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Specific Nitration at Tyrosine 430 Revealed by High Resolution Mass Spectrometry as Basis for Redox Regulation of Bovine Prostacyclin Synthase*

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Treatment of bovine aortic microsomes containing active prostacyclin synthase (PGI_2 synthase) with increasing concentrations of peroxynitrite (PN) up to 250 μM of PN yielded specific staining of this enzyme on Western blots with antibodies against 3-nitrotyrosine (3-NT), whereas above 500 μM PN staining of additional proteins was also observed. Following treatment of aortic microsomes with 25 μM PN, PGI_2 synthase was about half-maximally nitrated and about half-inhibited. It was then isolated by gel electrophoresis and subjected to proteolytic digestion with several proteases. Digestion with thermolysin for 24 h provided a single specific peptide that was isolated by high performance liquid chromatography and identified as a tetrapeptide Leu-Lys-Asn-Tyr(3-nitro)-COOH corresponding to positions 427–430 of PGI_2 synthase. Its structure was established by precise mass determination using Fourier transform-ion cyclotron resonance-nanoelectrospray mass spectrometry and Edman microsequencing and ascertained by synthesis and mass spectrometric characterization of the authentic Tyr-nitrated peptide. Complete digestion by Pronase to 3-nitrotyrosine was obtained only after 72 h, suggesting that the nitrated Tyr-430 residue may be embedded in a tight fold around the heme binding site. These results provide evidence for the specific inhibition of PGI_2 synthase by nitration at Tyr-430 that may occur already at low levels of PN as a consequence of endothelial co-generation of nitric oxide and superoxide.

The nitration of tyrosine residues in proteins has become a well recognized reaction, but has been heavily disputed with regard to the mechanisms involved and its physiological and/or pathophysiological significance (1–5). Peroxynitrite (PN)[1] generated from nitric oxide (NO) and superoxide ($O_2^{\cdot-}$) can react with Tyr or Tyr-containing proteins under formation of 3-nitrotyrosine (3-NT) (6–8) but in general the required concentrations are higher than expected to occur *in vivo*. Pfeiffer and Mayer (9–12) have even questioned the significance of PN as a cellular nitrating agent and have proposed nitrite/hydrogen peroxide as an alternative pathway with myeloperoxidase as a catalyst (11, 12), which may indeed apply for certain proteins. In the case of PN it has not been considered that PN can be activated by transition metal ions that may then catalyze the self-nitration of metalloproteins at low PN levels. We have recently provided evidence for this reaction for heme-thiolate (P450) proteins (13–15) that therefore may serve as a model for the P450 protein PGI_2 synthase.

PGI_2 synthase was inactivated by micromolar PN concentrations (16, 17) but also by a continuous generation of NO and $O_2^{\cdot-}$ from SIN-1 (18). In cellular systems the inhibition of nitration by a NO synthase inhibitor and polyethylene-glycolated superoxide dismutase provided evidence for the involvement of PN, whereas nitrite was ineffective (18). Because NO and PGI_2 are important for the endothelial barrier function the formation of PN and the nitration of PGI_2 synthase could play a role in the process of endothelial activation for adhesion and emigration of white blood cells into the tissue (19). Interestingly, PGI_2 synthase was found localized to the caveolae-like endothelial NO synthase (20) and hence PN formation may occur in close vicinity to PGI_2 synthase. This localization in a "quasi-extracellular" compartment may be a further important factor for efficient nitration by low concentrations of PN.

Beyond this physiological background no proof for the molecular basis of enzyme inhibition has been hitherto obtained by identification of nitrated tyrosine. Substrate analogs of prostaglandin-endoperoxide have been recently shown to inhibit the nitration (17), which suggested a proximity to the heme attached to the protein by the Cys-441 residue (21–23); however, previous attempts have been unsuccessful to detect and identify the nitrated tyrosine. In this study we present molecular evidence for the specific nitration of bovine PGI_2 synthase at tyrosine 430 by high resolution Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometry (24), and the presence of 3-NT upon extended Pronase digestion. We further show an unusually slow digestion by thermolysin, presumably because of a tight fold around the heme, to release a tetrapep-

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[1] The abbreviations used are: PN, peroxynitrite (oxoperoxonitrate (1−)); PGI_2, prostacyclin; PGI_2 synthase, prostacyclin synthase; NT, 3-nitrotyrosine; PGH_2, prostaglandin endoperoxide; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; EIA, enzyme immunoassay; NO, nitric oxide; PBS, phosphate-buffered saline; BSA, bovine serum albumin; $P450_{BM-3}$, bacterial monooxygenase-3 from *Bacillus megaterium* (CYP 102); FT-ICR, Fourier transform ion cyclotron resonance; HPLC, high performance liquid chromatography; PTH, phenylthiohydantoin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl.

tide by an unexpected specific cleavage adjacent to the nitrated tyrosine residue.

EXPERIMENTAL PROCEDURES

Materials—Pronase from *Streptomyces griseus* (lyophilized powder) was obtained from Roche Molecular Diagnostics. Thermolysin, type X from *Bacillus thermoproteolyticus rokko* was purchased from Sigma. All other chemicals were of analytical grade or highest purity available. PN was a gift from Dr. Koppenol (ETH Zürich, Switzerland) and was synthesized from NO and potassium superoxide according to Kissner *et al.* (25). $P450_{BM-3}$ (CYP 102), a F87Y variant from *Bacillus megaterium* was a kind gift from J. A. Peterson (Southwestern Medical School, Dallas, TX) and was purified as described (26).

A rabbit polyclonal antibody against PGI_2 synthase was produced according to Siegle *et al.* (27). A mouse monoclonal antibody against 3-NT (anti-NT, clone 1A6) was obtained from Upstate Biotechnology (Hamburg, Germany) as a stock solution of 1 mg/ml. Secondary antibodies (goat anti-mouse IgG and goat anti-rabbit IgG) were obtained from Pierce (stock solutions 0.8 mg/ml). The enhanced chemiluminescence (ECL) kit and nitrocellulose transfer membranes (Hybond C, pore size 0.5 μm) were purchased from Amersham Biosciences. PGH_2 was obtained from Cayman Chemical (Ann Arbor, MI).

Preparation of Bovine Aortic Microsomes—Endothelial and smooth muscle layers from 8 to 10 freshly received bovine aorta were isolated by dissection at 4 °C, rapidly frozen in liquid nitrogen, and stored at −70 °C. Frozen strips were homogenized at 0–4 °C in a Waring blender in 100 mM K-phosphate buffer, pH 7.5, containing 1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM butylated hydroxytoluene, and 44 mg/liter phenylmethylsulfonyl fluoride. The microsomal fraction was obtained by centrifugation as described (28) to a final volume of 75–100 ml, with a protein concentration of 10–20 mg/ml. The homogenization buffer contained 50 mM K_2HPO_4, pH 7.5, without additional protease inhibitors.

Peroxynitrite Treatment of Microsomes—Reaction with PN was carried out with microsomes because active enzyme is required for nitration and further purification steps involving denaturating detergents partially inactivate the protein. PN (10 μl) at a defined concentration was quickly added by thorough Vortex mixing to an ice-cold microsomal suspension (990 μl, total protein concentration 1 mg/ml in 50 mM K-phosphate buffer, pH 7.5). Controls were treated with decomposed PN (24 h at room temperature).

Activity Test for 6-Keto-$PGF_{1\alpha}$—The activity for PGI_2 formation of PN-treated microsomes was tested by incubation of 100 μl of microsomal suspension (1 mg of protein/ml) in 100 mM potassium phosphate buffer, pH 7.4, with PGH_2 (1 μg) for 1 min at 20 °C. To avoid cross-reactivities with PGH_2 degradation products the incubation mixture was stopped with 20 μl of 1 M citric acid and extracted two times with 300 μl of ethyl acetate and separated by TLC (Silica Gel 60, Merck, Darmstadt, Germany); solvent: ethyl acetate:2,2,4-trimethylpentane: acetic acid:water, 10:50:20:100). 6-Keto-$PGF_{1\alpha}$ was identified by an iodine-stained reference (R_F value about 0.18). The area of 6-keto-$PGF_{1\alpha}$ was excised, extracted with ethyl acetate, and evaporated to complete dryness. After addition of 100 μl of PBS three dilutions of 1:100, 1:1000, and 1:10000 were prepared and tested by EIA (Assay Designs Inc., BioTrend, Köln, Germany) according to the manufacturer's protocol.

Western Blot Analysis—The microsomal samples were treated for 5 min at 95 °C with Laemmli buffer and separated by 8% (v/v) SDS-PAGE (30 mA, 1 h). The proteins were then transferred onto a nitrocellulose membrane by a semidry blot procedure using a constant current of 50 mA for 90 min. The blotting buffer contained 48 mM Tris, 39 mM glycine, 20% (v/v) methanol, and 0.037% (w/v) SDS. Transfer efficiency of proteins was examined by staining with 0.1% Ponceau S in 5% (v/v) acetic acid. After destaining in PBS, the membrane was blocked with 5% (w/v) milk powder in PBS, pH 7.4, for 2 h at room temperature or at 4 °C overnight. The membrane was then incubated for 2 h with a polyclonal antibody against PGI_2 synthase (1 μg/ml PBS). After repeated washing with PBS, 0.1% Tween 20 the membrane was incubated for 45 min with a horseradish peroxidase-conjugated goat anti-rabbit antibody at a dilution of 1:7500 for 45 min, and ECL was used for detection of antibody binding according to the manufacturer's instructions.

Prior to staining with a second antibody the membrane was stripped by incubation in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 2% (w/v) SDS, 100 mM 2-mercaptoethanol) under gentle shaking for 60 min at 70 °C. After washing and blocking, the membrane was incubated with a mouse monoclonal antibody against 3-NT at a dilution of 1 μg/ml, followed by a horseradish peroxidase-conjugated goat anti-mouse antibody at a dilution of 1:7500. PGI_2 synthase samples were always stained first with the PGI_2 synthase antibody, then stripped one or two times before staining with the NT antibody, to ensure complete denaturation for recognition of nitrated protein.

Isolation of Nitrated Prostacyclin Synthase—Separation of PGI_2 synthase from microsomal membranes and solubilization was performed by adding 1% (v/v) Triton X-100 to aortic microsomes. The suspension was stirred for 2 h at 4 °C, then centrifuged for 1 h at 100,000 × *g* to yield a clear yellow supernatant. Because SDS-PAGE is hampered by the high actin concentration (about 80–90% of total protein) in microsomes, actin was partially removed by precipitation with 15 mM $CaCl_2$ for 1 h at 4 °C and centrifugation of the precipitate for 5 min at 10,000 × *g*. Because Triton X-100 interfered with SDS-PAGE in subsequent purification steps, detergent was removed by extracting with chloroform and Vortex mixing for a few seconds (29). After centrifugation for 30 min at 3000 rpm, the organic and aqueous phases were recovered. Proteins precipitated at the interphase as a solid white layer, and remaining chloroform was removed by evaporation. Proteins were then solubilized in SDS-containing electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS).

Preparative SDS-PAGE—Proteins were treated with Laemmli sample buffer for 5 min at 95 °C and then separated by 10% (v/v) SDS-PAGE (100 mA, 5 h) on preparative gels (160 × 165 mm, thickness 1.5 mm). Reverse staining by imidazole:zinc was applied for visualization of bands (30). Gels were equilibrated for 15 min in 100 ml of 0.2 M imidazole in water with gentle shaking, and then placed for 1 min in 100 ml of 0.3 M $ZnCl_2$. The staining solution was removed when the background became deep white showing the transparent protein bands. The band containing PGI_2 synthase was excised with a razor blade, immersed in 2% citric acid solution 2–3 times (10 min) to remove zinc ions from the gel matrix, and washed several times with SDS-PAGE buffer.

Recovery of PGI_2 synthase was performed by electroelution (ELUTRAP, Schleicher & Schuell, Dassel, Germany) (31). Protein bands were cut to slices, placed in the elution chamber and covered with SDS-PAGE buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS). A voltage of 200 V corresponding to the ~50 mA current was then applied. After an 8-h elution period proteins accumulated inside a trap (volume 800 μl) between two membranes were collected with a pipette. Protein solutions were finally concentrated with an Ultrafree-4 Centrifugal filter unit (Millipore Corp.) to a 5 μM solution (0.28 mg/ml). Protein concentrations were determined with the Bio-Rad DC assay (Bio-Rad).

Pronase Digestion of Nitrated PGI_2 Synthase and HPLC Analysis of 3-Nitrotyrosine—Samples of 100 μl of electroeluted PGI_2 synthase were treated with different concentrations of PN (0, 10, 50, 100, and 250 μM), heated for 10 min to 95 °C, and then mixed with 10 mM $CaCl_2$ to stabilize proteases. After addition of Pronase (1 mg/ml) the samples were incubated for 24 h at 40 °C, then another 1 mg/ml Pronase was added and incubated again for 24 h at 50 °C. Digestion was repeated with a third and fourth portion (0.5 mg/ml) for 12 h at 50 °C. Prior to HPLC separation the samples were filtered with the 10-kDa MICROCON centrifugal filter device (Millipore Corp.) by centrifuging for 30 min at 10,000 × *g*. Products were analyzed on a Jasco HPLC system consisting of a PU-980 pump, a Jasco UV-1575 and Spectra Physics spectra focus UV-visible detector, and a LG-980-02 low pressure mixing unit. A C_{18} Nucleosil 100-5 250 × 4.6 column from Macherey & Nagel (Düren, Germany) was used with a mobile phase gradient (0–15 min, 0% (v/v) B; 15–30 min, 0–90% (v/v) B; 30–40 min, 90% (v/v) B (A: 0.1% (v/v) trifluoroacetic acid, pH 2, B: 80% (v/v) acetonitrile with 0.08% (v/v) trifluoroacetic acid)). The flow rate was 1 ml/min and sample aliquots of 100 μl were injected. Tyrosine, phenylalanine, tryptophan, and 3-NT were identified and quantified at 270 and 360 nm by internal and external standards. The retention time of 3-NT was 12 min. As a control 3-NT was reduced with sodium dithionite to 3-aminotyrosine.

Thermolysin Digestion of Nitrated PGI_2 Synthase and HPLC Analysis of Peptides—Because of the large amount of SDS in the electroeluted protein solution (>1% SDS) in-gel digestion was more suitable than digestion in solution. Protein solutions (about 0.5 nmol of PGI_2 synthase isolated from treated (25 μM PN) or untreated microsomes) were incubated in Laemmli buffer for 5 min at 95 °C and separated by SDS-PAGE on a "Novex" 8% Tris glycine gel (10 wells, Invitrogen; 30 mA, 1 h). Protein bands were visualized by reverse staining with imidazole:zinc as described above. Proteolytic digestion in the gel matrix was carried out according to the procedure of Shevchenko *et al.* (32). The protein bands were excised from the gel, cut to pieces and washed with 2% citric acid, then with water to remove staining dye, gel buffers, and SDS, and dried at room temperature in a vacuum centrifuge. The washing step was repeated by dehydration of the gel pieces and discarding the solu-

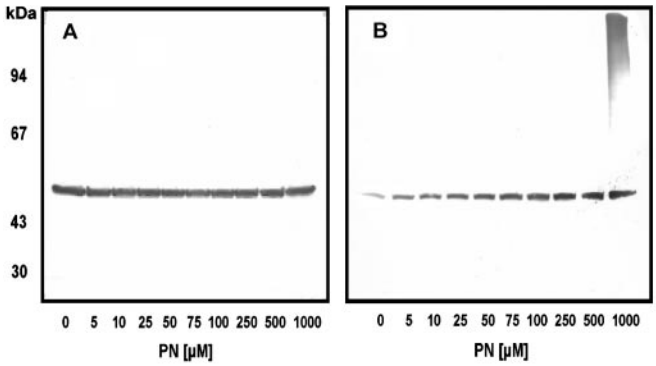

FIG. 1. **Western blot of aortic microsomes treated with increasing concentrations of PN.** *A*, immunodetection of PGI_2 synthase (52 kDa band) was performed with a polyclonal antibody against PGI_2 synthase. *B*, immunodetection of 3-NT-positive proteins was achieved with a monoclonal antibody against 3-NT from the same blot. Each lane of the preceding 8% Tris glycine gel contained 20 μg of protein.

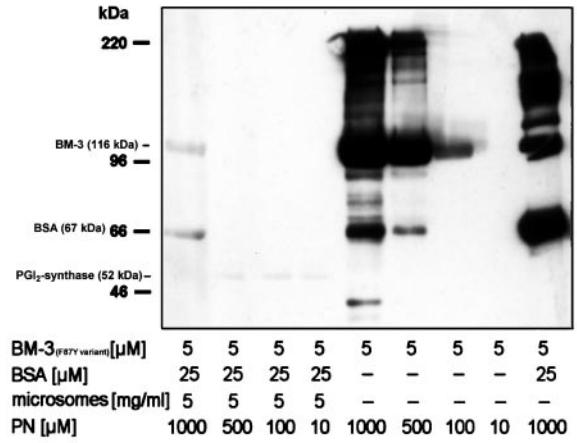

FIG. 2. **Effect of microsomal proteins on the nitration of BSA and $P450_{BM-3}$ by PN.** Anti-NT Western blot of $P450_{BM-3}$ (F87Y variant) (5 μM) and BSA (25 μM), which were treated with different concentrations of PN in the absence or presence of bovine aortic microsomes (5 mg/ml total protein). Protein loadings of the 8% Tris glycine gel were 3 μg of BM-3 for each lane, 8 μg of BSA, and 25 μg of microsomes according to their presence in the lane.

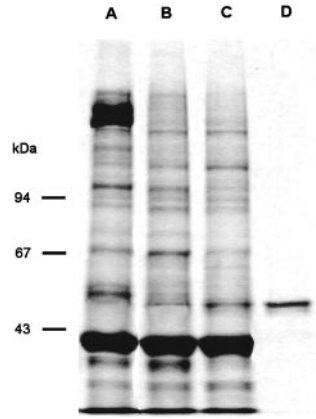

FIG. 3. **Isolation and purification steps of PGI_2 synthase on a Coomassie Blue-stained 8% Tris glycine gel.** *A*, microsomal fraction from bovine aorta. *B*, microsomes after solubilization with 1% Triton X-100. *C*, microsomes after precipitation with $CaCl_2$. *D*, PGI_2 synthase fraction after electroelution. *Lanes A–C* contained 35 μg of proteins; *lane D*, 3 μg of protein.

tion. After shrinking by vacuum centrifugation the gel pieces were reswollen in 200 μl of digest solution containing 50 mM Tris, pH 8.0, 5 mM $CaCl_2$, 10% (v/v) acetonitrile, and 25 ng/μl thermolysin, and the supernatant was removed. Proteolytic digestion was carried out for 24 h at 50 °C under gentle shaking. Peptides were extracted several times with 0.1% trifluoroacetic acid:acetonitrile for 24 h, lyophilized to dryness, and analyzed on the above described HPLC system. A C_{18} Nucleosil 100-3 125 × 4.6 column from Macherey & Nagel was used with a mobile phase gradient (0–5 min, 0% (v/v) B; 5–50 min, 0–60% (v/v) B (A: 0.1% (v/v) trifluoroacetic acid in water, pH 2, B: 80% (v/v) acetonitrile with 0.08% (v/v) trifluoroacetic acid)), at a flow rate of 0.8 ml/min. Peptide fragments were detected at 220 and 365 nm; peaks showing a strong absorption at 365 nm were collected and lyophilized for further analysis.

Sequence Determination—Sequence determination of the isolated peptide was achieved by Edman amino acid sequencing. NH_2-terminal Edman degradation was performed on an Procise HT sequencing system, model 494 (PerkinElmer Life Sciences, Weiterstadt, Germany), fitted with an online, narrow-bore HPLC-based amino acid analyzer that utilized a 220 × 2.1-mm C_{18} reversed-phase column held at 55 °C in a column heater oven. Released phenylthiohydantoin (PTH)-derivatives from each cycle were separated under the recommended binary gradient conditions using 3.5% tetrahydrofuran in water (buffered with sodium phosphate, pH 4.5; solvent A) and 10% 2-propanol in acetonitrile (unbuffered; solvent B). Prior to sequence determination, samples of peptides were applied to a biobrene-treated glass fiber disk and allowed to dry in a stream of argon. Reagents, operating software, and protocols were used as described from the instrument manufacturer. Chromatographic identification of the UV signals was done by reference to the retention times and the absorbance of a PTH standard run. PTH-derivatives display characteristic UV spectra with an absorbance maximum at 269 nm.

Peptide Synthesis—The nitrated tetrapeptide LKNY(nitro) was synthesized on a semiautomated peptide synthesizer (EPS-221, Abimed) using solid-phase peptide synthesis Fmoc chemistry methods (33) with all chemicals of analytical grade or highest available purity. Fmoc amino acids, NovaSyn TGR resin, PyBop, and other reagents were obtained from Novabiochem (Laufelfingen, Switzerland). To synthesize the peptide with COOH-terminal 3-NT carboxamide the TGR resin was employed with 40 min coupling time and 5 min deprotection in 20% piperidine in *N*,*N*-dimethylformamide. Purification of the peptide was performed with preparative HPLC on a Grom-Sil ODS-4Me column.

Mass Spectrometry—High resolution mass spectrometry was performed with a 7T Bruker Daltonik (Bremen, Germany) Apex II FT-ICR mass spectrometer equipped with an actively shielded 7.0 tesla superconducting magnet (Magnex, Oxford, UK), an APOLLO (Bruker Daltonik) electrospray ionization source and nano-electrospray system, an API1600 ESI control unit, and a UNIX based Silicon Graphics O_2 work station. Details of the instrumental conditions of ESI-FT-ICR-MS were as previously reported (34). The mass spectra were obtained by collecting 32–124 single scans. Experimental conditions were: full scan mode; 45–70 V capillary exit voltage; setting of skimmer 1, 10; setting of skimmer 2, 7; RF amplitude, 500; offset 0.9; trap, 10; extract, 10; ionization pulse time, 2500 ms; ionization delay time, 0.001 s; excitation sweep pulse 1, 2 ms; excitation sweep attenuation, 1:2.16 dB. Acquisition of spectra was performed with the Bruker Daltonik software XMASS and corresponding programs for mass calculation, data calibration, and processing. Peptide samples were dissolved in a solution of 3% acetic acid in 50% methanol:water.

MALDI-time of flight mass spectrometry was performed with a Bruker BiFlex-DE mass spectrometer equipped with a Scout MALDI source and video system, a nitrogen UV laser (337 nm), and a dual channel plate detector. Sample preparation was performed with 1 μl of a freshly prepared saturated solution of α-cyano-4-hydroxycinnamic acid in acetonitrile, 0.1% trifluoroacetic acid (2:1), which was mixed with 0.5 μl of the peptide solution (34). Spectra were recorded at an accelerating voltage of 25 kV and were averaged over 40 single laser shots.

RESULTS

Upon treatment of isolated PGI_2 synthase with PN no nitrated tryptic peptide(s) could be initially found although immunoprecipitation of the enzyme with a monoclonal NT antibody, as well as conventional acid hydrolysis of the nitrated enzyme, indicated the presence of nitrated tyrosine. In previous work with other P450 proteins a nitration with PN resulted in proteolytic peptides with characteristic absorbance at 365 nm, from which the position of the 3-NT could be identified (13, 14).

TABLE I

Expected cleavage pattern of PGI_2 synthase after complete thermolysin digestion

Only the expected fragments containing tyrosine are indicated. Moreover fragments representative of all identified Tyr-containing fragments by MALDI-TOF-MS and MALDI-FTI-CR-MS from several tryptic digestions are listed. Except for Tyr positions 430 and 446 all Tyr-containing peptides were found.

Possible Tyr-containing thermolysin fragments	Tyrosine position	Tryptic fragments found
78–84 LDPHS**Y**D	83	73–91 HVTVLLDPHS**Y**DAVVWEPR
98–99 A**Y**	99	92–106 SRLDFHA**Y**AVFLMER
110–123 VQLPH**Y**NPGDEKSK	115	107–121I FDVQLPH**Y**NPGDEK
140–143 M**Y**TN	141	132–145 ELQALTDAM**Y**TNLR
167–170 FS**Y**G	189	146–174 TVLLGDTVEAGSGWHEMGLLEFS**Y**GFLLR
175–177 AG**Y**	177	175–197 AG**Y**LTQYGVEAPPHTQESQAQDR
178–182 LTQ**Y**G	181	175–197 AGYLTQ**Y**GVEAPPHTQESQAQDR
256–259 LES**Y**	259	253–275 SRWLES**Y**LLHLEEMGVSEEMQAR
397–402 I**Y**TDPE	398	383–408 LLLFPFLSPQKDPEI**Y**TDPEVFKYNR
404–408 FK**Y**NR	406	383–408 LLLFPFLSPQKDPEIYTDPEVF**KY**NR
420–426 F**Y**KDGKR	421	409–422 FLNPDGSEKKDF**Y**K
427–436 LKN**Y**SLPWG	430	
442–446 LGKG**Y**	446	
478–482 LSR**Y**G	481	481–495 **Y**GFGLMQPEHDVPVR
494–497 VR**Y**R	496	481–497 YGFGLMQPEHDVPVR**Y**R

Because the postulated mechanism (35, 36) suggested that only active PGI_2 synthase can be nitrated, bovine aortic microsomes were first nitrated with increasing concentrations of PN and then the enzyme was isolated by gel electrophoresis (Fig. 1). Western blot analyses shown in Fig. 1 provided identical, specific bands at approximately 52 kDa up to a concentration of 500 μM PN that were stained by a polyclonal antibody against PGI_2 synthase, and a monoclonal antibody against 3-NT, whereas higher concentrations than 500 μM caused unspecific additional staining of other proteins. The control also stained weakly, which probably was because of the presence of some atherosclerotic plaques in bovine arteries (37).

A clear concentration dependence of PN on the extent of nitration was found up to 250 μM, which was at variance with the high affinity seen with the isolated enzyme (16, 17), but may be explained by competitive targets for PN in the microsomal fraction. Indeed, when aortic microsomes were added to P450 BM-3 (M_r 116,000) as a model protein for PGI_2 synthase its Tyr-nitration was sharply decreased (Fig. 2). Because the concentration of PGI_2 synthase on the gel is very low its nitration hardly shows. If microsomes were treated with 5,5′-dithiobis(2-nitrobenzoic acid) to block SH groups their inhibitory effect is much less (data not shown), indicating that in microsomes protein thiols compete for PN and therefore higher PN concentrations are required as with the isolated enzyme.

Previous studies with other P450 proteins and with model proteins (13, 14, 38) had indicated that multiple Tyr nitrations may occur; therefore, in this study a PN concentration of 25 μM was selected that appeared most suitable to yield selective modification of a single Tyr residue. The inhibition of 6-keto-$PGF_{1\alpha}$ formation was about 50 ± 15% compared with the inhibition at 250 μM, thus matching the NT staining intensities at 25 *versus* 250 μM. Treatment and isolation of bovine aortic microsomes under these conditions (see "Experimental Procedures") provided ~20 μg of PGI_2 synthase isolated on SDS-PAGE from the 52-kDa band (Fig. 3, *lane D*). The protein band was excised and subjected to proteolytic digestion using trypsin and mass spectrometric proteome analysis by MALDI-TOF as well as high resolution FT-ICR (39) (data not shown), which yielded unequivocal peptide fragment identification of the PGI_2 synthase sequence. However, no NT-containing peptides or other modified peptide sites were detected by these mass spectrometric data (see Table I). With low abundance, ATP synthase (56 kDa) and peptide ions because of additional (unidentified) contaminating proteins in very low amounts were found by protein sequence data base analyses (SwissProt data base; data not shown). The contaminating proteins were estimated to account for maximally 20–30% of the protein band (see Fig. 3).

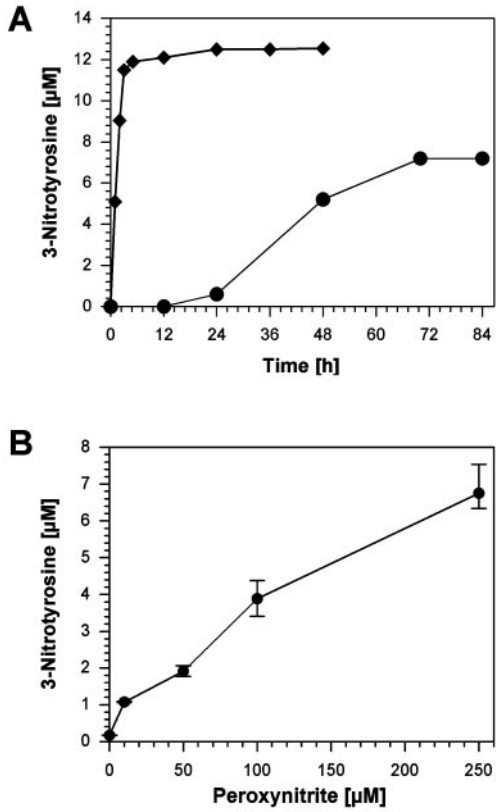

FIG. 4. **Time- and PN concentration-dependent 3-NT yield from nitrated BSA or PGI_2 synthase after digestion by Pronase.** *A*, BSA (20 μM) and PGI_2 synthase fraction (26 μg/100 μl, isolated from bovine microsomes after nitration), both nitrated by 250 μM PN, were treated with equal concentrations of Pronase (0.5 mg/ml every 12 h). 3-NT was detected and quantified by HPLC. *Diamonds* represent values for BSA, *circles* for PGI_2 synthase. *B*, the PGI_2 synthase fraction (26 μg/100 μl) was isolated from microsomes after treatment with different concentrations of PN. All samples were digested by Pronase for 5 days.

The isolated protein was subjected to Pronase digestion under conditions that should lead to quantitative release of the 3-NT residue for HPLC analysis. Initial digestion for 12 h provided positive Western blot staining with an NT antibody but did not yield detectable 3-NT by HPLC, although nitrated BSA as a reference protein was completely digested under

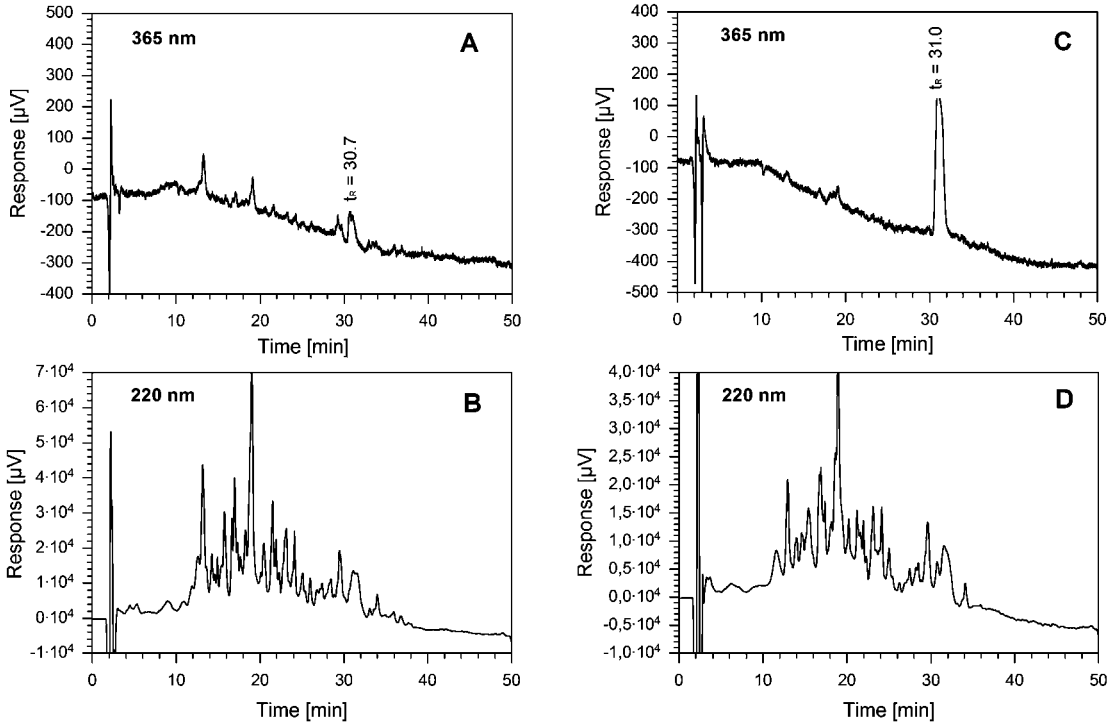

FIG. 5. **HPLC detection of NT-positive peptides in thermolysin digests of PN-treated microsomes.** *A* and *B*, HPLC of thermolysin-digested un-nitrated PGI_2 synthase monitored at 365 and 220 nm; *C* and *D*, nitrated PGI_2 synthase (25 μM PN) monitored at 365 and 220 nm. The fraction corresponding to the peak at 31.0 min was separated and used for sequencing and mass analysis.

these conditions (Fig. 4*A*). However, prolonged digestion for 72 h provided the complete liberation and HPLC detection of 3-NT after the lag phase of about 12 h, suggesting a decreased accessibility for degradation in the microenvironment of the nitration site (Fig. 4*A*). A quantitative estimation of 3-NT (Fig. 4*B*) yielded ~1.5 μM 3-NT at a PN concentration of 25 μM, with the assumption of a pure protein band of PGI_2 synthase. Correcting for 20–30% of contaminating proteins (ATP-synthase fragments were found) and a nitration of about 50% one could estimate a nitrotyrosine concentration of less than 2 μM with 25 μM PN-treated microsomes. This value would agree with the result in Fig. 4*B*, which also indicates that at higher PN concentrations (>100 μM) secondary Tyr nitrations may occur. Therefore, at 25 μM PN only the specific nitration site could be expected.

The isolated PGI_2 synthase was subjected to proteolytic digestion with trypsin under a variety of conditions, followed by mass spectrometric peptide mapping using both MALDI-TOF-MS and MALDI-FT-ICR-MS (Table I). These studies provided the detailed characterization of the primary structure of the protein; however, HPLC analysis of the digestion mixture did not result in any peptide with an absorption at 365 nm indicative for nitrated tyrosine. Experiments with other proteases (Asp-N, Lys-C, or pepsin) had also shown not to be successful. Based on the experience with Pronase digestion, thermolysin was then selected as a protease under comparatively extensive digestion conditions (24 h, 50 °C). At these conditions a distinct, abundant peak was found in the PN-treated protein by HPLC at 365 nm with a retention time of approximately 31 min (Fig. 5*C*). Typical peptide patterns were obtained at 220 nm, suggesting that a large portion of the protein had been digested (Fig. 5, *B* and *D*). A small peak was also observed in the untreated control enzyme, confirming a small basal nitration of PGI_2 synthase (Fig. 5*A*).

The HPLC-isolated peptide was analyzed by ESI-FT-ICR-MS (Fig. 6*A*) and Edman sequencing, which resulted in the unequivocal structure determination and identification of the nitration site. The ESI-FT-ICR mass spectrum yielded a single major protonated molecular ion at *m/z* 582.29073, corresponding to the monoisotopic composition of the tetrapeptide, LKNY(nitro)-COOH (PGI_2 synthase (427–430)); in addition a less abundant $(M + Na)^+$ was obtained. Several less abundant ions were also found indicating some contamination of the HPLC peak, but did not interfere with the precise mass determination of the tetrapeptide. The specificity of the FT-ICR-MS analysis was ascertained by comparison with all possible thermolysin fragments and their tyrosine-containing products, none of which could account for the MS data of the nitrated peptide (Table I). Additional proof for the nitration at Tyr-430 came from Edman microsequencing, which yielded the sequence LKN-Y(nitro), using 3-NT as a standard and the FT-ICR mass spectrum of the synthetic tetrapeptide in the carboxamide form (Fig. 6*B*).

DISCUSSION

In this study we present the definite, molecular identification of the nitration of PGI_2 synthase at tyrosine 430 as an unusual post-translational modification that occurs with the so far highest reported affinity for PN. Unequivocal identification of this nitrated peptide, as well as previously studied Tyr-nitrated peptides, was obtained by high resolution electrospray-FT-ICR-MS, in combination with microsequence analysis of 3-NT; in contrast, MALDI-MS of nitrated peptides yields extensive fragmentation by cleavage of the nitro group with elimination of NO and O, hence the resulting ions may obscure the assignment of nitration sites in complex proteolytic peptide mixtures (40). Although the specificity of the NT antibodies has been found sufficiently high to exclude cross-reactivity with other oxidatively modified amino acids, it was essential to define the tyrosine residue responsible for the inhibition of enzyme activity. The proteolytic degradation of the Tyr-nitrated domain was hampered by the high stability in the microenvironment at the nitration site, whereas the previously used acid hydrolysis is ambiguous in its specificity because

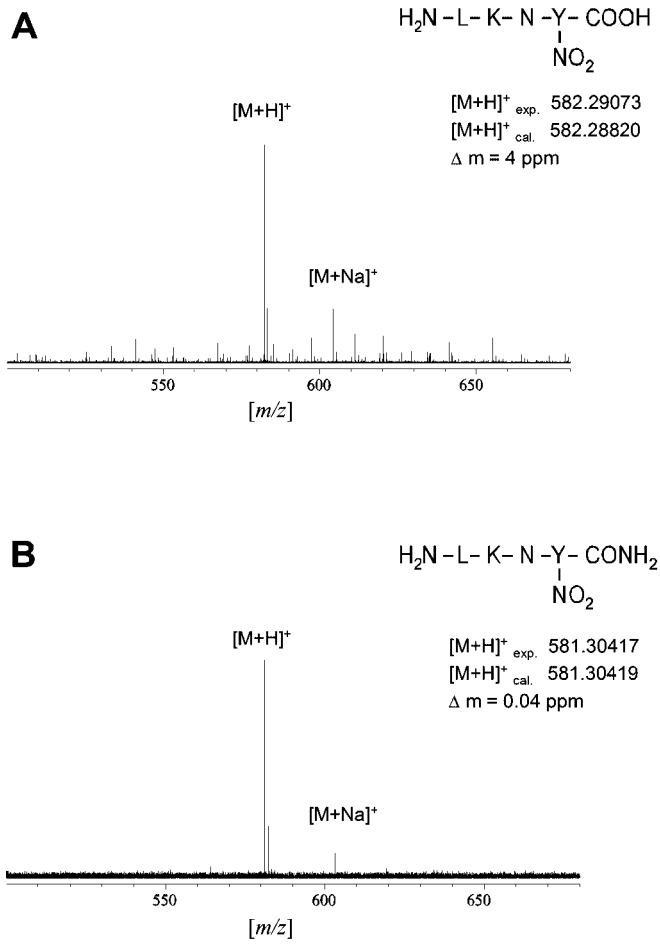

FIG. 6. *A*, nano-ESI-FT-ICR-MS spectrum of the isolated 365-nm absorbing peptide. The spectrum shows a major peak at 582.29073 atomic mass units. The calculated $(M + H)^+$ of the nitrated LKNY(nitro) peptide (582.28820) fits to the experimental data ($\Delta m = 0.00253$, 4 ppm). The peak at 604.27360 corresponds to the sodium adduct of the same nitrated peptide with quasimolecular ion $(M + Na)^+$. *B*, FT-ICR mass spectrum of the synthetic tetrapeptide in the carboxamide form LKNY(nitro)-CO-NH_2.

traces of nitrite may yield artifacts and false-positive results. Using prolonged Pronase digestion we could establish in this study a PN concentration-dependent increase of the 3-NT formation providing qualitative and quantitative evidence for the nitration reaction. By treatment of aortic microsomes with only 25 μM PN a specific nitration at Tyr-430 was established that was important in view of the possibility that at higher concentrations a heme-catalyzed formation of a ferryl complex and the $^{\cdot}NO_2$ radical may lead to secondary modification of other Tyr residues in PGI_2 synthase. Under the conditions employed for the nitration of PGI_2 synthase by PN only about half of the enzyme was nitrated as judged from the staining intensity in the Western blots and about half of the PGH_2 conversion to 6-keto-$PGF_{1\alpha}$ was lost.

From previous experiments with aortic microsomes (16) we also know that the untreated enzyme contains a basal level of about 5% nitration (100% activity) and that with 150–250 μM PN about 85% inhibition is reached. Higher PN concentrations did not result in more inhibition but from Fig. 4*B* a higher 3-NT formation is apparent that probably reflects Tyr residues other than Tyr-430. If only 25 μM PN at a given concentration of microsomal protein are used it is likely that only Tyr-430 is being nitrated. Indeed no other nitrated peptides were found. A direct correlation of the 50% inhibition of activity under these conditions with 50% of enzyme being nitrated is difficult to obtain, because the gel region of 52 kDa isolated contained not only PGI_2 synthase, as judged from other peptides (found *e.g.* from ATP synthase). But assuming about 70% purity the agreement is satisfactory (1.75 μM PGI_2 synthase for 1.5 μM 3-NT).

Our results provide definite proof for Tyr-nitration by PN that has been questioned repeatedly (9–12). The specificity of this post-translational modification may be provided by the heme catalysis involved that allows the exclusive nitration of Tyr residues closely located to the heme (17). The low levels of PN originating from the reaction of NO and O_2^- under cellular conditions may therefore be sufficient for this nitration reaction. It should be pointed out that PGI_2 synthase nitration occurs independently of myeloperoxidase/H_2O_2/NO_2^-, whereas a nitration of cyclooxygenase-2 has been found to also occur in a peroxidatic fashion autocatalyzed by the intrinsic peroxidase activity of the heme (15).

The most puzzling finding was the low accessibility of Tyr-430 that hampered the identification of its nitration for some time. The previously observed poor yield of sequence analysis of tryptic peptides (approximately up to 50% at best per experiment of the expected peptide yield) may be explained by the high resistance of the core protein to protease digestion. Accordingly, we postulate that Tyr-430 must be close to the heme because substrate analogs block the nitration; furthermore, because Cys-441 binds heme at its fifth coordination site the domain, Tyr-430 to Cys-441 is expected to form a loop around the heme and this structure may be embedded in a tightly folded conformation. This structure would explain the high resistance to proteases, and is probably also the reason for the incomplete transfer of the enzyme to blotting membranes and the difficulties in obtaining reproducible Western blots, caused by refolding on the blotting membrane. Also the incomplete incorporation of the heme into the protein in expression systems may be associated with a tight secondary structure at the active site.[2] Obviously mutation of the Tyr-430 residue should give further clues to the importance of this amino acid. Such experiments are ongoing but hampered by the difficulty of incorporating the heme. However, first data show that a substitution of Tyr-430 by phenylalanine does not block the activity suggesting that the nitro group may only block sterically access to the active site.[3] This would be in agreement with the observation that nitration could never block the activity by more than ~80% (17).

Our results also shed some light on the reactivity of PN in biological systems that has been a matter of debate in recent literature. Because of its short lifetime of less than 1 s under physiological conditions one cannot define an affinity for PN and a given target because the effective concentration for PN varies with other potential targets present and their concentration. Hence, using isolated PGI_2 synthase it was easy to obtain nitration and inhibition of activity at submicromolar levels of PN (16, 17), however, with a complex mixture of macromolecules present in microsomes substantially higher PN concentrations are required. This is evident from our data that show in microsomes no Tyr-nitration other than in PGI_2 synthase up to about 250 μM. By eliminating reactive thiol groups by 5,5′-dithiobis(2-nitrobenzoic acid) the sensitivity of PGI_2 synthase for nitration was increased. It is known that PN is an efficient oxidant for zinc finger proteins (41) and in the presence of metal ions further potential targets may compete for reaction with PN. Despite this straightforward explanation, the observation that micromolar PN can nitrate PGI_2 synthase in whole endothelial (17, 42) or mesangial cells (42) is not

[2] M. Wada and T. Tanabe, personal communication.
[3] M. Wada, T. Tanabe, and V. Ullrich, unpublished results.

readily understood. A possible clue to this phenomenon may be the recent observation that PGI_2 synthase is mainly located to the caveolae at the outer cell membrane (20). Thus, added PN may find PGI_2 synthase as a target before the complex and antioxidative environment of the cell will compete. The fact that endothelial NO synthase is found in the same compartment suggests that the generation of NO and its reaction with O_2^- is confined to a restricted area and low fluxes of both radicals may be sufficient to generate PN for the catalytic process of PGI_2 synthase nitration.

Recently we could demonstrate the nitration of PGI_2 synthase by endotoxin exposure of aortic rings and found nitration, loss of activity, and tissue contraction correlated (43). Inhibition of NO synthesis and the presence of superoxide dismutase could prevent all three effects.

Another still open question is the reversibility of the nitration. The presence of a denitratase has been postulated but could not yet be established (44, 45). From own observations[4] there may be indeed a recovery of PGI_2 synthase activity that is faster than new protein synthesis. If this would turn out as a process involving denitration a new redox-regulatory mechanism would be established. But even without this reversibility the superoxide-mediated trapping of NO with a concomitant down-regulation of PGI_2 synthase, followed by thromboxane A_2/PGH_2 receptor activation, represents a key event in our understanding of endothelial activation (5).

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REFERENCES

1. Cohen, R. A. (1995) *Prog. Cardiovasc. Dis.* **38,** 105–128
2. Moncada, S., and Vane, J. R. (1979) *Pharmacol. Rev.* **30,** 293–331
3. Moncada, S., and Vane, J. R., (1979) *N. Engl. J. Med.* **300,** 1142–1147
4. Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991) *Pharmacol. Rev.* **43,** 109–142
5. Zou, M. H., Jendral, M., and Ullrich, V. (1999) *Br. J. Pharmacol.* **126,** 1283–1292
6. Crow, J. P., and Beckman, J. S. (1995) *Adv. Pharmacol.* **34,** 17–43
7. Beckman, J. S., and Koppenol, W. H. (1996) *Am. J. Physiol.* **271,** C1424–C1437
8. Viner, R. I., Williams, T. D., and Schöneich, C. (1999) *Biochemistry* **38,** 12408–12415
9. Pfeiffer, S., and Mayer, B. (1998) *J. Biol. Chem.* **273,** 27280–27285
10. Pfeiffer, S., Schmidt, K., and Mayer, B. (2000) *J. Biol. Chem.* **275,** 6346–6352
11. Pfeiffer, S., Lass, A., Schmidt, K., and Mayer, B. (2001) *J. Biol. Chem.* **276,** 34051–34058
12. Pfeiffer, S., Lass, A., Schmidt, K., and Mayer, B. (2001) *FASEB J.* **15,** 2355–2364
13. Daiber, A., Schöneich, C., Schmidt, P., Jung, C., and Ullrich, V. (2000) *J. Inorg. Biochem.* **81,** 213–220
14. Daiber, A., Herold, S., Schöneich, C., Namgaladze, D., Peterson, J. A., and Ullrich, V. (2000) *Eur. J. Biochem.* **267,** 6729–6739
15. Heinz, K.; Dünstl, G., Bachschmid, M., Daiber, A., Nüsing, R., and Ullrich, V. (2002) *Nitric Oxide* **6,** 400
16. Zou, M. H., and Ullrich, V. (1996) *FEBS Lett.* **382,** 101–104
17. Zou, M. H., Martin, C., and Ullrich, V. (1997) *Biol. Chem.* **378,** 707–713
18. Zou, M. H., Klein, T., Pasquet, J.-P., and Ullrich, V. (1998) *J. Biochem.* **336,** 507–512
19. Ullrich, V., Zou, M. H., and Bachschmid, M. (2001) *Biochim. Biophys. Acta* **1532,** 1–14
20. Spisni, E., Griffoni, C., Santi, S., Riccio, M., Marulli, R., Bartolini, G., Toni, M., Ullrich, V., and Tomasi, V. (2001) *Exp. Cell Res.* **266,** 31–43
21. Hatae, T., Hara, S., Yokoyama, C., Yabuki, T., Inoue, H., Ullrich, V., and Tanabe, T. (1996) *FEBS Lett.* **389,** 268–272
22. Shyue, S., Ruan, K., Wang, L., and Wu, K. (1997) *J. Biol. Chem.* **272,** 3657–3662
23. Ullrich, V., and Brugger, R. (1994) *Angew. Chem. Int. Ed. Engl.* **33,** 1911–1919
24. Rossier, J. S., Youhnovski, N., Lion, N., Damoc, E., Reymond, F., Girault, H. H., and Przybylski, M. (2003) *Angew. Chem. Int. Ed. Engl.* **42,** 53–58
25. Kissner, R., Nauser, T., Bugnon, P., Lye, P. G., and Koppenol, W. H. (1997) *Chem. Res. Toxicol.* **10,** 1285–1292
26. Graham-Lorence, S., Truan, G., Peterson, J. A., Falck, J. R., Wei, S., Helvig, C., and Capdevila, J. H. (1997) *J. Biol. Chem.* **272,** 1127–1135
27. Siegle, I., Nüsing, R., Brugger, R., Sprenger, R., Zecher, R., and Ullrich, V. (1994) *FEBS Lett.* **347,** 221–225
28. Ullrich, V., Castle, L., and Weber, P. (1981) *Biochem. Pharmacol.* **30,** 2033–2036
29. Horikawa, S., and Ogawara, H. (1979) *Anal. Biochem.* **97,** 116–119
30. Fernandez-Patron, C., Castellanos-Serra, L., and Rodriguez, P. (1992) *BioTechniques* **12,** 564–573
31. Jacobs, E., and Clad, A. (1986) *Anal. Biochem.* **154,** 583–589
32. Shevchenko, A., Jensen, O. N., Podtelejnikov, A. V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Shevchenko, A., Boucherie, H., and Mann, M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14440–14445
33. Michels, J., Geyer, A., Mocanu, V., Welte, W., Burlingame, A. L., and Przybylski, M. (2002) *Protein Sci.* **11,** 1565–1574
34. Bauer, S. H., Wiechers, M. F., Bruns, K., Przybylski, M., and Stuermer, C. A. (2001) *Anal. Biochem.* **298,** 25–31
35. Mehl, M., Daiber, A., Herold, S., Shoun, H., and Ullrich, V. (1999) *Nitric Oxide* **3,** 142–152
36. Zou, M. H., Daiber, A., Peterson, J. A., Shoun, H., and Ullrich, V. (2000) *Arch. Biochem. Biophys.* **376,** 149–155
37. Zou, M. H., Leist, M., and Ullrich, V. (1999) *Am. J. Pathol.* **154,** 1359–1365
38. Jiao, K., Mandapati, S., Skipper, P. L., Tannenbaum, S. R., and Wishnok, J. S. (2001) *Anal. Biochem.* **293,** 43–52
39. Fligge, T. A., Reinhard, C., Harter, C., Wieland, F. T., and Przybylski, M. (2000) *Biochemistry* **39,** 8491–8496
40. Sarver, A., Scheffler, N. K., Shetlar, M. D., and Gibson, B. W. (2001) *J. Am. Soc. Mass. Spectrom.* **12,** 439–448
41. Kröncke, K.-D., Klotz, L.-O., Suschek, C. V., and Sies, H. (2002) *J. Biol. Chem.* **277,** 13294–13301
42. Zou, M., Yesilkaya, A., and Ullrich, V. (1999) *Drug Metab. Rev.* **31,** 343–349
43. Bachschmid, M., Thurau, S., Zou, M. H., and Ullrich, V. (2003) *FASEB J.*, in press
44. Kamisaki, Y., Wada, K., Bian, K., Balabanli, B., Davis, K., Martin, E., Behbod, F., Lee, Y. C., and Murad, F. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 11584–11589
45. Bian, K., and Murad, F. (2001) *Free Radical Biol. Med.* **31,** 421–429

[4] P. Schmidt, M. Bachschmid, and V. Ullrich, unpublished data.

Specific Nitration at Tyrosine 430 Revealed by High Resolution Mass Spectrometry as Basis for Redox Regulation of Bovine Prostacyclin Synthase

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