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Nitric oxide donors activate the cyclo-oxygenase and peroxidase activities of prostaglandin H synthase

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Abstract Prostaglandin H synthase (PHS) is a dual enzyme with cyclo-oxygenase and peroxidase activities. The nitric oxide (NO) donors, sodium nitroprusside (SNP), *S*-nitroso-*N*-acetylpenicillamine (SNAP) and spermine NONOate (SPER/NO), activated both cyclo-oxygenase and peroxidase activities of PHS. SNP activated PHS by increasing V_{max} without affecting K_m, the activation constants being 1.0 mM for cyclo-oxygenase and 1.3 mM for peroxidase. Analysis of progress curves and absorption spectra of PHS suggested that NO released from SNP interacted with the heme at the active site of the enzyme. Moreover, SNP counteracted the peroxide-induced inactivation of PHS, suggesting that the interplay between the intracellular peroxide and NO is critical in tuning PHS activity in cells.

Key words: Cyclo-oxygenase; Peroxidase; Nitric oxide; Arachidonate cascade

1. Introduction

Arachidonic acid can be converted into prostanoids (i.e., prostaglandins, prostacyclin, thromboxane) or leukotrienes and lipoxins, all biologically active compounds, via the cyclo-oxygenase pathway or the lipoxygenase pathway (Scheme 1). Prostaglandin H (prostaglandin endoperoxide) synthase (E.C. 1.14.99.1; PHS) is a heme-containing oxygenase which catalyses the first committed step in prostanoid formation [1–3]. PHS is a dual enzyme, because two catalytic activities copurify with a single protein molecule: cyclo-oxygenase and peroxidase. The cyclo-oxygenase activity catalyzes the addition of two molecules of oxygen to one molecule of arachidonic acid, thus forming a cyclic endoperoxide hydroperoxide, prostaglandin G_2 (PGG_2). The peroxidase activity reduces the hydroperoxide to the corresponding alcohol, prostaglandin H_2 (PGH_2), at the expense of a reducing co-substrate, DH. These reactions can be schematically represented as follows:

$$\text{Arachidonic acid} + 2O_2 \rightarrow PGG_2 \text{ (cyclo-oxygenase)}$$

$$PGG_2 + 2DH \rightarrow PGH_2 + 2D^{\cdot} + H_2O \text{ (peroxidase)}$$

Nitric oxide (NO) is a regulatory molecule involved in several processes, as different as platelet aggregation and neurotransmission [4,5]. Recently, we found that NO donors inhibit the lipoxygenase pathway of arachidonate cascade by acting as competitive inhibitors of enzyme activity [6]. Our results provided a biochemical background to the decrease of lipoxygenase metabolites observed in cells challenged with NO [7]. On the other hand, the effect of NO on PHS has been studied so far only in cellular systems and has led to conflicting reports showing either activation [8–10] or inhibition [11–13] of cyclo-oxygenase activity. Therefore, the possible role of NO in modulating the cyclo-oxygenase pathway of arachidonate cascade remains unclear. In this study, we used pure PHS to investigate the interaction of NO with both cyclo-oxygenase and peroxidase activities of the enzyme. Because peroxide tone is a critical factor in modulating PHS [14] and it can represent a constraint in prostaglandin biosynthesis in tissues [15], we studied the possible interplay between NO and peroxides in tuning PHS activity. Results presented in this paper help to clarify the overall picture of the role of NO in the control of arachidonate cascade, suggesting that nitric oxide inhibits the lipoxygenase [6,7] while activating the cyclo-oxygenase pathway.

2. Materials and methods

Chemicals were of the purest analytical grade. Arachidonic (eicosatetraenoic) acid, hemin, hydrogen peroxide (30%, w/w), phenol, sodium nitroprusside (sodium nitroferricyanide, SNP) and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) were from Sigma Chemical Co. (St. Louis, MO, USA). *S*-Nitroso-*N*-acetylpenicillamine (SNAP) was purchased from Research Biochemicals International (Natick, MA, USA). Spermine NONOate (SPER/NO, (*Z*)-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]-amino}-diazen-1-ium-1,2-diolate) was from Alexis Corporation (Läufelfingen, Switzerland). 5-, 12- and 15-Hydroperoxyeicosatetraenoic acids (5-, 12- and 15-HPETEs), a kind gift from Mr. Guus van Zadelhof (Bijvoet Center for Biomolecular Research, Utrecht University, The Netherlands), were biosynthesized by incubating arachidonic acid with 5-, 12- or 15-lipoxygenase, respectively. HPETEs were extracted from reaction mixtures on octadecyl-SPE columns (Baker, Deventer, The Netherlands) and purified by RP-HPLC, performed on a Perkin Elmer 1022 LC Plus liquid chromatograph (Norwalk, CO, USA) as reported [16]. Prostaglandin H synthase (E.C. 1.14.99.1; PHS), purified from ram seminal vesicles, was purchased from ICN Biochemicals (Cleveland, OH, USA). The enzyme preparation was electrophoretically pure, migrating as a single band of 71 kDa in 12% SDS-PAGE. Pure PHS showed a cyclo-oxygenase activity of 37 nmol $O_2 \cdot min^{-1}$ and a peroxidase activity of 14 mU, assayed as described below.

2.1. Cyclo-oxygenase assay

PHS was assayed for cyclo-oxygenase activity at 30°C by means of a polarographic electrode [17]. The standard reaction mixture contained 0.1 M potassium phosphate (pH 7.2), 1 mM phenol and various amounts (0–100 μM) of arachidonate. The reaction was started by adding 35 nM PHS. Cyclo-oxygenase activity was expressed as nmol O_2 consumed per min. Progress curves of the oxygenation of 75 μM arachidonic acid by 35 nM PHS were recorded by following

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Abbreviations: 5-, 12-, 15-HPETEs, 5-, 12-, 15-hydroperoxyeicosatetraenoic acids; NO, nitric oxide; PHS, prostaglandin H synthase; RP-HPLC, reversed phase–high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; SPER/NO, spermine NONOate; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine

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polarographically the oxygen consumption. The lag phase of the cyclo-oxygenase reaction (i.e., the time required to reach maximal velocity) was determined from the chart record tracing of oxygen consumption versus time [18].

2.2. Peroxidase assay

PHS was assayed for peroxidase activity at 30°C spectrophotometrically, as described [19]. The reaction mixture consisted of 0.1 M Tris-HCl (pH 8.1), 170 μM TMPD, 1 μM hemin and various amounts (0–100 μM) of arachidonate. Reaction was started by addition of 35 nM PHS. After 5 min incubation at 30°C, absorbance values at 590 nm were measured, defining one unit (U) of peroxidase activity as the amount of PHS necessary to generate a change of 1 A_{590} unit during the 5 min incubation [19].

2.3. Kinetic analysis

The effect of SNP on the cyclo-oxygenase and peroxidase activities of PHS was analysed by double reciprocal plots, using two different concentrations of freshly prepared NO donor (0.75 mM and 1.50 mM) to calculate the kinetic parameters of each enzymatic activity. Fitting of the experimental points by a linear regression programme (Kaleidagraph) yielded straight lines with r-values >0.93.

2.4. PHS inactivation by peroxides

The ability of peroxides to inactivate the cyclo-oxygenase and peroxidase activities of PHS was determined by pre-incubating 35 nM PHS with various amounts (0–1.0 μM) of each peroxide (H_2O_2, 5-, 12- or 15-HPETEs) for 2 min at room temperature [14]. Enzyme activity was then measured using 75 μM arachidonate as substrate, in the presence or absence of freshly prepared SNP (0.50 mM) in the reaction mixture.

2.5. Absorption spectra of PHS

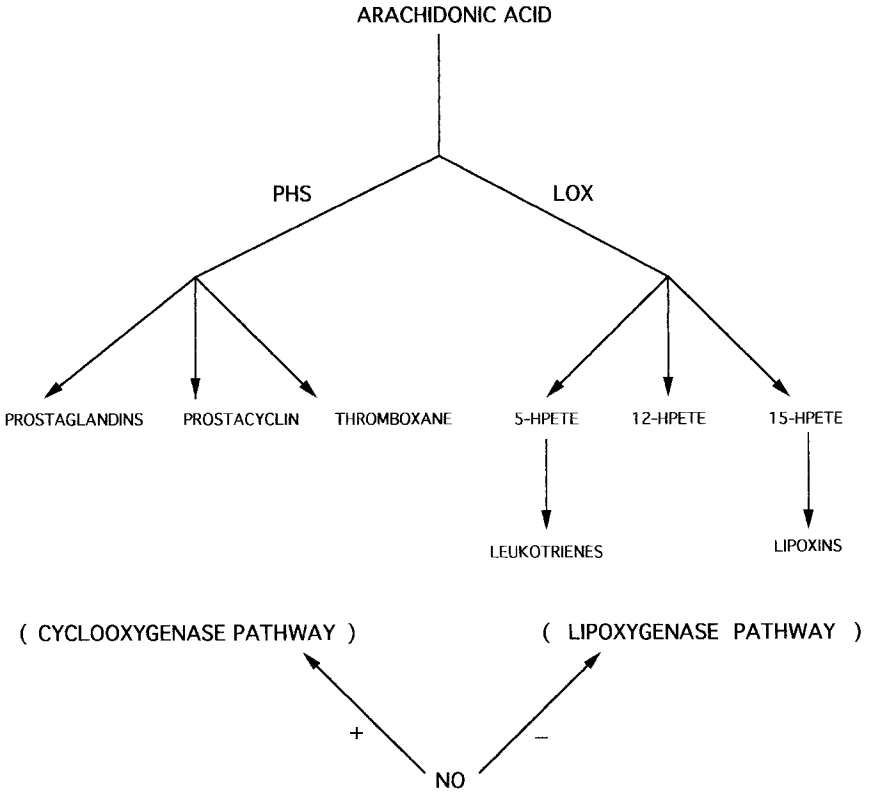
Absorption spectra of PHS were recorded in the wavelength range 320–620 nm using 14 μM PHS in 0.1 M Tris-HCl (pH 8.1) [20]. The effect of NO on PHS spectral properties was investigated by adding freshly prepared SNP up to 200 mM. Absorption spectra were recorded at room temperature in a UV/VIS spectrometer Lambda 18 (Perkin Elmer, Norwalk, CO, USA).

2.6. Data analysis

Data reported in this paper are the mean (±S.D.) of at least 3 independent determinations, each performed in duplicate. Reported drawings are also representative of triplicate experiments. Statistical analysis was performed by the Student t-test, considering significant the difference between control and each treatment at least at 5% level ($P<0.05$).

3. Results and discussion

Prostaglandin H (prostaglandin endoperoxide) synthase (PHS) is a dual enzyme, which catalyzes the cyclo-oxygenase and peroxidase reactions leading to prostaglandins, prostacyclin and thromboxane synthesis from arachidonic acid [1–3]. The cyclo-oxygenase and peroxidase active sites, though residing on a single protein, are distinct [21]. Nevertheless, both activities of PHS were similarly enhanced by different NO donors in a dose-dependent manner (Fig. 1). SNP, SNAP and SPER/NO are structurally dissimilar compounds which share only the ability of releasing nitric oxide in aqueous solutions [22]. The different NO donors affected PHS activity in a similar manner (Fig. 1), suggesting that they acted by providing NO rather than directly. The release of NO from these widely used NO donors has been investigated [6,22,23], showing that millimolar concentrations of NO donors release free nitric oxide at concentrations in the physiological range and that free NO is no longer detectable in 12-h-old solutions of SNP, SNAP or SPER/NO. Interestingly, 12-h-old solutions



Scheme 1. Arachidonic (eicosatetraenoic) acid metabolism is directed towards prostanoids (i.e., prostaglandins, prostacyclin and thromboxane) or leukotrienes and lipoxins (through hydroperoxyeicosatetraenoic acid (HPETE) intermediates). Biosynthesis of prostanoids is catalyzed by prostaglandin H synthase (PHS), via the so-called 'cyclo-oxygenase pathway'. Lipoxygenase (LOX) synthesizes leukotrienes and lipoxins in the so-called 'lipoxygenase pathway'. Nitric oxide (NO) inhibits (−) the lipoxygenase while activating (+) the cyclo-oxygenase pathway.

of all NO donors lost their ability to activate PHS (data not shown), corroborating the hypothesis that SNP, SNAP and SPER/NO were acting on PHS by releasing NO. SNP was slightly more efficient than SNAP or SPER/NO in activating PHS; therefore the effect of NO on PHS was further characterized using SNP. Double reciprocal plots of cyclo-oxygenase and peroxidase activities of PHS were drawn, in the presence or absence of SNP (Fig. 2). From these plots the V_{max} and K_m values of the PHS reaction with arachidonate were calculated, and were 44.2 ± 4.4 nmol $O_2 \cdot min^{-1}$ and 14.1 ± 1.4 μM for the cyclo-oxygenase, 22.2 ± 2.0 mU and 36.7 ± 3.5 μM for the peroxidase. The double reciprocal plots showed SNP to activate PHS by increasing apparent V_{max} without affecting K_m values (Fig. 2). Therefore, the activation constant (K_a) was calculated from the apparent V_{max} in the presence of SNP ($V_{max,app}$), using the formula:

$$V_{max,app} = V_{max}(1 + [SNP]/K_a).$$

The K_a values of SNP for the cyclo-oxygenase and the peroxidase activities were 1.0 ± 0.1 and 1.3 ± 0.1 mM, respectively. We have recently measured the amount of NO released from

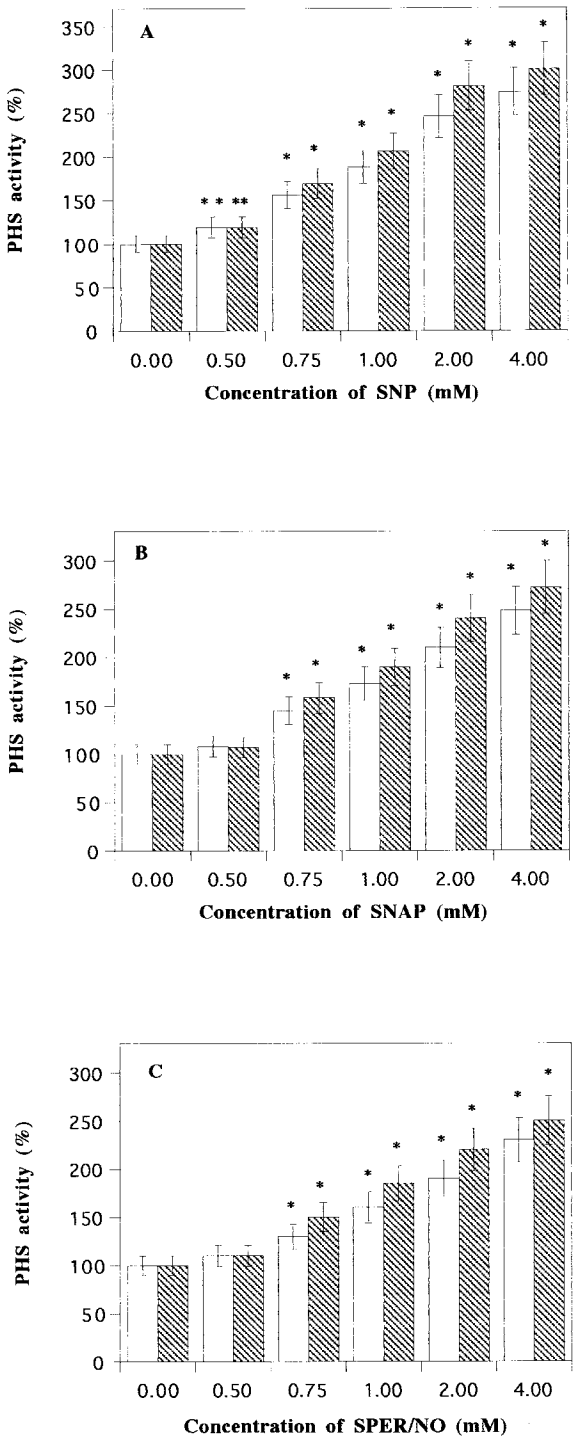


Fig. 1. Effect of SNP (A), SNAP (B) and SPER/NO (C) on the cyclo-oxygenase (open bars) and peroxidase (hatched bars) activities of PHS. Both PHS activities were measured using 75 μM arachidonate as substrate, 100% corresponding to 37 nmol $O_2 \cdot min^{-1}$ and 14 mU, respectively, for the cyclo-oxygenase and the peroxidase activity. Data are reported ± S.D. values ($n = 6$). $^*P < 0.01$ compared to control. $^{**}P < 0.05$ compared to control.

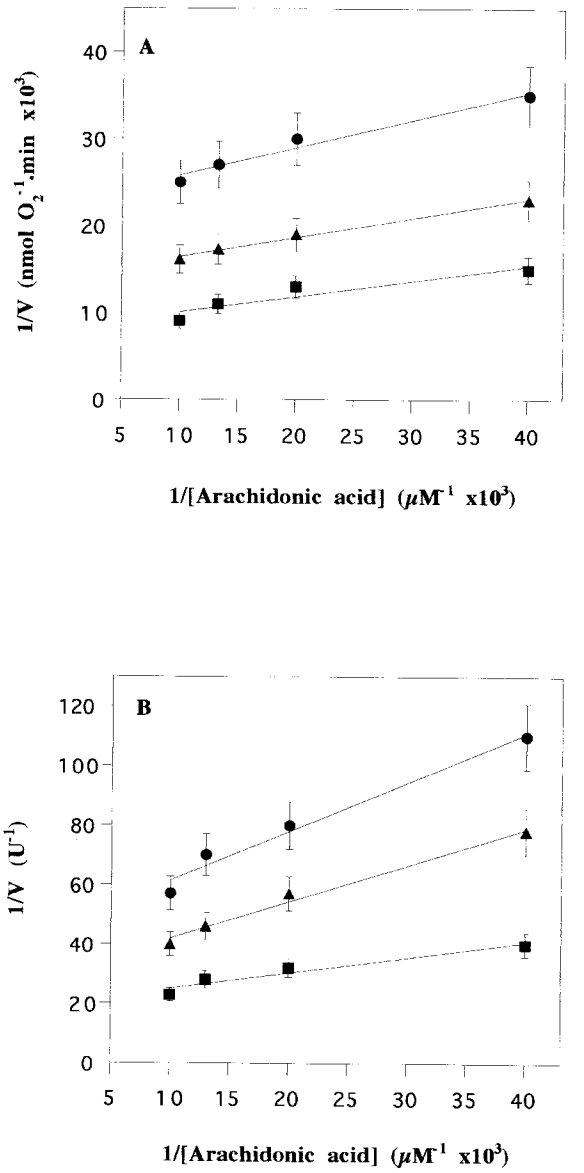


Fig. 2. Double reciprocal plots of the cyclo-oxygenase (A) and peroxidase (B) activity of PHS, in the absence (circles) or presence of 0.75 mM SNP (triangles) or 1.50 mM SNP (squares). Data are reported ± S.D. values ($n = 8$).

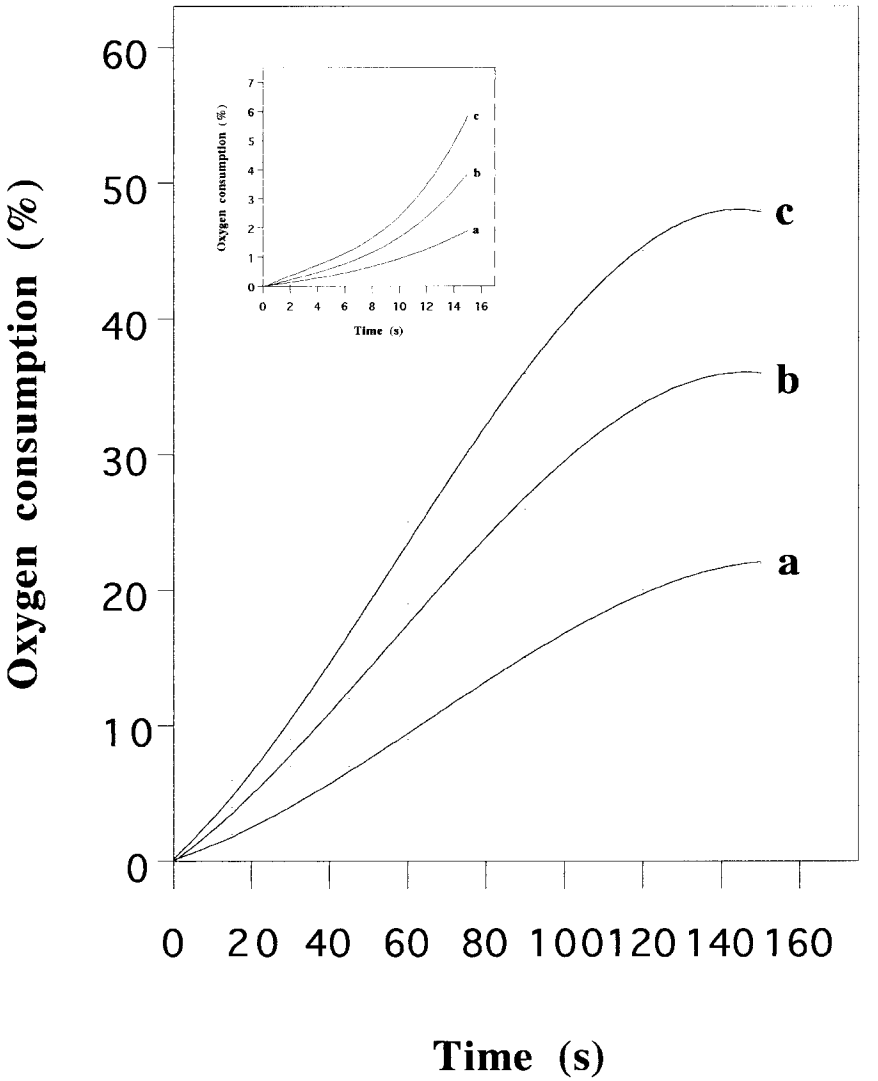


Fig. 3. Progress curves of the cyclo-oxygenase reaction using 75 μM arachidonate as substrate, in the absence (a) or presence of 0.75 mM SNP (b) or 1.50 mM SNP (c). Inset: Lag phase of the cyclo-oxygenase reaction.

SNP in aqueous solutions [6]. Based on the release of nitric oxide from SNP, it was possible to estimate that K_a values of NO for PHS were approximately 0.65 μM for cyclo-oxygenase and 0.85 μM for peroxidase, respectively. The observation that SNP modulates in a similar way both activities of PHS is in keeping with the intimate correlation between these activities in prostanoid biosynthesis. In order to characterize in more detail the activation of PHS by SNP, progress curves of the cyclo-oxygenase activity were recorded (Fig. 3). It is shown that SNP reduced the lag phase of the reaction (Fig. 3, inset) in a dose-dependent manner, from approximately 8 s of the untreated control to approximately 4 s of the samples treated with 1.50 mM SNP. PHS is a heme-protein which requires ferriprotoporphyrin IX for both cyclo-oxygenase and peroxidase activities [1–3,14]. NO can bind to ferrous complexes and assist the oxidation of iron from ferrous to ferric form [24]. Indeed, the ability of NO to activate ferrous enzymes by oxidizing them to ferric forms has been reported [25,26]. Therefore, the shortening of the reaction lag phase might be indicative of a direct oxidation of inactive Fe(II) to active Fe(III) in the heme moiety. Also the analysis of the absorption spectra of PHS favours the hypothesis of a direct interaction of NO with heme. In Fig. 4 it is shown that addition of SNP increased the absorption of PHS in the region around 400 nm, where the spectrum is mainly contributed by the heme moiety [20,24]. These spectral changes might be assigned to the formation of NO adducts such as iron–nitrosyl complexes, which have indeed been observed by EPR spectroscopy in PHS treated with gaseous NO [20]. On the other hand, cyclo-oxygenase and peroxidase activities of PHS are inactivated by pre-incubation with hydrogen peroxide (Fig. 5A,B). The peroxide-induced inactivation of cyclo-oxygenase was paralleled by an increase in the lag phase of the reaction (Fig. 5C). The interaction of peroxides with PHS is known to generate higher oxidation states of the heme iron, such as Fe(IV) and Fe(V), which can undergo internal rearrangements or side-reactions, ultimately leading to enzyme inactivation [14,15,27]. Interestingly, the effect of H_2O_2 was prevented by SNP (Fig. 5). In particular, 0.5 mM SNP, which released 0.4 μM NO in the buffer [6], fully counteracted the inactivation of PHS caused by 0.4 μM hydrogen peroxide. Also the lag phase of the cyclo-oxygenase reaction returned

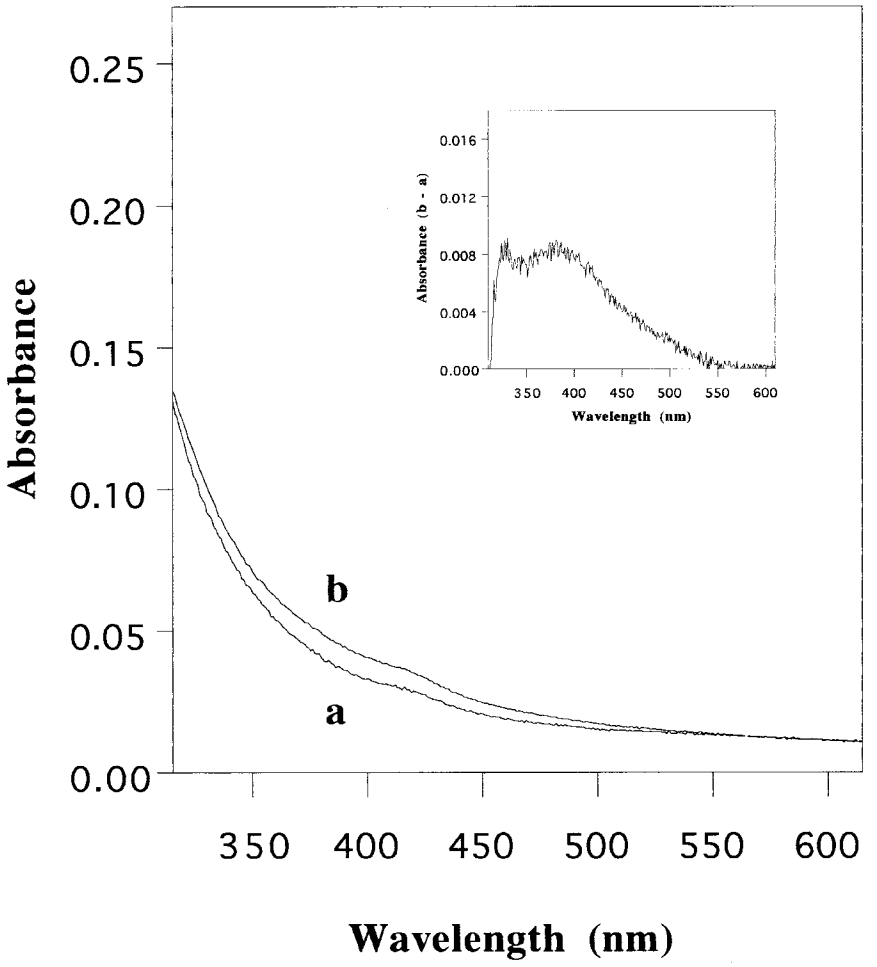


Fig. 4. Absorption spectra of 14 μM PHS in the absence (a) or presence (b) of 200 mM SNP. Inset: Difference spectrum calculated by subtracting spectrum (a) from (b).

to the control value when 0.5 mM SNP was added to the H_2O_2-treated enzyme (Fig. 5). Thus, it can be proposed that nitric oxide, which can behave also as a reducing agent [28], counteracts H_2O_2 by reducing Fe(IV) or Fe(V) to active Fe(III). NO might also protect PHS from peroxide inactivation by supporting peroxide reduction, which has been shown to occur when PHS is incubated with reducing substrates [14].

Interestingly, lipoxygenase-derived hydroperoxides 5- and 12-HPETEs were more effective than H_2O_2 in inactivating PHS, whereas 15-HPETE was less effective (Table 1). SNP also counteracted the effect of these hydroperoxides, though to a different extent (Table 1). The potency of HPETEs in inactivating PHS might correlate with the tridimensional structure of the active site, although more structural data are needed

Table 1
Inactivation of PHS by lipoxygenase products

Peroxide	Cyclo-oxygenase activity of PHS (nmol O_2·min^{-1})		Peroxidase activity of PHS (mU)	
	−SNP	+SNP	−SNP	+SNP
None	37.0 ± 3.5 (100%)	44.0 ± 4.2 (100%)	14.0 ± 1.3 (100%)	16.7 ± 1.6 (100%)
5-HPETE	13.6 ± 1.2 (37%)*	33.4 ± 3.2 (76%)**	4.6 ± 0.5 (33%)*	11.7 ± 1.2 (70%)**
12-HPETE	16.3 ± 1.5 (44%)*	34.8 ± 3.5 (79%)**	5.5 ± 0.5 (39%)*	10.9 ± 1.1 (65%)*
15-HPETE	25.2 ± 2.5 (68%)**	46.6 ± 4.5 (106%)	10.1 ± 1.0 (72%)**	19.2 ± 1.9 (115%)
H_2O_2	19.2 ± 1.8 (52%)*	35.0 ± 3.3 (79%)**	6.4 ± 0.6 (46%)*	15.0 ± 1.5 (90%)

Hydroperoxy derivatives of arachidonic acid (HPETEs) were used at a final concentration of 0.4 μM in the presence or absence of 0.50 mM SNP. The effect of HPETEs was compared to that obtained with 0.4 μM H_2O_2 (data from Fig. 5). Data ± S.D. are reported ($n = 8$) and values in brackets represent percentages of the controls, arbitrarily set to 100.
*$P < 0.01$ compared to control. **$P < 0.05$ compared to control.

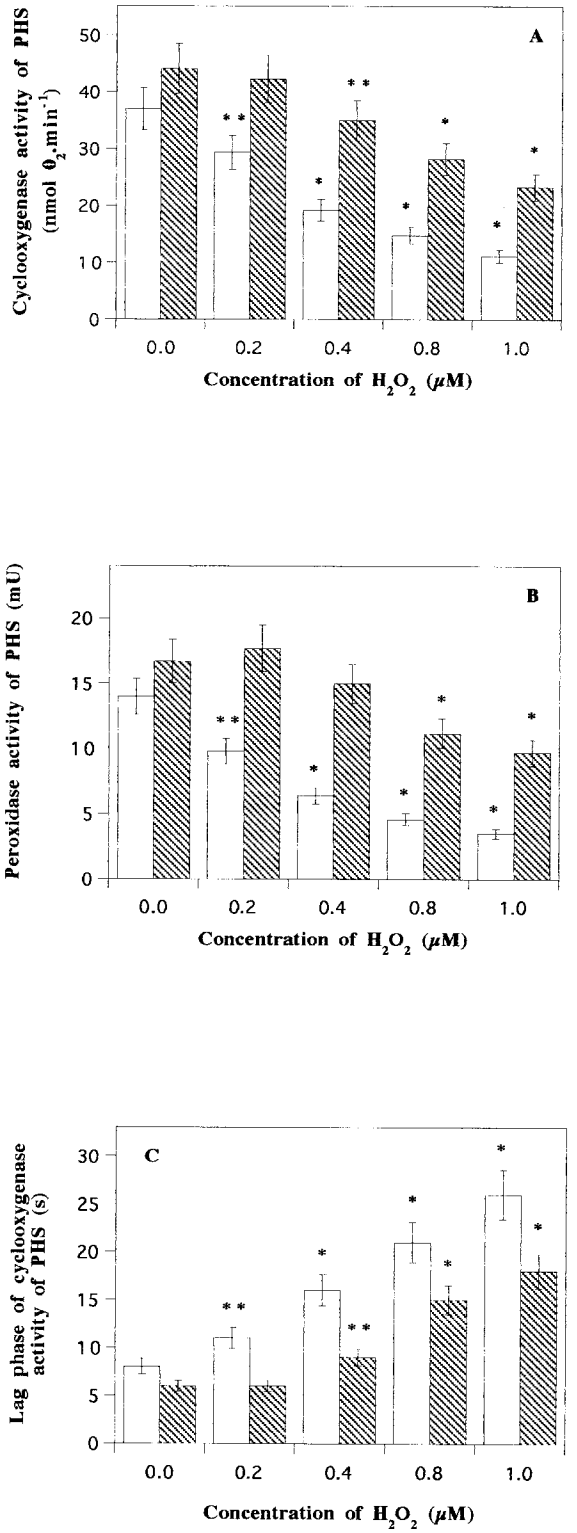


Fig. 5. Inactivation of the cyclo-oxygenase (A) and peroxidase (B) activities of PHS by H_2O_2, and effect of H_2O_2 on the lag phase of the cyclo-oxygenase reaction (C). Measurements were performed in the absence (open bars) or presence (hatched bars) of 0.50 mM SNP, using 75 μM arachidonate as substrate. Data are reported ±S.D. values ($n=6$). $^{*}P<0.01$ compared to control. $^{**}P<0.05$ compared to control.

for making more conclusive statements [29]. It is noteworthy that the effect of hydroperoxides on PHS occurs at micromolar concentrations, which are in the physiological range [18]. This points out the key-role of the intracellular peroxide level in tuning the cyclo-oxygenase and peroxidase activities of PHS in the presence of NO [15,30]. Difference in peroxide levels might reconcile conflicting reports describing the ability of NO to activate [8–10] or inhibit [11–13] PHS in cellular extracts. Finally, it seems interesting that concentrations of SNP shown here to activate PHS have been reported to inhibit lipoxygenase activity, with an inhibition constant (K_i) of NO for lipoxygenase of 0.4 μM [6]. Therefore, it is tempting to suggest that nitric oxide modulates the arachidonate cascade by inhibiting one branch (i.e., the lipoxygenase pathway) while activating the other (i.e., the cyclo-oxygenase pathway) at the same time (Scheme 1). Inhibition of lipoxygenase activity reduces the intracellular amount of HPETEs, thus increasing the stimulatory effect of NO on PHS. Altogether, these findings might be relevant in (patho)physiological processes such as inflammation, neurotransmission, neurotoxicity and cell death [5,6,31–35], where nitric oxide could play its role by deranging the arachidonate cascade in favour of prostanoids.

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