Peroxynitrite formed by simultaneous generation of nitric oxide and superoxide selectively inhibits bovine aortic prostacyclin synthase

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Abstract The effect of various oxidants on bovine aortic prostacyclin synthase was tested with ^{14}C-labelled prostaglandin endoperoxide as substrate. No sensitivity against hydrogen peroxide, superoxide or hydroxyl radicals was observed but hypochlorite inhibited with an IC_{50} value of 7 µM. Among the reactive nitrogen species nitric oxide and nitrogen dioxide radicals were ineffective, but peroxynitrite irreversibly blocked prostacyclin biosynthesis with an IC_{50} value of 50 nM. Peroxynitrite acted within seconds whereas hypochlorite required up to 30 min for completion. Simultaneous generation of nitric oxide and superoxide also caused inhibition which suggested that under pathological conditions like ischemia-reperfusion not only the vasodilatory effects of nitric oxide but also those of prostacyclin could be eliminated.

Key words: Peroxynitrite; Hypochlorite; Prostacyclin synthase; Ischemia-reperfusion; Irreversible inhibition; Circulation

1. Introduction

Prostacyclin (PGI$_2$) and nitric oxide (NO) are two endothelium-derived vasodilators which by a synergistic action on vascular smooth muscle cells guarantee blood flow in large vessels and microcirculation [1]. Since the biosynthesis of both compounds depends on molecular oxygen, hypoxia may reduce the formation of PGI$_2$ and NO which in turn will lead to vessel contraction and tissue ischemia. Reperfusion after a prolonged period of ischemia causes tissue damage and it has been postulated that reactive oxygen and nitrogen species contribute to the subsequent tissue damage. Superoxide anion radicals ($^{\bullet}O_2^-$) are extensively produced during ischemia/reperfusion. A number of different mechanisms for the production of superoxide radicals under these conditions has been suggested, e.g. altered mitochondrial electron flow [2], conversion of xanthine dehydrogenase to a xanthine oxidase [3] or leukocytes stimulated by their adherence to a pathologically modified endothelium [4]. Superoxide radicals are not very reactive in general, but they rapidly combine with NO radicals to form peroxynitrite in a nearly diffusion-controlled process [5,6]. Thus, in the presence of excessive O_2^- concentrations the vasodilatory action of NO would be eliminated. Here we show that the product of this process, i.e. peroxynitrite, blocks prostacyclin synthase (PGIS) at very low concentrations. Therefore, superoxide appears to interfere with two important vasodilators: it neutralises NO and interferes with the formation of PGI$_2$. The selectivity of peroxynitrite for this process was compared to that of other oxidants.

2. Materials and methods

2.1. Materials

[^{14}C]Arachidonic acid (53 mCi/mmol) was purchased from Dupont (Dreieich, Germany). Organic solvents were obtained from Merck (Darmstadt, Germany). Prostaglandin H$_2$, 3-morpholinosydnonimine-N-ethylcarbamide (SIN-1), ethanamine, N-ethyl-, compd. with 1,1-diethyl-2-hydroxy-2-nitrosohydrazine (diethylamine NONOate), and 1,3-propanediamine-N-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl] (spermine NONOate) were obtained from Cayman SPI (Massy, France) Other reagents, if not specified, were obtained from Sigma Biochemicals (Deisenhofen, Germany). Nitrogen dioxide was purchased as N$_2$O$_4$ in a tank from Fluka (Neu-Ulm, Germany).

2.2. Peroxynitrite synthesis

Peroxynitrite was prepared using a quenched-flow reaction as described by Reed et al. [7]. Briefly, an aqueous solution of 0.6 M sodium nitrite was rapidly mixed with an equal volume of 0.7 M hydrogen peroxide containing 0.6 M HCl and immediately quenched with the same volume of 1.5 M NaOH. All reactions were performed on ice. The concentration of peroxynitrite was determined spectrally in 0.7 M NaOH ($\varepsilon_{302} = 1670$ M^{-1} cm^{-1}). The stock solution of peroxynitrite was diluted in 0.5 M NaOH to give working solutions.

2.3. Biosynthesis of [^{14}C]PGH$_2$

Microsomes from sheep seminal vesicles were prepared according to the method of Kulmacz and Lands [8]. [^{14}C]PGH$_2$ was prepared by incubation of [^{14}C]arachidonic acid with sheep seminal microsomes followed by normal phase HPLC preparations as described [9].

2.4. Assay of prostacyclin synthase activity

Bovine aortic microsomes were prepared according to [10]. Prostacyclin synthase activity was assayed as follows: An aliquot of [^{14}C]PGH$_2$ at a final concentration of 5 µM was incubated with aortic microsomes (100 µg protein) in 100 µl of 100 mM KPi buffer, pH 7.4, for 3 min at 25°C after preincubation for 10 min with the indicated agents. The reaction was stopped with 20 µl of 1 M HCl and the radioactive products were extracted with 4 volumes of ethyl acetate, evaporated under N$_2$ and subsequently separated by thin-layer chromatography (ethyl acetate/H$_2$O/isooctane/acetic acid, 90:100:50:20). The amount of 6-keto-PGF$_{1\alpha}$ was quantified with a phosphoimaging system (Image Quant, Molecular Dynamics).

2.5. Generating system for superoxide, OH radicals and nitric oxide

Superoxide (O$_2^-$) was measured by the reduction of cytochrome c and was generated at a constant rate of 0.34 µmol min^{-1} ml^{-1} in a system of 100 µM hypoxanthine and 1 mU ml xanthine oxidase. The rate increased with 10 mU xanthine oxidase to 1.81 µmol min^{-1} ml^{-1} in the first 5 min and to 1.55 µmol min^{-1} at 7–10 min. 100 mU min^{-1} xanthine oxidase initially generated superoxide at a rate of 9 µM min^{-1}, 3.5 µM min^{-1} at 4 min, 1.13 µM min^{-1} at 6 min, 0.31 µM min^{-1} at 8–10 min. All reactions were carried out in 100 mM K-phosphate buffer, pH 7.4.

Nitric oxide (NO) was formed from diethylamine NONOate and quantified polarographically by a modified oxygen electrode applying an oxidizing potential of 800 mV. 1 mM diethyl NONOate released 2.87 µmol min^{-1} initially, 1.55 µmol min^{-1} at 3 min, 1 µM min^{-1} at 5 min, 0.33 µM min^{-1} at 7–10 min. The velocities with 100 µM diethylamine NONOate were 0.8 µmol min^{-1} within the first 3 min, 0.57 µM min^{-1} at 5 min, 0.34 µM min^{-1} after 7–10 min. With 10 µM diethylamine NONOate, the rates were 0.019 µmol min^{-1}, 0.010 µM min^{-1} at 3 min, and 0.007 µM min^{-1} at 5 min.
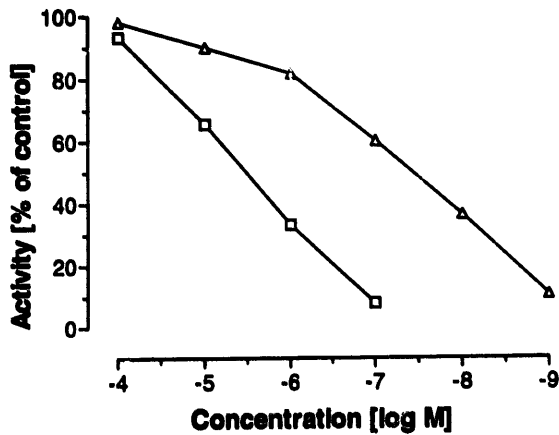
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Fig. 1. IC_{50} for peroxynitrite- and hypochlorite-induced inhibition of aortic microsomal PGI-synthase. △, peroxynitrite, □, hypochlorite.

3. Results

Bovine aortic microsomes that contain PGI-synthase (PGIS) were preincubated for the times indicated with various oxidants at different concentrations. Then [^{14}C]PGH$_2$ was added and the incubation was continued for 3 min. The reaction was stopped and extracted mixtures were analysed for 6-keto-PGF$_{1\alpha}$ (Table 1).

Hydrogen peroxide (H_2O_2) at concentrations up to 10 μM did not influence 6-keto-PGF$_{1\alpha}$ production. When the concentration was increased to 10 mM a slight increase (130%) in PGIS activity was observed. Superoxide anions generated by the hypoxanthine/xanthine oxidase system as well as conditions under which OH radicals were formed failed to inhibit PGIS. In contrast, hypochlorite started to inhibit PGIS at 1 μM and caused almost complete inhibition at 100 μM.

Among the reactive nitrogen species neither NO generated from diethylamine NONOate nor NO$_2$ had an inhibitory effect on PGIS activity, rather a small stimulation by these compounds was noted. Peroxynitrite, however, caused the strongest inhibition of PGIS of all oxidants tested, starting at 1 nM and being almost complete at 1 μM. Fig. 1 compares the potency of peroxynitrite (IC$_{50}$ value of 50 nM) and hypochlorite (7 μM) with respect to their ability to inhibit PGIS.

Both agents inhibited the enzyme irreversibly since repeated addition of PGH$_2$ or dilution of the inactivated enzyme failed to restore activity of the enzyme. Inhibition of PGIS by peroxynitrite was already complete 5 s after addition of the agent (Fig. 2). In contrast to this very rapid inactivation of the enzyme, the blocking action of hypochlorite developed over a longer time and was maximal only after 30 min. In control experiments with decomposed peroxynitrite or with the degradation products nitrite and nitrate no inhibition of PGIS was observed.

When peroxynitrite was generated by recombination of NO and O$_2^-$ radicals (by coincubation of the two generating sys-

Table 1
Effect of reactive oxygen and nitrogen species on the activity of aortic microsomal PGI-synthase

Reactive species	% change of activity
Hydrogen peroxide	
10 μM	+4.5±1.3
100 μM	+5.7±2.7
1 mM	+9.4±4.7
Superoxide anion	
1 mU/ml X.O.	−1.7±2.9
10 mU/ml X.O.	−3.3±4.2
100 mU/ml X.O.	−11.4±10.7
OH radicals	
Fenton reaction	−10.0±9.1
Haber-Weiss reaction	−11.3±10.5
Hypochlorite	
1 μM	−33.1±11.2
10 μM	−65.4±8.9
100 μM	−93.2±5.6
Nitric oxide (diethylamine NONOate)	
10 μM	+20.7±4.6
100 μM	+20.1±5.7
1 mM	+42.8±5.7
Nitrogen dioxide (1 mM)	−21.3±4.7
Peroxynitrite	
0.01 μM	−36.2±9.7
0.1 μM	−59.8±6.8
1 μM	−81.4±7.9

Aortic microsomal PGI-synthase activity was assayed by the formation of 6-keto-PGF$_{1\alpha}$ as described in section 2. The enzyme was preincubated with the reactive species for 10 min. Nitrogen dioxide was from compressed N$_2$O$_4$ gas diluted in dinitrogen and equilibrated by shaking at 37°C. Sodium hypochlorite was standardized by absorbance measurements using ε_{290nm} = 350 mol^{-1} cm^{-1}. The data represent 10 samples (means ± S.E.M.) from 4 different experiments. "+" and "−" represent an increase or a decrease of prostacyclin synthase activity compared with untreated aortic microsomes.

Table 2
Effects of combined generating systems for NO and superoxide as well as SIN-1 on PGH_2-induced 6-keto-$PGF_{1\alpha}$ formation by aortic PGI-synthase

Agents	% inhibition
Nitric oxide (1 mM diethylamine NONOate)	
+100 mU X.O.	−85.6±11.7
+10 mU X.O.	−96.2±2.0
+1 mU X.O.	−93.3±4.0
Nitric oxide (0.1 mM diethylamine NONOate)	
+100 mU X.O.	−66.3±9.5
+10 mU X.O.	−6.4±5.1
+1 mU X.O.	−3.1±4.7
Nitric oxide (0.01 mM diethylamine NONOate)	
+100 mU X.O.	−4.7±2.1
+10 mU X.O.	−3.6±4.3
+1 mU X.O.	−3.5±2.9
SIN-1 (1 mM)	−56.7±6.9

Aortic microsomes were preincubated with generating systems for NO and superoxide for 10 min or SIN-1 for 2 h and assayed as described in

tems employed in Table 1) a clear inhibition of PGIS was observed that was dependent on the rate of formation of both radicals (Table 2). In addition, in the presence of SIN-1, a compound that simultaneously generates NO and O_2^- [11], a strong inhibition of 6-keto-$PGF_{1\alpha}$ formation was observed.

4. Discussion

Peroxynitrite is known as a potent oxidant and the inhibition of different enzymes by this agent has been reported [12–14]. The concentrations of peroxynitrite that were required, however, were well above 10 μM and thus probably higher than those that can arise from physiological concentrations of NO and O_2^-. Moreover, to our knowledge, the combined generation of NO and O_2^- was never effective in inhibition of an enzyme in these experiments. In contrast, the inhibition of PGI-synthase occurred at low concentrations of peroxynitrite and was also observed when NO and O_2^- were generated simultaneously. The reaction was selective in comparison with all other oxidants tested. The only other agent that was inhibitory was hypochlorite, the IC_{50} value of which was about 100-fold higher than that of peroxynitrite. The finding that some oxidants stimulated 6-keto-$PGI_{1\alpha}$ forma-

Fig. 2. Time-course of peroxynitrite- and hypochlorite-induced inhibition of aortic microsomal PGI-synthase. △, peroxynitrite, □, hypochlorite.

tion may be due to blocking of other competing PGH_2 pathways leaving more PGH_2 for the isomerization by PGIS. Further work will be concerned with the mechanisms of PGIS inhibition by peroxynitrite but it is clear already that no SH-group oxidation is involved since PGIS has only one cysteine residue which forms the ligand to the heme–iron ("P450-enzyme") and this heme–thiolate linkage is affected only at concentrations above 10 μM (Pasquet, J.P., unpublished). Alternatively peroxynitrite may decompose to a reactive nitrating species and give rise to nitro forms of tyrosine or tryptophane [6]. Preliminary results with an antibody against nitrotyrosine showed a positive reaction of prostacyclin synthase under our incubation conditions (Zou et al., in preparation). The rapid time course of the peroxynitrite effect in comparison with the slow reaction of hypochlorite would also be in agreement with a potent nitrating agent as the active species.

The inhibition of PGIS by hypochlorite and peroxynitrite may be of pathophysiological significance. Hypochlorite is a product from leukocytes that could be formed through the action of NADPH-oxidase and myeloperoxidase upon adhesion and activation of these cells at a modified endothelium [15,16]. The expression of adhesion factors and receptors on both cell types which occurs under conditions of severe oxidative stress [17] may contribute to this process. For peroxynitrite a similar scenario can be envisaged: inflammatory conditions through the action of cytokines induce NO synthesis which greatly enforces the basal NO production in the vessels. Ischemia-reperfusion events provide conditions under which O_2^- formation by xanthine oxidase, mitochondria or leukocytes commences and in severe cases approaches, and finally compensates, the rate of NO formation. This not only eliminates the vasodilating actions of NO but also blocks PGI-synthase. The latter effect may occur at two stages: a rapid inhibition by peroxynitrite followed by an inhibition that is slower in onset caused by leukocyte-generated hypochlorite. The resulting endothelial dysfunction will lead to a collapse of the microcirculation, subsequent ischemia and finally a loss of organ function. Thus, peroxynitrite could be considered a messenger molecule arising from inflammatory or traumatic conditions which by oxidative posttranslational protein mod-
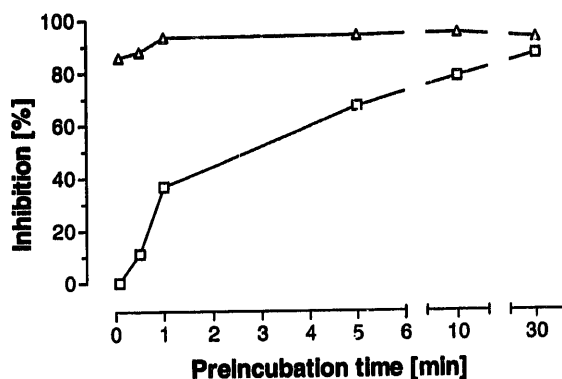
ification would be able to coordinate the tissue response to the pathological situation.

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