activity. Values are means ± SD ($n = 4$). *$P < 0.05$ vs. concentration = 0.

tion at 100 μM spermine-NONOate may have indirectly occurred via formation of NO_2^-. It was puzzling to find NO_2^- alone sufficient for PGHS-2 inhibition but catalase released both the inhibition as well as 3-NT formation, thus indicating the presence of low levels of H_2O_2 formed by autoxidation. This would be in agreement with the low levels of peroxides (2 nM) as the saturating peroxide tone reported for PGHS-2 [51]. According to these results the simplest interpretation would be a competitive reaction of nitrite and arachidonate with the ferryl-Tyr-radical enzyme intermediate. This assumption could be verified by preincubation with unlabeled AA for 1 min followed by 1 min NO_2^- or vice versa (Fig. 5). Preincubation of purified PGHS-2 with AA prevented the enzyme from nitration by NO_2^-. In contrast, when PGHS-2 was incubated with NO_2^- first, a dose-dependent inhibition could be observed.

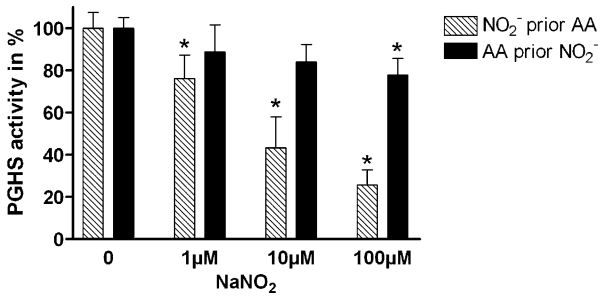

Fig. 5. Competition between AA and NO_2^-. Purified PGHS-2 was preincubated with NO_2^- for 1 min followed by addition of 10μM unlabeled AA for 1 min and 17.2μM ^{14}C-AA for 2 min (open bars). Alternatively, PGHS-2 was incubated with 10 μM unlabeled AA for 1 min followed by 1 min NO_2^- and 2 min 17.2 μM ^{14}C-AA (black bars). Preincubation with AA significantly prevented nitrite-dependent inhibition of PGHS-2 activity. Values are means ± SD ($n = 4$). *$P <$ vs. $NaNO_2 = 0$.

To confirm that nitration of PGHS-2 by nitrite can also be observed under cellular conditions, RAW 264.7 macrophages were stimulated with LPS in the presence of the NOS-inhibitors AMT or L-NMMA. This experiment however led to a significant inhibition of PGHS-2 expression in the LPS-stimulated RAW 264.7 macrophages by the NOS-inhibitors. Although an involvement of ·NO-synthesis in the induction process for PGHS-2 represented an interesting new aspect of interactions between the ·NO and the prostanoid pathway, this topic was not further studied here. Therefore, RAW 264.7 macrophages were treated with LPS for 5 h for PGHS-2 induction and their homogenates were supplied with increasing levels of nitrite and

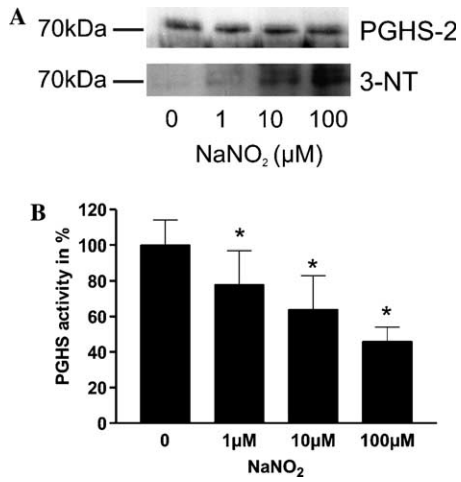

Fig. 6. Nitration of PGHS-2 by NO_2^- in RAW 264.7 homogenates. (A) Cells were incubated with LPS for 5 h for PGHS-2 induction. Aliquots of a pooled homogenate were incubated with NO_2^- in the concentrations indicated for 10 min followed by the addition of 17.2μM AA. 3-NT staining intensified with elevated NO_2^- concentrations. (B) PGHS activity of RAW 264.7 homogenates is inhibited with increasing concentrations of NO_2^-. Samples were treated as described in (A). After preincubation with NO_2^- for 10 min, ^{14}C-AA (17.2 μM) was added for 2 min. Total prostaglandin formation was integrated for evaluation. Values are means ± SD ($n = 4$). *$P < 0.05$ vs. $NO_2^- = 0$.

analyzed for PGHS-2 nitration. A Tyr-nitration of PGHS-2 could be achieved at 10 μM nitrite which was further enhanced at 100 μM nitrite (Fig. 6A). PGHS-2 activity declined only to about 50% which may be explained by the presence of cellular free arachidonate that could have protected the enzyme from higher inhibition (Fig. 6B).

Discussion

The present study was initiated by the observation that long-term exposure of RAW 264.7 macrophages to LPS resulted in Tyr-nitration of a 72 kDa protein, which by immunoprecipitation with anti-3-NT antibodies yielded PGHS-2 as the main nitrated protein. Nitration of PGHS-2 had already been reported for the LPS-stimulated cell line J774.A1 [49] and positive staining for 3-NT was also demonstrated in LPS-stimulated RAW 264.7 macrophages [31] however the nitrated protein(s) were not identified.

The observations of this work clarify several issues on the interaction of the ·NO-pathway with prostanoid biosynthesis. Tyr-nitrations can be a consequence of peroxynitrite formation which requires the simultaneous generation of ·NO and $·O_2^-$. Whereas peroxynitrite is unstable and only formed transiently, nitrite as the stable end product of ·NO synthesis was reported to be utilized by the myeloperoxidase/H_2O_2 pathway to cause nitrations at Tyr-residues [50]. This appears as an unspecific oxidation mechanism during phagocytosis or autodestructive processes in tissues while regulatory functions have not yet been found associated with this pathway. As the main novel finding, we herein provide evidence that PGHS-2 is autocatalytically inactivated in the presence of nitrite by a heme/peroxide-catalyzed formation of the $·NO_2$ radical which subsequently nitrates and inactivates the enzyme. In accordance with the literature [51], the peroxide requirements for PGHS-2 were detected to be very low since no additions of peroxides were necessary for the peroxidatic activation of NO_2^- although catalase was inhibitory. In the absence of arachidonate, nitrite levels of around 10 μM were sufficient for the nitration of PGHS-2. However, in the presence of arachidonate the inhibition was effectively suppressed thus allowing to conclude on a rather simple mechanism of nitrite activation as depicted in Fig. 7. Since peroxides generate a Tyr-radical at the active site-located Tyr^{371} it is very likely, although still not proven, that this residue becomes nitrated by combination with the $·NO_2$ radical [52,53].

From this mechanism a new interpretation of related data in the literature could be derived. First, the reported nitration of this Tyr^{371}-residue by high concentrations of ·NO (1–2 mM) [54–56] under aerobic conditions could follow the suggested pathway since nitrite readily forms from ·NO and dioxygen. This would also apply for the detection of nitrated PGHS-2 in LPS/INF-γ/·NO-treated J774.A1 macrophages [49]. Second, the observed increase of 3-NT in LPS-stimulated RAW 264.7 macrophages could be assumed to originate also from a self-catalyzed nitration of PGHS-2 without any involvement of MPO. There is no doubt that PGHS-2 can also be nitrated by peroxynitrite, but from earlier data indicating peroxynitrite levels of more than 100 μM as necessary for an inhibition [57] as well as from the time course of NOS-2 induction and PGHS-2 inhibition found in this work, nitrite as the precursor of the Tyr-nitrating process appears as a rather convincing alternative explanation.

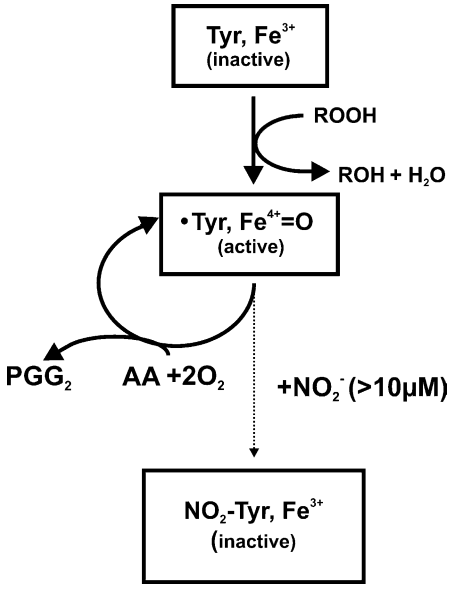

Fig. 7. Proposed mechanism of PGHS nitration by nitrite. Resting ferric PGHS-2 is first activated by peroxides to form ·Tyr, Fe^{4+}=O at the active site of the enzyme. On the left, the normal catalytic cycle of arachidonate oxygenation and reformation of the active state by PGG_2 is depicted. In the presence of NO_2^-, Fe^{4+}=O can activate NO_2^- to form the $·NO_2$ radical which subsequently recombines with a ·Tyr radical yielding the nitrated and inactivated enzyme.

A physiological function of nitrite as a regulator of prostanoid formation however still remains a matter of speculation. A strong activation of macrophages for massive induction of NOS-2 is certainly required to generate sufficient amounts of ·NO. This can be converted to nitrite by several pathways and will accumulate with time until it reaches concentrations that allow the self-catalyzed nitration of PGHS-2. Since this process was found to be competitive with arachidonate oxygenation, the levels of nitrite have to rise well above the threshold of 10 μM as detected for isolated PGHS-2. Indeed, levels as high as 50–80 μM have been measured in inflamed tissue and more than 70 μM can accumulate in the supernatants of LPS-stimulated macrophages. If at the resolution of the inflammatory phase a decline of phospholipase A_2 activity would lower the levels of free arachidonate, a synergistic effect would arise for a rapid inhibition of prostanoid formation. The requirement for a high output of ·NO may limit the significance of PGHS-nitration to the PGHS-2 isoform which usually is associated with NOS-2 induction. PGHS-1 can become nitrated by peroxynitrite [58,59] and

also by nitrite (unpublished) but under physiological conditions the levels of ·NO from NOS-1 or NOS-3 activity could be regarded as too low for an effective inhibition by nitrite formation.

In summary, our data support an autocatalytic inhibition of PGHS-2 mediated by the peroxidatic generation of the $\cdot NO_2$ radical which subsequently nitrates an essential Tyr residue at the active site of the enzyme.

Acknowledgments

This work was supported by the DFG (Deutsche Forschungsgemeinschaft) and the Forschergruppe "Endogene Gewebszerstörung".

References

[1] D.J. Stuehr, M.A. Marletta, Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide, Proc. Natl. Acad. Sci. USA 82 (1985) 7738–7742.
[2] G.M. Rosen, S. Pou, C.L. Ramos, M.S. Cohen, B.E. Britigan, Free radicals and phagocytic cells, FASEB J. 9 (1995) 200–209.
[3] E. Linares, S. Giorgio, R.A. Mortara, C.X. Santos, A.T. Yamada, O. Augusto, Role of peroxynitrite in macrophage microbicidal mechanisms in vivo revealed by protein nitration and hydroxylation, Free Radic. Biol. Med. 30 (2001) 1234–1242.
[4] D.J. Perkins, D.A. Kniss, Blockade of nitric oxide formation downregulates cyclooxygenase-2 and decreases PGE_2 biosynthesis in macrophages, J. Leukoc. Biol. 65 (1999) 792–799.
[5] A. Habib, C. Bernard, M. Lebret, C. Creminon, B. Esposito, A. Tedgui, J. Maclouf, Regulation of the expression of cyclooxygenase-2 by nitric oxide in rat peritoneal macrophages, J. Immunol. 158 (1997) 3845–3851.
[6] D.C. Goodwin, L.M. Landino, L.J. Marnett, Effects of nitric oxide and nitric oxide-derived species on prostaglandin endoperoxide synthase and prostaglandin biosynthesis, FASEB J. 13 (1999) 1121–1136.
[7] J.R. Vane, Y.S. Bakhle, R.M. Botting, Cyclooxygenases 1 and 2, Annu. Rev. Pharmacol. Toxicol. 38 (1998) 97–120.
[8] W.L. Smith, D.L. DeWitt, R.M. Garavito, Cyclooxygenases: structural, cellular, and molecular biology, Annu. Rev. Biochem. 69 (2000) 145–182.
[9] D. Picot, P.J. Loll, R.M. Garavito, The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1, Nature 367 (1994) 243–249.
[10] R. Dietz, W. Nastainczyk, H.H. Ruf, Higher oxidation states of prostaglandin H synthase. Rapid electronic spectroscopy detected two spectral intermediates during the peroxidase reaction with prostaglandin G_2, Eur. J. Biochem. 171 (1988) 321–328.
[11] R.J. Kulmacz, R.B. Pendleton, W.E. Lands, Interaction between peroxidase and cyclooxygenase activities in prostaglandin-endoperoxide synthase. Interpretation of reaction kinetics, J. Biol. Chem. 269 (1994) 5527–5536.
[12] N. Ogino, S. Ohki, S. Yamamoto, O. Hayaishi, Prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. Inactivation and activation by heme and other metalloporphyrins, J. Biol. Chem. 253 (1978) 5061–5068.
[13] T. Shimokawa, R.J. Kulmacz, D.L. DeWitt, W.L. Smith, Tyrosine 385 of prostaglandin endoperoxide synthase is required for cyclooxygenase catalysis, J. Biol. Chem. 265 (1990) 20073–20076.
[14] A. Tsai, R.J. Kulmacz, Tyrosyl radicals in prostaglandin H synthase-1 and -2, Prostaglandins Other Lipid Mediat. 62 (2000) 231–254.
[15] L.G. Cleland, Oxy radicals, "peroxide tone" and inflammation, J. Rheumatol. 11 (1984) 725–726.
[16] M.B. Grisham, D. Jourd'Heuil, D.A. Wink, Physiological chemistry of nitric oxide and its metabolites: implications in inflammation, Am. J. Physiol. 276 (1999) G315–G321.
[17] R.E. Huie, S. Padmaja, The reaction of NO with superoxide, Free Radic. Res. Commun. 18 (1993) 195–199.
[18] P.C. Ford, D.A. Wink, D.M. Stanbury, Autoxidation kinetics of aqueous nitric oxide, FEBS Lett. 326 (1993) 1–3.
[19] C. Mitaka, Y. Hirata, K. Yokoyama, H. Wakimoto, M. Hirokawa, T. Nosaka, T. Imai, Relationships of circulatory nitrite/nitrate levels to severity and multiple organ dysfunction syndrome in systemic inflammatory response syndrome, Shock 19 (2003) 305–309.
[20] Y. Ersoy, E. Ozerol, O. Baysal, I. Temel, R.S. MacWalter, U. Meral, Z.E. Altay, Serum nitrate and nitrite levels in patients with rheumatoid arthritis, ankylosing spondylitis and osteoarthritis, Ann. Rheum. Dis. 61 (2002) 76–78.
[21] S.D. Chauhan, G. Seggara, P.A. Vo, R.J. Macallister, A.J. Hobbs, A. Ahluwalia, Protection against lipopolysaccharide-induced endothelial dysfunction in resistance and conduit vasculature of iNOS knockout mice, FASEB J. 17 (2003) 773–775.
[22] H. Ischiropoulos, Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species, Arch. Biochem. Biophys. 356 (1998) 1–11.
[23] K. Bian, K. Davis, J. Kuret, L. Binder, F. Murad, Nitrotyrosine formation with endotoxin-induced kidney injury detected by immunohistochemistry, Am. J. Physiol. 277 (1999) F33–F40.
[24] D.M. Fries, E. Paxinou, M. Themistocleous, E. Swanberg, K.K. Griendling, D. Salvemini, J.W. Slot, H.F. Heijnen, S.L. Hazen, H. Ischiropoulos, Expression of inducible nitric-oxide synthase and intracellular protein tyrosine nitration in vascular smooth muscle cells: role of reactive oxygen species, J. Biol. Chem. 278 (2003) 22901–22907.
[25] M. Bachschmid, S. Thurau, M.H. Zou, V. Ullrich, Endothelial cell activation by endotoxin involves superoxide/NO-mediated nitration of prostacyclin synthase and thromboxane receptor stimulation, FASEB J. 17 (2003) 914–916.
[26] M.H. Zou, M. Bachschmid, Hypoxia-reoxygenation triggers coronary vasospasm in isolated bovine coronary arteries via tyrosine nitration of prostacyclin synthase, J. Exp. Med. 190 (1999) 135–139.
[27] M.H. Zou, M. Leist, V. Ullrich, Selective nitration of prostacyclin synthase and defective vasorelaxation in atherosclerotic bovine arteries, Am. J. Pathol. 154 (1999) 1359–1365.
[28] M.H. Zou, C. Shi, R.A. Cohen, High glucose via peroxynitrite causes tyrosine nitration and inactivation of prostacyclin synthase that is associated with thromboxane/prostaglandin H(2) receptor-mediated apoptosis and adhesion molecule expression in cultured human aortic endothelial cells, Diabetes 51 (2002) 198–203.
[29] K.M. Pittman, L.A. MacMillan-Crow, B.P. Peters, J.B. Allen, Nitration of manganese superoxide dismutase during ocular inflammation, Exp. Eye Res. 74 (2002) 463–471.
[30] L.A. MacMillan-Crow, J.A. Thompson, Tyrosine modifications and inactivation of active site manganese superoxide dismutase mutant (Y34F) by peroxynitrite, Arch. Biochem. Biophys. 366 (1999) 82–88.
[31] S. Pfeiffer, A. Lass, K. Schmidt, B. Mayer, Protein tyrosine nitration in cytokine-activated murine macrophages. Involvement of a peroxidase/nitrite pathway rather than peroxynitrite, J. Biol. Chem. 276 (2001) 34051–34058.
[32] S. Pfeiffer, A. Lass, K. Schmidt, B. Mayer, Protein tyrosine nitration in mouse peritoneal macrophages activated in vitro and in vivo: evidence against an essential role of peroxynitrite, FASEB J. 15 (2001) 2355–2364.
[33] M.G. Espey, S. Xavier, D.D. Thomas, K.M. Miranda, D.A. Wink, Direct real-time evaluation of nitration with green fluorescent protein in solution and within human cells reveals the impact of nitrogen dioxide vs. peroxynitrite mechanisms, Proc. Natl. Acad. Sci. USA 99 (2002) 3481–3486.
[34] S. Pfeiffer, B. Mayer, Lack of tyrosine nitration by peroxynitrite generated at physiological pH, J. Biol. Chem. 273 (1998) 27280–27285.

[35] A.M. Cassina, R. Hodara, J.M. Souza, L. Thomson, L. Castro, H. Ischiropoulos, B.A. Freeman, R. Radi, Cytochrome *c* nitration by peroxynitrite, J. Biol. Chem. 275 (2000) 21409–21415.
[36] A. Alayash, B.A. Ryan, R.E. Cashon, Peroxynitrite-mediated heme oxidation and protein modification of native and chemically modified hemoglobins, Arch. Biochem. Biophys. 349 (1998) 65–73.
[37] A. Daiber, S. Herold, C. Schoneich, D. Namgaladze, J.A. Peterson, V. Ullrich, Nitration and inactivation of cytochrome P450BM-3 by peroxynitrite, stopped-flow measurements prove ferryl intermediates, Eur. J. Biochem. 267 (2000) 6729–6739.
[38] S. Baldus, J.P. Eiserich, M.L. Brennan, R.M. Jackson, C.B. Alexander, B.A. Freeman, Spatial mapping of pulmonary and vascular nitrotyrosine reveals the pivotal role of myeloperoxidase as a catalyst for tyrosine nitration in inflammatory diseases, Free Radic. Biol. Med. 33 (2002) 1010.
[39] J.B. Sampson, Y. Ye, H. Rosen, J.S. Beckman, Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and hydrogen peroxide, Arch. Biochem. Biophys. 356 (1998) 207–213.
[40] J.L. Bourassa, E.P. Ives, A.L. Marqueling, R. Shimanovich, J.T. Groves, Myoglobin catalyzes its own nitration, J. Am. Chem. Soc. 123 (2001) 5142–5143.
[41] K. Kilinc, A. Kilinc, R.E. Wolf, M.B. Grisham, Myoglobin-catalyzed tyrosine nitration: no need for peroxynitrite, Biochem. Biophys. Res. Comm. 285 (2001) 273–276.
[42] S. Herold, F. Rehmann, Kinetic and mechanistic studies of the reactions of nitrogen monoxide and nitrite with ferryl myoglobin, J. Biol. Inorg. Chem. 6 (2001) 543–555.
[43] J.P. Eiserich, M. Hristova, C.E. Cross, A.D. Jones, B.A. Freeman, B. Halliwell, A. van der Vliet, Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils, Nature 391 (1998) 393–397.
[44] K. Bian, Z. Gao, N. Weisbrodt, F. Murad, The nature of heme/iron-induced protein tyrosine nitration, Proc. Natl. Acad. Sci. USA 100 (2003) 5712–5717.
[45] M. Galinanes, B.M. Matata, Protein nitration is predominantly mediated by a peroxynitrite-dependent pathway in cultured human leucocytes, Biochem. J. 367 (2002) 467–473.
[46] H. Schweer, B. Watzer, H.W. Seyberth, Determination of seven prostanoids in 1 ml of urine by gas chromatography-negative ion chemical ionization triple stage quadrupole mass spectrometry, J. Chromatogr. 652 (1994) 221–227.
[47] T. Klein, F. Reutter, H. Schweer, H.W. Seyberth, R.M. Nusing, Generation of the isoprostane 8-epi-prostaglandin F2alpha in vitro and in vivo via the cyclooxygenases, J. Pharmacol. Exp. Med. 282 (1997) 1658–1665.
[48] H. Schmidt, M. Kelm, in: M. Feelisch, J.S. Stammler (Eds.), Methods in Nitric Oxide Research, Wiley, Chichester, UK, 1996, pp. 491–497.
[49] R. Clancy, B. Varenika, W. Huang, L. Ballou, M. Attur, A.R. Amin, S.B. Abramson, Nitric oxide synthase/COX cross-talk: nitric oxide activates COX-1 but inhibits COX-2-derived prostaglandin production, J. Immunol. 165 (2000) 1582–1587.
[50] J.P. Gaut, J. Byun, H.D. Tran, W.M. Lauber, J.A. Carroll, R.S. Hotchkiss, A. Belaaouaj, J.W. Heinecke, Myeloperoxidase produces nitrating oxidants in vivo, J. Clin. Invest. 109 (2002) 1311–1319.
[51] R.J. Kulmacz, L.H. Wang, Comparison of hydroperoxide initiator requirements for the cyclooxygenase activities of prostaglandin H synthase-1 and -2, J. Biol. Chem. 270 (1995) 24019–24023.
[52] L.C. Hsi, C.W. Hoganson, G.T. Babcock, W.L. Smith, Characterization of a tyrosyl radical in prostaglandin endoperoxide synthase-2, Biochem. Biophys. Res. Commun. 202 (1994) 1592–1598.
[53] A.L. Tsai, G. Palmer, G. Xiao, D.C. Swinney, R.J. Kulmacz, Structural characterization of arachidonyl radicals formed by prostaglandin H synthase-2 and prostaglandin H synthase-1 reconstituted with mangano protoporphyrin IX, J. Biol. Chem. 273 (1998) 3888–3894.
[54] M.R. Gunther, L.C. Hsi, J.F. Curtis, J.K. Gierse, L.J. Marnett, T.E. Eling, R.P. Mason, Nitric oxide trapping of the tyrosyl radical of prostaglandin H synthase-2 leads to tyrosine iminoxyl radical and nitrotyrosine formation, J. Biol. Chem. 272 (1997) 17086–17900.
[55] D.C. Goodwin, M.R. Gunther, L.C. Hsi, B.C. Crews, T.E. Eling, R.P. Mason, L.J. Marnett, Nitric oxide trapping of tyrosyl radicals generated during prostaglandin endoperoxide synthase turnover, Detection of the radical derivative of tyrosine 385, J. Biol. Chem. 273 (1998) 8903–8909.
[56] A.L. Tsai, C. Wei, R.J. Kulmacz, Interaction between nitric oxide and prostaglandin H synthase, Arch. Biochem. Biophys. 313 (1994) 367–372.
[57] M.H. Zou, C. Martin, V. Ullrich, Tyrosine nitration as a mechanism of selective inactivation of prostacyclin synthase by peroxynitrite, Biol. Chem. 378 (1997) 707–713.
[58] R.S. Deeb, M.J. Resnick, D. Mittar, T. McCaffrey, D.P. Hajjar, R.K. Upmacis, Tyrosine nitration in prostaglandin H(2) synthase, J. Lipid Res. 43 (2002) 1718–1726.
[59] C. Boulos, H. Jiang, M. Balazy, Diffusion of peroxynitrite into the human platelet inhibits cyclooxygenase via nitration of tyrosine residues, J. Pharmacol. Exp. Ther. 293 (2000) 222–229.