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Ebselen as a Peroxynitrite Scavenger *In Vitro* and *Ex Vivo*

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ABSTRACT. We have previously shown that peroxynitrite (PN) selectively impaired prostacyclin (PGI_2)-dependent vasorelaxation by tyrosine nitration of PGI_2 synthase in an *in situ* model (Zou MH, Jendral M and Ullrich V, *Br J Pharmacol* **126:** 1283–1292, 1999). By using this established model, we tested whether or not ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one), which reacts rapidly with the anionic form of PN, affected PN inhibition of PGI_2 synthase. Administration of ebselen (1 to 50 μM) to bovine coronary strips 5 min prior to PN (1 μM) treatment neither prevented PN-triggered vasoconstriction nor the inhibition of PGI_2 release. In line with these results, ebselen affected neither PN inhibition of the conversion of [^{14}C]-PGH_2 into 6-keto-$PGF_{1\alpha}$ nor the nitration of PGI_2 synthase in bovine aortic microsomes. Following the hypothesis that a reaction of ebselen with cellular thiols could have caused the inefficiency of ebselen, we observed that free ebselen quickly reacted with thiols in both coronary strips and in aortic microsomes to form two metabolites, one of which was identified as the ebselen–glutathione adduct, whereas the other had a similar retention time to that of the ebselen–cysteine adduct. The nitration of phenol by PN in a metal-free solution could be blocked more efficiently in the presence of ebselen or glutathione alone than in the presence of both, indicating that like selenomethionine and other selenocompounds, ebselen–thiol adducts were less reactive towards PN than ebselen itself. Further evidence came from the results that ebselen became effective in preventing the inhibition and nitration of PGI_2 synthase after thiol groups of microsomal proteins were previously oxidized with Ellman's reagent. We conclude that in cellular systems ebselen is present as thiol adducts and thus loses its high reactivity towards PN, which is required to compete with the nitration of PGI_2 synthase. BIOCHEM PHARMACOL **59**;2: 153–160, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. prostacyclin synthase; tyrosine nitration; glutathione adduct; selenocompounds; antioxidants

NO† has been established as a potent messenger in biological signaling pathways which are mostly linked to a down-regulation of cellular activity [1, 2]. Due to its almost diffusion-limited reaction with superoxide (O_2^-) [3, 4], this radical becomes an efficient antagonist of NO. Whereas each radical alone has rather weak oxidizing properties, the resulting product, PN, has been described as a strong oxidizing species either by one-electron acceptance or as an oxene donor equivalent to a two-electron acceptor. It is mainly the chemical structure of the reactant that will determine the mechanism by which PN will oxidize a given substrate. Since PN can be formed in biological systems, its reactivity with cellular constituents has often been investigated, and many destructive reactions with macromolecules such as DNA [5], lipids [6], or proteins [7] have been reported (for review see [8, 9]). Typical reactions of proteins include thiol and methionine oxidations [10, 11] or tyrosine nitrations [12, 13], which have been observed under pathological conditions such as tissue graft rejection [14], ischemic events [15], or neurodegenerative diseases [9]. Therefore, efforts have been undertaken to block PN reactions either by preventing its formation or by quenching it with antioxidants [16–18]. Indeed, several reports on efficient scavengers for PN suggest that antioxidants are promising tools for suppressing PN-mediated oxidations and could be potential drugs against its presumed damaging effects in tissues [19].

We recently reported a very sensitive inhibition by PN of PGI_2 synthase, which was paralleled by a tyrosine nitration at the active site [13, 20]. Using intact cells and tissues, this modification could also be observed *ex vivo* [21], which at first sight seemed incompatible with the fact that the high cellular antioxidant potential should have eliminated such low PN concentrations already effective as 0.1 μM bolus additions. A closer investigation into the chemical mechanism of tyrosine nitration of PGI_2 synthase revealed a catalytic action of its heme active site, allowing the PN anion to directly and rapidly form the nitrating species [22]

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† *Abbreviations:* PN, peroxynitrite; PGI_2, prostacyclin; PCS, prostacyclin synthase; NO, nitric oxide; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent); PGH_2, 15-hydroxy-11 alpha, 9 alpha-(epoxymethano)prosta-5, 13-dienoic acid; A II, angiotensin II; PMSF, phenylmethanesulfonyl fluoride; ECL, enhanced chemiluminescence; and LPS, lipopolysaccharide.

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and not be converted to its excited protonated state, which is considered to be the active form of PN in the absence of transition metals [3, 23]. This could also explain the failure of antioxidants such as ascorbate, glutathione, vitamin E, or uric acid to block the inhibition of PGI_2 synthase by PN in rat mesangial cells [21]. As a result of such findings, we set out to look for inhibitors that directly attack and quench the PN anion in order to compete with PGI_2 synthase. An ideal candidate should be the drug ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one), since its second-order reaction constant with PN was found to be $2*10^6$ $M^{-1}s^{-1}$ [24] and it should therefore be able to effectively block the inhibition of PGI_2 synthase *in vitro* and *in vivo*. However, we report herein that such expectations were not fulfilled in an *ex vivo* model which, on the other hand, provided interesting insights into the mechanism of ebselen action.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma or Fluka at the highest purity available. Ebselen was a gift from Prof. Wendel (University of Konstanz). Bovine aortic microsomes were prepared as described in [25]. PN was prepared from NO and KO_2 according to Koppenol and Kissner [4]. Two HPLC systems were used: 1) Jasco PU-980 pump, UV-975 variable-wavelength detector, and LG-980-02 low-pressure gradient mixer; and 2) LKB 2150 pump, Spectra Physics spectra focus detector, and SP4290 integrator. In both systems solvents were gassed with helium. Kinetics were recorded on an Aminco DW-2 UV/Vis spectrophotometer in the dual-wavelength mode, equipped with a magnetic stirrer.

Separation of Ebselen, GSH–Ebselen, and Ebselenoxide

System I was used with a Nucleosil 300-5 C_4 column 250*4.6 (Macherey–Nagel), and the mobile phase consisted of 80% 0.1 M K-phosphate buffer pH 4.5 and 20% acetonitril. The flow was 1.2 mL/min and the products were detected at 270 nm. Spectra were taken from each peak. Solutions of 500 μM ebselen (from a 10-mM stock solution of ebselen in acetonitril) in 0.1 M K-phosphate buffer at pH 7 and 9 were treated with different concentrations of GSH (0.05–5 mM) and were then reacted with 500 μM PN. Fifty microliters of each sample were injected on the HPLC.

Determination of Free Ebselen Concentrations in Bovine Aortic Microsomes and Tissue-containing Solutions

System II was used with a Nucleosil 300-5 C_4 column 250*4.6 (Macherey–Nagel), with the mobile phase consisting of 80% 0.1 M K-phosphate buffer pH 4.5 and 20% acetonitril for the tissue-samples (1) and 75% water and 25% acetonitril with 0.1% added trifluoroacetic acid in the microsomal experiments (2). The flow was 1.2 mL/min and the products were detected at 270 nm. (1) Ebselen (4.3 μM) was incubated with 0.134 g dry weight (first sample) and 0.077 g (second sample) of bovine coronary tissue in 10 mL PBS buffer and aliquots of 50 μL were taken after 1, 15, 30, and 35 min and injected on HPLC. Ebselen and GSH–ebselen were identified by external standards. (2) Five hundred microliters of bovine aortic microsomes (1 mg/mL protein) was incubated with 500 μL of 1 mM DTNB for 10 min in 0.2 M K-phosphate buffer pH 7.5. The solution turned yellow, indicating the reaction of DTNB with free thiol groups. After centrifugation, the supernatant was removed and 1 mL fresh K-phosphate buffer was added. The sample was vortexed and then 20 or 50 μM ebselen was added and incubated for 5 min. Fifty microliters of this solution was injected on HPLC. The same procedure was performed for the non-DTNB-treated microsomes. Concentrations of ebselen were quantified by external standards.

Protective Effects of Ebselen, GSH, and Ebselen-GSH on Phenol and BSA Nitration

Solutions of 5 mM phenol in 0.1 M K-phosphate buffer at pH 7 or 9 were supplemented with ebselen, GSH, or both, and were reacted with 1 mM PN. The yield of nitrophenols (pH 7) or 4-nitrosophenol (pH 9) was determined from the absorption at 400 nm (after addition of NaOH(aq)). PN and ebselen decomposition kinetics were recorded at 302 against 370 nm. At this wavelength, both PN and ebselen absorb, but not ebselenoxide, and the ebselen–GSH adduct has a very low absorption. PN was always added last to the stirred solution in the cuvette by a syringe through a septicum. The decomposition of 100 μM PN and 50 μM ebselen with 0, 20, or 40 μM BSA in 0.2M K-phosphate buffer at pH 10 was recorded as was the decomposition of 250 or 500 μM PN and 200 μM ebselen with 0, 200, and 400 μM GSH in 0.2 M K-phosphate buffer pH 8, 9, and 10.

Ebselen Effects on PN-Treated Bovine Coronary Arteries

Bovine hearts were obtained from the local slaughterhouse. The epicardial coronary arteries of the left ventricle were quickly dissected, cleaned of adhering fat and connective tissues, and placed in an ice-cold Krebs-bicarbonate solution consisting of (in $mmolL^{-1}$): NaCl 118; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25; EDTA 0.016; glucose 11.1. The tissues were kept in ice-cold Krebs buffer for up to 24 hr with frequent buffer changes. For the isometric measurement of tension in bovine coronary arteries, a spiral (20 × 5 mm) cut from bovine coronary arteries was suspended in a tissue bath filled with 15 mL of Krebs buffer, gassed with 95% O_2, 5% CO_2 (37°; pH 7.4) and attached to a force–displacement TFT6V5 transducer coupled to a polygraph for the measurement of isometric tension. Passive tension was adjusted to approximately 5 g over a 30-min equilibrating period and then coronary arteries were constricted by addition of the thromboxane

mimetic U46619 (0.001 ~ 0.01 $\mu molL^{-1}$). The vasorelaxation after acetylcholine (1 $\mu molL^{-1}$) was used to demonstrate a functional endothelium. Throughout the experiment, care was taken to avoid any injury to the endothelium. After rinsing the tissue with fresh buffer and allowing the mechanical tension to obtain a steady-state value, a reference response of vasocontraction–relaxation was obtained for each spiral by addition of A II (50 $nmolL^{-1}$). Thirty minutes after A II stimulation, the medium was collected for prostanoid analysis and the tissue was treated with $ONOO^-$ as follows: the vessel was placed into a 2-mL Eppendorff tube containing 0.9 mL prewarmed Krebs buffer with 0.1–50 mM ebselen mixed thoroughly with 0.1 mL $ONOO^-$ (0.1 to 10 mM) to give the indicated concentration of $ONOO^-$, and was quickly and thoroughly mixed. Control vessels were treated with the same volume of alkaline Krebs buffer medium or decomposed $ONOO^-$ (24 hr at room temperature). Two minutes after treatment, the tissue was resuspended in the organ bath without adjusting the tension. Where indicated, the pharmacological agents indomethacin, SQ29548, CGS 13080, and U51605 were added to the organ bath immediately after treatment. After 60 min of re-equilibration, the tissue was stimulated with the same concentration of A II for another 30 min. At the end of the experiment, vessels were collected for immunoprecipitation and incubation media were collected and stored at −20° for prostaglandin and thromboxane analysis. The percentage of vasoconstriction/relaxation was calculated as the percentage of relaxation in the secondary stimulation with A II relative to that in the first stimulation. Similarly, the amounts of prostanoids were expressed as the percentage of prostanoids formed by the second stimulation compared to those after the first stimulation with A II. In order to evaluate the effects of $ONOO^-$ on the receptor-dependent or receptor-independent vasoconstriction mediated by U46619, PGH_2, or KCl, a reference contractile response for each compound was initially obtained for each spiral. Subsequently, the tissues were treated with $ONOO^-$ or alkaline Krebs solution as described above and then resuspended in the organ bath containing fresh Krebs buffer. Once the tension had returned to baseline, the vessels were treated by addition of the same concentration of U46619 (5×10^{-8} $molL^{-1}$), PGH_2 (10^{-6} $molL^{-1}$), or KCl (6×10^{-4} $molL^{-1}$). Agonist-induced vasconstriction of coronary arteries was calculated as the percentage of vasoconstriction in the secondary stimulation compared to the reference contractile response.

Extraction and Analysis of Prostanoids

After acidification with 1 N HCl to pH 3.5, the collected medium was extracted with 3 volumes of ethyl acetate. The organic phases were collected and evaporated to dryness under nitrogen. Samples were resuspended in PBS containing 0.1% BSA. Prostanoids and thromboxane B_2 were analyzed by enzyme-linked immunoassay kits, according to the instructions provided by the supplier.

Activity Assay of PGI_2 Synthase

Bovine aortic microsomes were treated with the indicated concentrations of $ONOO^-$ and were quickly and thoroughly mixed. Two minutes after treatment, 100 $\mu molL^{-1}$[^{14}C]-PGH_2 was added and the samples were incubated for an additional 3 min. The reaction was stopped by acidification with 1 N HCl to pH 3.5. The incubation media were extracted with 3 volumes of ethyl acetate and after centrifugation, the organic phases were evaporated to dryness under nitrogen. Samples were then resuspended in 60 μL of ethyl acetate and subsequently separated by TLC (ethyl acetate/water/isooctane/acetic acid; 90:100:50:20). Prostanoids were quantified with a phosphor imager system (Image Quant, Molecular Dynamics) as previously described.

Immunoprecipitation of 3-Nitrotyrosine-containing Proteins

Bovine aortic microsomes (5 mg/mL) were diluted (1 to 1) in 50 $mmolL^{-1}$ Tris buffer containing 1 $mmolL^{-1}$ PMSF, 5 $mmolL^{-1}$ EDTA, 150 $mmolL^{-1}$ NaCl, and 0.5% Nonidet P-40 (pH 8.0) and for 30 sec in a Branson Sonifier 250 (Schwäbisch Gmünd), setting 5, at 50% duty cycle and centrifuged (14,000 *g*) at 4° for 5 min to remove cellular debris. Protein concentrations were determined using the Bradford assay. Solubilized proteins (3 mg) were precleared by addition of 40 μL of protein A-Sepharose CL-4B and the supernatant was incubated (18 hr, 4°) with 10 μg of monoclonal anti-nitrotyrosine antibody (Upstate Biotechnology). Immune complexes were precipitated with 30 μL of protein A CL-4B and washed with 0.5 mL SNNTE (0.5% sucrose, 1% Nonidet P-40, 0.5 $molL^{-1}$ NaCl, 50 $mmolL^{-1}$ Tris, and 5 $mmolL^{-1}$ EDTA, pH 7.4). Resuspended protein pellets were then precipitated by centrifugation (14,000 *g*, 1 min) and resuspended in 40 μL Laemmli sample buffer/β-mercaptoethanol (9:1), heated at 95° for 5 min and kept on ice.

Western Blots

Protein precipitates were separated by 7.5% SDS–PAGE (30 mA, 1 hr) and blotted for 1 hr with a constant current of 200 mA onto a nitrocellulose membrane in a semidry blot procedure (48 $mmolL^{-1}$ Tris/ 39 $mmolL^{-1}$ glycine/ 20% methanol/ 0.037% SDS). Proteins were visualized with a 0.1% Ponceau S solution in 5% acetic acid to check transfer efficiency. After destaining, the membrane was blocked with 5% milk powder in PBS/ 0.1% Tween 20 for 2 hr at room temperature and incubated with a polyclonal antibody directed against PGI_2-synthase [13, 20] (1 μg/mL) overnight at 4°. After washing several times with PBS/0.1% 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 visualized by the ECL technique, according to the instructions of the supplier (Amersham).

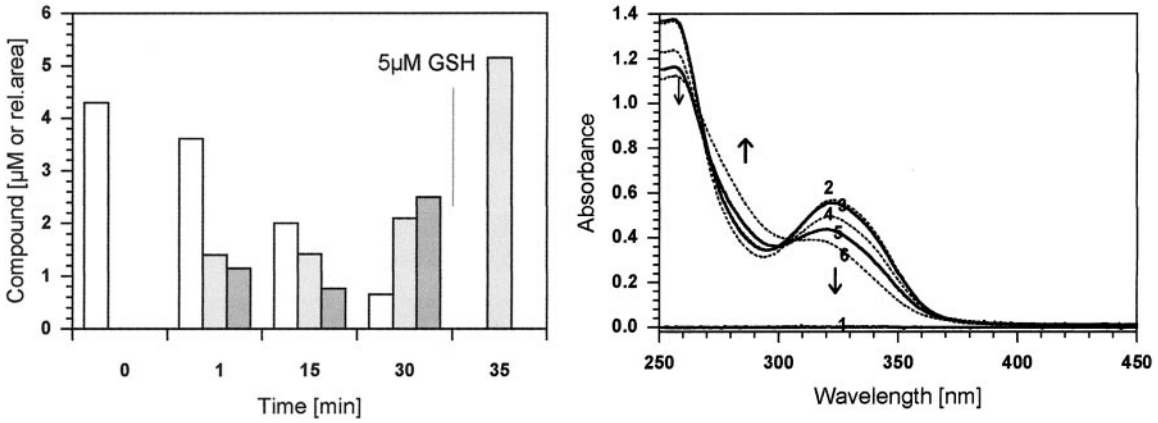


FIG. 1. (A) Time-dependent loss of 4.3 µM "free" ebselen (white bars) in a solution containing 0.134 g (dry weight) bovine coronary tissue and parallel formation of the ebselen–GSH adduct (grey, area/1000) and an unknown product (black, area/1000) (similar retention time as the ebselen–cysteine adduct). Thirty minutes after addition of 5 µM GSH, all the ebselen and the unknown product were converted to the ebselen–GSH adduct. Separation and quantification was done by HPLC. This diagram shows the results of one out of two almost identical experiments. (B) UV/Vis spectra of 100 µM ebselen incubated for 5 min with 0 (2), 50 (3), 100 (4), 200 (5), and 400 µM (6) GSH in 0.2 M K-phosphate buffer pH 7. (1)-buffer without ebselen and GSH.

The blots which had been stained with a polyclonal antibody against PGI_2 synthase were then stripped by incubating the membrane in stripping buffer (100 mmolL^{-1} 2-mercaptoethanol, 2% SDS, 62.5 mmolL^{-1} Tris–HCl, pH 6.7) at 50° for 30 min with agitation. The membranes were washed, blocked, and incubated overnight at 4° with a monoclonal antibody against 3-nitrotyrosine at a dilution of 1 µg/mL and with a goat anti-mouse antibody for 45 min at a dilution of 1:7500. Antibody binding was visualized as described above.

RESULTS

Effects of Ebselen on PN Action in Bovine Aortic Rings

PGI_2 is an effective mediator and relaxant of vascular tone and can be studied via its pharmacological actions in bovine aortic rings [26]. Using this model, we showed a sensitive inhibition by PN of the relaxation phase after angiotensin II stimulation of this tissue, paralleled by a decrease in PGI_2 formation [27]. In this *ex vivo* model of PGI_2 action, it was also possible to measure decreased PGI_2 levels as a consequence of PGI_2 synthase inhibition by PN and by parallel immunoprecipitations to quantitate the amount of nitrated enzyme. In a similar series of experiments, we used LPS as a presumed trigger of PN and indeed were able to mimic the effects of PN in this *in situ* model of vascular tone (unpublished result). When looking for a pharmacological tool to inhibit PN action, we knew that the selenocompound ebeselen had been reported to react with PN at a second-order rate constant of $2.*10^6$ M^{-1} sec^{-1} [24], suggesting that it might be an efficient scavenger. However, additions of up to 10 µM ebselen to aortic rings failed to show any effect (results not shown). On the basis of the reported kinetic constants, 10 µM ebselen should have been sufficient to quench the inhibitory action of PN at the submicromolar level. A possible explanation for the lack of effect of ebselen could have been its reaction with sulfhydryl groups in the tissue during the preincubation phase, as was the case for albumin [28]. This was tested by setting up an HPLC system for the detection of ebselen and its possible reaction products.

The Fate of Ebselen in Tissues

The fact that ebselen forms adducts with thiols, especially glutathione (GSH) [29], could have been responsible for the lack of the expected ebselen effect. Indeed, ebselen disappeared from the bath solution time dependently and two metabolite peaks were formed concomitantly, as seen in Fig. 1A. According to the optical spectra, the band with a retention time of 7 min proved to be identical to the spectra of the ebselen–GSH adduct as seen from a titration of ebselen with GSH (Fig. 1B). The second metabolite was not identified, but it did have a similar retention time to that of the ebselen–cysteine adduct and disappeared after excess addition of GSH to the bath solution. Not only GSH but also reactive thiols in proteins can bind ebselen, as had been shown for albumin and lipoate [28, 29]. Using a microsomal fraction from aortic rings, it was possible by HPLC to observe a decrease in "free" ebselen after addition of this membrane fraction (Expt 1:50 mM to 13; Expt 2:20 mM to 0). However, if the protein-bound thiols had been oxidized with Ellman's reagent (DTNB), more ebselen was recovered (Expt 1:50 mM to 38; Expt 2:20 mM to 4 mM). Since this microsomal fraction also contained PGI_2 synthase, it was possible to conduct an experiment with PN and ebselen with and without previous treatment with DTNB. Additions of 1 and 10 µM PN caused the expected inhibitions of 6-keto $PGF_{1\alpha}$ formation in the presence of [^{14}C]-PGH_2, with DTNB having no significant modulatory effect. However, when 10 µM ebselen was added before PN, the inhibition was largely prevented (Table 1). A qualitative confirmation of this result was obtained by

TABLE 1. Protection of prostacyclin synthase from inhibition by peroxynitrite in DTNB-treated and non-treated bovine aortic microsomes

Microsomes (PCS)	% inhibition of PCS (control)	% inhibition of PCS (DTNB)
+ 1 μM PN	57.3 ± 7.1	50.4 ± 6.4
+ 10 μM PN	73.6 ± 6.9	64.2 ± 8.7
+ 1 μM PN + ebselen	55.4 ± 5.6	11.3 ± 4.7
+ 10 μM PN + ebselen	74.1 ± 6.9	19.9 ± 5.6

Effect of ebselen on peroxynitrite inhibition of the conversion of [^{14}C]-PGH_2 into 6-keto-PGF1α in bovine aortic microsomes with and without DTNB treatment. One hundred micrograms of bovine aortic microsomes was treated with the indicated concentrations of PN 10 min after the addition of ebselen or ebselen plus DTNB. Five minutes after PN treatment, 100 μM[^{14}C]-PGH_2 was added and further incubated for 3 min. The reaction was stopped by acidification to pH 3.5. Prostanoids were extracted and analyzed as described under Methods. Results are means ± SEM of seven separate experiments.

immunoprecipitation experiments with a monoclonal anti-nitrotyrosine antibody [20, 27]. Without DTNB pretreatment of aortic microsomes, 1 and 10 μM PN caused a massive nitration not influenced by ebselen (Fig. 2B) whereas the DTNB-mediated modification of microsomes allowed ebselen to partially trap PN, leading to a diminished nitration of PGI_2 synthase (Fig. 2A). It is interesting to note that control microsomes already contained a certain amount of nitrated enzyme, as was consistently observed in previous experiments [13, 20, 21].

The Reaction of PN with Ebselen in the Absence and Presence of Thiols

Ebselen and the PN anion rapidly react to give the corresponding selenoxide and nitrite [30]. When this reaction was carried out at pH 9, the stability of PN was high enough to lead to an almost quantitative conversion of ebselen to its selenoxide after equimolar mixing with PN. Only a minor amount of ebselen remained, due to some isomerization of PN to nitrate [3] or its dismutation to nitrite and dioxygen [31] (Fig. 3). At pH 7, only about 50% of the ebselen was converted to the oxide. When the same experiment was carried out when GSH was added at a slight molar excess over ebselen 1 min before PN, only a small amount of ebselen oxide appeared at pH 7 and a larger amount at pH 9 (Fig. 3). The peak of the ebselen–GSH adduct formed under these conditions suggested that this adduct does not undergo a rapid reaction with PN to its corresponding selenoxide. At pH 9, a small peak with a lower retention time than the ebselen–GSH adduct could be consistent with such a selenoxide, confirming the better stability of PN at pH 9, which allowed formation of this product at pH 9 but not at pH 7. Such results point to a slower reaction of PN with the ebselen–GSH adduct than with ebselen itself. Direct kinetic measurements are difficult to perform since the absorbancies of PN and that of the adduct are in the same region, i.e. between 290 and 340 nm. However, when choosing 320/370 nm as a wavelength pair and pH 10 when the stability of PN is high enough, one can observe kinetics of PN decomposition with ebselen (Fig. 4). At the same wavelength pair, a preincubated equimolar mixture of ebselen and GSH decomposes PN with slower kinetics, although the maximum absorbance change is somewhat larger (Fig. 4A). This is at least a qualitative confirmation of the slower reaction of the adduct. As a control, a secondary addition of PN resulted only in a minor change due to the absorbance of PN at 302 nm [32]. A corresponding experiment was performed with the ebselen–albumin adduct (Fig. 4B). Additions of 20 and 40 μM albumin to 100 μM ebselen at pH 10 slowed down the kinetics of the ebselen reaction. Again, a second addition of PN did not alter the absorbance difference significantly, proving that in the initial reaction the disap-

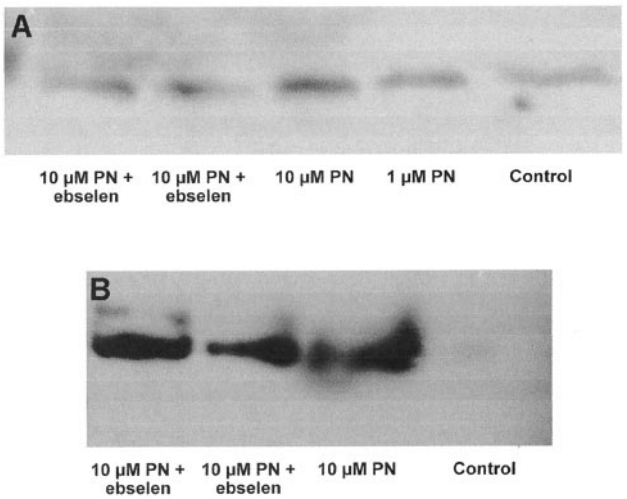


FIG. 2. Western blot analysis of the peroxynitrite-mediated nitration of prostacyclin synthase in bovine aortic microsomes. Bovine aortic microsomes were treated with the indicated concentrations of PN in the presence (A) or absence (B) of DTNB. Nitrated PGI_2 synthase was immunoprecipitated with a monoclonal antibody against 3-nitrotyrosine and immunoblotted with polyclonal antibodies against 3-nitrotyrosine or against PGI_2 synthase. Results are representative of four experiments.

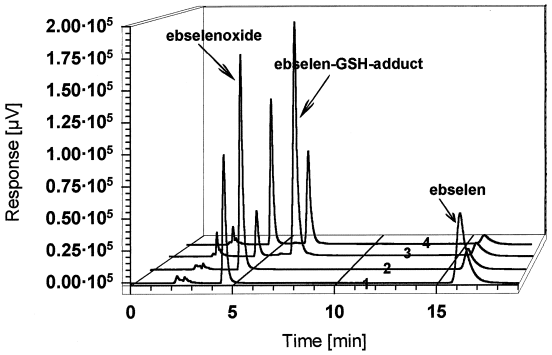


FIG. 3. Chromatograms of the reaction mixtures of (1) 500 μM ebselen and 500 μM PN at pH 7, (2) 500 μM ebselen and 500 μM PN at pH 9, (3) 500 μM ebselen, 500 μM GSH, and 500 μM PN at pH 7, and (4) 500 μM ebselen, 500 μM GSH, and 500 μM PN at pH 9. Incubation times were 5 min for all reactions. Ebselen peak retention times: t_R = 16 min; ebselen–GSH adduct, t_R = 6.6 min; and ebselenoxide, t_R = 4.5 min.

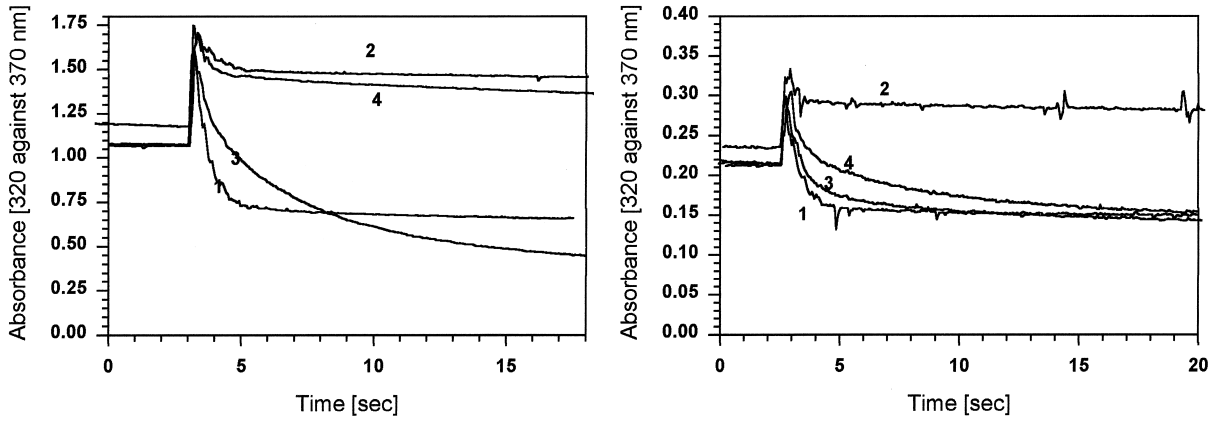


FIG. 4. Kinetics of the decomposition of peroxynitrite and disappearance of ebselen. (A) The system consisted of: (1) 400 μM ebselen and 500 μM PN in 0.2M K-phosphate buffer at pH 10; (2) second addition of 500 μM PN to the reaction solution of (1); (3) 400 μM ebselen, 200 μM GSH, and 500 μM PH; and (4) second addition of 500 μM PN to the reaction solution of (3). (B) The system consisted of: (1) 100 μM ebselen and 100 μM PN in 0.2 M K-phosphate buffer at pH 10; (2) second addition of 100 μM PN to the reaction solution of (1); (3) 100 μM ebselen, 20 μM BSA, and 100 μM PN; and (4) 100 μM ebselen, 40 μM BSA, and 100 μM PN.

pearance of the adduct through oxidation by PN had been monitored. A weaker scavenging effect of the ebselen–GSH adduct than with GSH alone could also be observed during the nitration of 5 mM phenol with 650 μM PN. The corresponding hydroxylated and nitrated phenol metabolites were separated by HPLC as described [23]. In this system, 100 μM ebselen caused 21 ± 2% inhibition, 100 μM GSH gave 88 ± 1%, and the combination of 100 μM ebselen/100 μM GSH yielded only 83 ± 3% inhibition (mean values of five measurements). With tyrosine, the corresponding differences were even more distinct (results not shown).

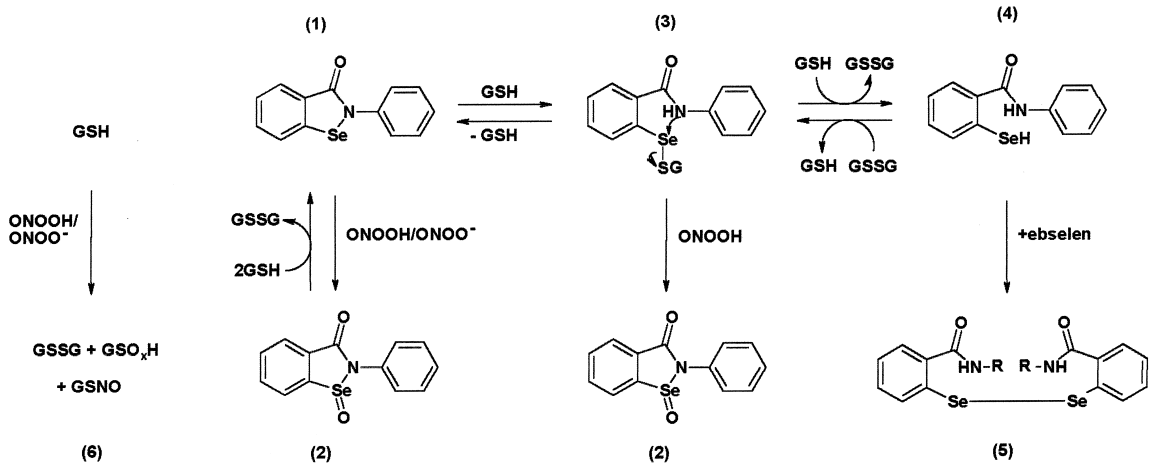
The Effect of Ebselen on PN-mediated Inactivation and Nitration of PGI_2 Synthase

According to these data, ebselen failed to inhibit the nitration of PGI_2 synthase, because the cellular content of GSH and protein thiols favored its conversion to adducts which reacted too slowly to scavenge PN for the obviously rapid reaction of PN with PGI_2 synthase. This may be different for the free radical pathway of PN oxidations and nitrations, which are thought to involve a protonated excited state of PN [3, 23]. A reaction of this species with GSH, ascorbate, uric acid, or other antioxidants has been reported [16–18, 33]. Using phenol as a model substrate, one can test this one-electron oxidation pathway by following the formation of nitrophenols and phenolic dimerization products [23, 34, 35]. In this system at pH 7, the absorbance increase due to the formation of 2- and 4-nitrophenol could be effectively blocked by ebselen, even if its concentration was only 50% of that of PN. An equimolar mixture of ebselen and GSH, which would lead to adduct formation, was also quenching although to a somewhat lower extent (not shown). Thus, if PN performs nitrations or oxidations by a mechanism either different or slower than the heme-catalyzed nitration of PGI_2 synthase, many antioxidants, as well as ebselen or even its thiol adducts, can act as scavengers.

DISCUSSION

There are several aspects to the results presented. First, the unexpected lack of protection by ebselen of PGI_2 synthase nitration and inhibition is consistent with the high reactivity of ebselen with thiol compounds [28, 29]. Second, given to *in vitro* preparations of aortic rings, ebselen is present almost exclusively in the form of its adducts to thiols. Due to its high concentration of about 5 mM in cells, GSH is a favorite reactant, but some protein-bound thiols/thiolates can be even more nucleophilic and therefore could also form adducts. Third, ebselen adducts can scavenge PN in a slower process as may occur in methionine or sulfhydryl oxidations.

Our results can be summarized by Scheme 1, which also includes the known complex chemistry that ebselen can perform under physiological conditions. The numerous reactions and equilibria that take place in this system are also the reason why we could not determine a kinetic constant for the reaction of PN with ebselen–GSH adduct. There are several species absorbing in the same wavelength region and thus complicating such kinetic measurements, but at least one can qualitatively see a much slower reaction compared with ebselen itself (Fig. 4, A and B). As shown in Scheme 1, GSH also reacts with PN (6), as already described [33, 36, 37]. Consequently, it is also impossible to distinguish between kinetic effects which originate from ebselen, GSH, or ebselen–GSH adduct. We could not determine whether the reactivity towards PN in an ebselen- and GSH-containing system results from the small amount of "free" ebselen (1) which is formed by a slow equilibrium from the adduct (3) (if so it must be very slow, or else one would not get a peak for the adduct in HPLC), or if it comes directly from the adduct. The latter would mean that a



SCHEME 1. Chemistry of the system ebselen, glutathione and peroxynitrite.

reaction from the adduct (3) to the ebselenoxide (2) is possible, but then the question arises as to what happens to the ebselen-bound GSH. Ebselenoxide (2) can be converted back to ebselen (1) by two molecules of GSH [38]. Another possibility is the conversion of the adduct (3) to the corresponding selenol (4) by one molecule of GSH followed by the reaction with another molecule of ebselen to give the diselenid (5) [29]. Of course (4) and (5) may also react with PN [19].

In summary, the mechanism of ebselen inactivation towards PN is complex, but this scheme fits the observations made in this study: ebselen binds to thiols and forms isolatable adducts. These systems show a decreased reactivity towards PN anion at least comparable to that of other tested selenocompounds such as Se–methionine [39]. Therefore, the loss of reactivity of ebselen towards peroxynitrite in biological systems seems to be due to this thiol binding. Hence, in the absence of any thiols as shown after preincubation with Ellman's reagent, ebselen can strongly compete with microsomal PGI_2 synthase for PN. Although the ebselen adducts reacted too slowly to protect PGI_2 synthase, they did interact with PN and therefore at least could function as antioxidants in cells. Therefore, the free radical nitration of phenol could be inhibited. In the context of PN nitration of PGI_2 synthase, the results lend support to our mechanistic proposal that PN reacts, as does its anion, directly at the ferric heme and that the nitrated tyrosine must be at the active site, as judged from the protection against nitration by quasi-substrates blocking the substrate binding site [20]. Thus, for kinetic and steric reasons, none of the known antioxidants was able to interfere with this process. In the case of an initially assumed inhibitory action of ebselen, it might have been possible to decide whether the nitration of PGI_2 synthase, which now has been identified to occur during atherosclerosis [40] and endotoxin exposure of aortic rings [41], is only a pathological and damaging process or whether the simultaneous trapping of NO and PGI_2 by superoxide generation is a physiological reaction required for host defense and cellular activation.

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