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Nitric Oxide-Donor Compounds Inhibit Lipoxygenase Activity

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The nitric oxide (NO)-releasing agents sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) inhibit dioxygenase activity of lipoxygenase in human platelets and human CHP100 neuroblastoma cells, leading the latter to necrosis. The effect of both NO-donors on the dioxygenase reaction was investigated by using soybean lipoxygenase type II (LOX-2) as a model for the mammalian enzyme. SNP and SNAP were competitive inhibitors of LOX-2, with inhibition constants of 525 μM and 710 μM, respectively. Both compounds inactivated LOX-2 by reducing the catalytic iron to the inactive Fe(II) form and counteracted the H_2O_2-mediated activation of the LOX-2-catalyzed dioxygenase reaction. Similarly, the co-oxidative and peroxidative activities of LOX-2 were also inhibited by the NO-releasing agents. These findings suggest that the biological role played by NO can be mediated, at least in part, by the inactivation of lipoxygenase, a key-enzyme for the arachidonic acid metabolism in human cells.

Nitric oxide (NO) plays several regulatory roles, such as the control of blood pressure, neurotransmission, platelet aggregation and the cytostatic action of macrophage cells [1]. NO is considered a cytotoxic species, although other evidences suggest a protective role against cellular damage [2, 3]. Such cytoprotection should occur by preventing membrane damage mediated by hydroxyl radicals and other reactive oxygen species [2, 3]. Recently, attention has been drawn to the possible interaction of NO with lipoxygenase (LOX), an enzyme which activates the arachidonic acid cascade leading to leukotrienes [4]. Lipoxygenase activity gives also rise to hydroxyl radicals and superoxide anions [5], which are known to be involved in NO neurotoxicity [3]. Moreover, LOX plays a role in modelling the membranes of animal cells [6]. It has been already reported that NO can inhibit lipid oxidation catalyzed by lipoxygenase, but such inhibition has not been explained [7, 8]. Here, the effect of nitric oxide on the dioxygenase activity of LOX was studied in two cellular systems responsive to NO modulation, *i.e.* human platelets and human CHP100 neuroblastoma cells [8,9]. The interaction of NO with LOX was investigated in more detail using soybean lipoxygenase type II (LOX-2), which has optimum activity at neutral pH, at variance with other lipoxygenases. Soybean lipoxygenases (linoleate:oxygen oxidoreductase, E.C. 1.13.11.12) are widely used as models for mammalian LOXs in structural and mechanistic studies [10, 11]. Since lipoxygenases are multifunctional enzymes showing co-oxidative and peroxidative activities besides the oxygenating activity [12, 13], the effect of NO-donors on these activities was investigated as well.

MATERIALS AND METHODS

Materials. Chemicals were of the purest analytical grade. Linoleic acid, arachidonic acid, sodium nitroprusside (sodium nitroferricyanide; SNP), all *trans*-β-carotene (type I; BC) and nordihydroguaiaretic acid (NDGA) were from Sigma Chemi-

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Abbreviations: BC, β-carotene; HPETE, hydroperoxyeicosa-6,8,11,14-tetraenoic acid; LOX, lipoxygenase; NDGA, nordihydroguaiaretic acid; NO, nitric oxide; SNAP, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside; TMB, tetramethylbenzidine.

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cal Co. (St. Louis, MO, U.S.A.). S-Nitroso-N-acetylpenicillamine (SNAP) was purchased from RBI (Research Biochemicals International; Natick, MA, U.S.A.). Oxyhemoglobin was a kind gift from Dr. M.E. Clementi (University of Rome 'Tor Vergata'). Human blood was collected from healthy volunteers which were kept drug-free for two weeks prior to blood sampling.

Cell cultures. Human CHP100 neuroblastoma cells were cultured as reported [14]. Prior to addition of NO-donors, the medium was removed and the cell cultures were washed with the controlled saline solution (CSS) already described [14], *i.e.* 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 10 mM glucose, 25 mM Tris/HCl (pH 7.4). Cells were then incubated in CSS containing either SNP or SNAP (500 μM final concentration) for 5 min at room temperature. Paired controls were exposed for 5 min to CSS without NO-donors. After the treatment, cell cultures were washed, trypsinized, pelleted and resuspended in 0.1 M sodium phosphate buffer (pH 7.0), 0.01% Tween-20. Finally, cells were subjected to three cycles of rapid freezing and thawing (−80°C/+25°C), then they were centrifuged for 15 min at 14000 ×*g* and the supernatants were used for assaying lipoxygenase (LOX) activity. Human platelets were isolated from blood according to [15]. Isolated platelets were washed with Ca^{2+}-free CSS and incubated in the same solution in the presence of either SNP or SNAP (500 μM final concentration) for 5 min at room temperature. Paired controls were incubated for the same period of time in Ca^{2+}-free CSS, in which the NO-donors were omitted. After drug exposure, platelets were washed and homogenized as described for the CHP100 cells.

Lipoxygenase assays. Soybean (*Glycine max* (L.) Merrill) lipoxygenase type II (LOX-2) was purified from seeds as reported earlier [16]. LOX-2 dioxygenase activity was determined spectrophotometrically by recording the formation of conjugated hydroperoxides from linoleic acid at 234 nm [17]. A stock solution of linoleic acid (30 mM), dissolved in 1% Tween-20 in water, was diluted to the appropriate final concentration in 0.1 M sodium phosphate buffer (pH 7.0), according to [18]. The effect of inhibitors on linoleic acid dioxygenation by LOX-2 was analysed by Lineweaver-Burk double reciprocal plots, using two different concentrations of each NO-donor to calculate inhibition constants. Enzyme inhibition was also analysed by Yoshino's graphical method [19], in order to distinguish partial from complete inhibitors. The effect of NO-releasing agents on the formation of side products of the LOX-2-catalyzed reaction was assessed by following the formation of oxodienes (*i.e.*, 13-oxo-octadeca-9,11-dienoic plus 13-oxo-trideca-9,11-dienoic acids) at 285 nm [20]. Progress curves of the dioxygenation of 100 μM linoleic acid by LOX-2 were recorded by following polarographically the oxygen consumption [21]. Dioxygenation of linoleic acid by LOX-2 in the presence of hydrogen peroxide was assessed according to [22]. Co-oxidative activity of LOX-2 was determined by the β-carotene (BC) bleaching assay [7]. Briefly, LOX-2 was incubated in 0.1 M sodium phosphate buffer (pH 7.2) containing 40 μM BC, 2 mM linoleic acid, 0.05% Tween-20, and the decrease in absorbance at 460 nm was followed. BC bleaching was quantitated by using the molar absorption coefficient 23000 $M^{-1}\cdot cm^{-1}$ at 460 nm [18]. Peroxidative activity of LOX-2 was determined by tetramethylbenzidine (TMB) oxidation,

TABLE 1
Cell death and Specific Activity of Lipoxygenase (LOX) in Human Platelets and Human CHP100 Neuroblastoma Cells

Sample	Cell death[a]	LOX specific activity[b] (pmol HPETE.min^{-1}.mgP^{-1})	
		Cell homogenate	Intact cell
Human platelets (no addition)	N.D.	340 ± 30 (100)	340 ± 30 (100)
Human platelets (SNP)	N.D.	140 ± 12 (41)	155 ± 15 (45)
Human platelets (SNAP)	N.D.	221 ± 25 (65)	200 ± 18 (59)
CHP100 cells (no addition)	3.9 ± 0.6 (100)	374 ± 35 (100)	374 ± 34 (100)
CHP100 cells (SNP)	12.9 ± 1.3 (331)	221 ± 22 (59)	225 ± 23 (60)
CHP100 cells (SNAP)	9.3 ± 0.3 (238)	254 ± 23 (68)	258 ± 26 (69)
CHP100 cells (NDGA)	12.2 ± 0.7 (313)	197 ± 20 (53)	280 ± 30 (75)

Values in brackets represent percentages of the controls, arbitrarily set to 100.

[a] Cell death is expressed as number of necrotic cells counted (at least in triplicate) every 100 cells (±S.D.). N.D., not determined.

[b] Inhibitors were added up to 500 μM (SN(A)P) and 10 μM (NDGA) final concentrations.

followed at 450 nm [12]. LOX-2 was incubated in 50 mM Tris. HCl buffer (pH 5.7) containing 0.15 mM TMB. Reaction was started by addition of H_2O_2 (1.25 mM final concentration) and the increase of A_{460} was followed. Lipoxygenase activity of human platelets and CHP100 cells was determined on supernatants of cell homogenates centrifuged for 15 min at 14000 × *g*. Cell supernatants were incubated with 80 μM arachidonic acid in 0.1 M sodium phosphate buffer (pH 7.0) at 37°C for 10 min. The reaction products (*i.e.*, hydroperoxyeicosa-6,8,11,14-tetraenoic acid, HPETE) were then extracted as described [23] and quantitated by using the molar absorption coefficient 29500 $M^{-1}\cdot cm^{-1}$at 237 nm [24]. Protein concentration was determined according to [25], using bovine serum albumin as a standard.

Cytotoxicity study. The effect of SNP, SNAP and NDGA on CHP100 cells death was determined as previously reported [14].

NO release. The amount of NO released from sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) was determined by the hemoglobin method [26], and by direct measurements of NO release, performed with the Iso-NO meter according to the manufacturer's instructions (WPI, World Precision Instruments, Inc.; Sarasota, FL, U.S.A.). The reported data are the mean of at least three independent determinations, with S.D. < 10% if not otherwise stated.

RESULTS AND DISCUSSION

Sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) are NO-donors widely used for studying the effect of nitric oxide on cells [2, 3] and enzymes [27]. In agreement with previous data [8] lipoxygenase activity was inhibited by SNP in intact human platelets (Table 1). We extended such observation, showing that also SNAP is an effective inhibitor of platelet LOX,

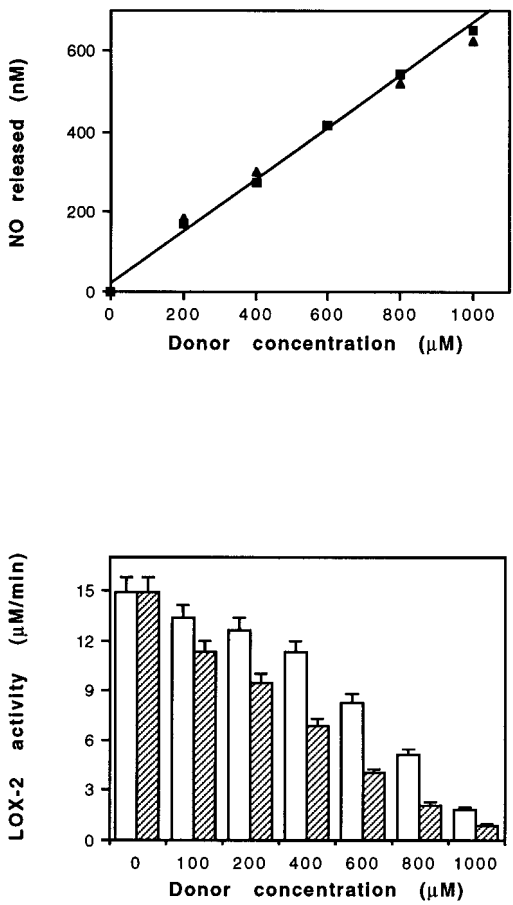


FIG. 1. Upper panel, NO released from SNP (triangles) and SNAP (squares). The values are the mean of three independent determinations (S.D.<10%). Lower panel, inhibition of LOX-2 dioxygenase activity by SNP (hatched bars) and SNAP (empty bars).

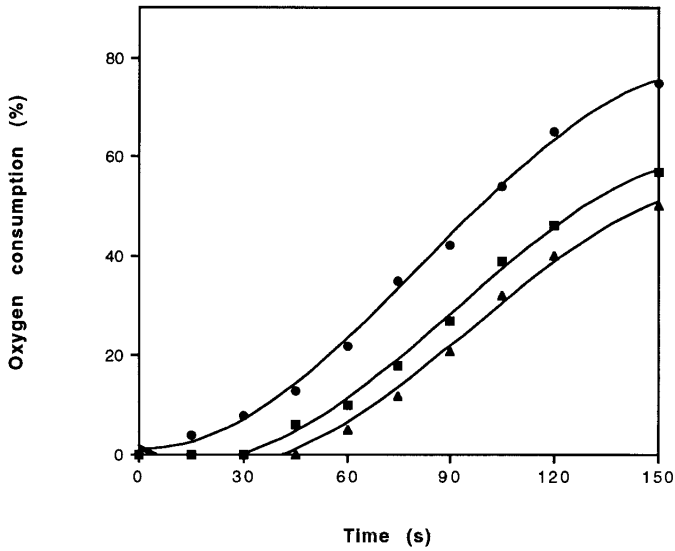


FIG. 2. Progress curves of the linoleic acid dioxygenation catalyzed by LOX-2, in the absence (circles) or in the presence of either 500 μM SNP (triangles) or 500 μM SNAP (squares). The values are the mean of three independent determinations (S.D.<10%).

and that both NO-donors inhibit LOX activity in CHP100 neuroblastoma cells as well (Table 1). In addition, the exposure of platelet and CHP100 cell homogenates to SNP and SNAP yielded a significant inhibition of LOX, which was almost identical to that observed in intact cells (Table 1). This is in keeping with the free diffusion of NO through biological membranes, which allows this radical to reach intracellular targets. Interestingly, the inhibition of LOX in CHP100 cells was obtained at concentrations of NO-donors which cause cell necrosis (Table 1). The sensitivity of the CHP100 cell line to NO [28] may suggest that LOX can be indeed an important target through which NO produces its cytotoxic effects. This hypothesis is supported by the observation that treatment with NDGA, a specific LOX inhibitor [29], yielded a similar CHP100 cell death (Table 1). These results are also consistent with the antiproliferative effects of LOX inhibitors in various human cell lines, often attributable to their ability to suppress mitogenesis [30].

The mechanism of NO interaction with cellular LOX was investigated in more detail by using purified soybean lipoxygenase type II (LOX-2), which has a pH optimum in the cellular physi-

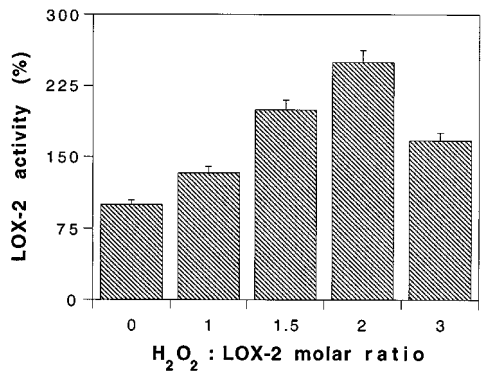


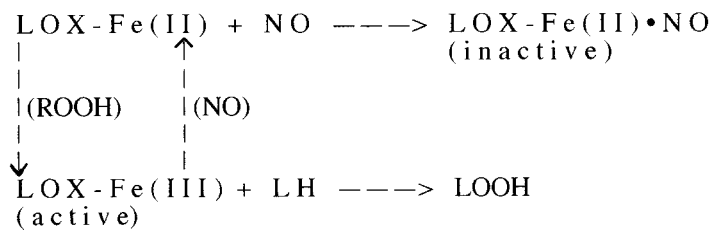
FIG. 3. Effect of hydrogen peroxide on the dioxygenation of 90 μM linoleic acid catalyzed by LOX-2 (0.5 nM). The values are the mean of three independent determinations and are expressed as percentages of the LOX-2 activity.

TABLE 2
Effect of NO-Donors SNP and SNAP on the Cooxidative and Peroxidative Activities of LOX-2, Determined by β-Carotene (BC) Bleaching and Tetramethylbenzidine (TMB) Oxidation, Respectively

Sample	LOX-2 specific activity	
	Co-oxidase (nmol BC.min^{-1}.mgP^{-1})	Peroxidase (nmol TMB.min^{-1}.mgP^{-1})
LOX-2 (no addition)	850 ± 40 (100)	69.4 ± 4.2 (100)
LOX-2 (SNP)	570 ± 30 (67)	39.1 ± 2.3 (56)
LOX-2 (SNAP)	470 ± 25 (55)	34.7 ± 2.1 (50)

Both NO-donors were used at 500 μM final concentration. Values in brackets represent percentages of the controls, arbitrarily set to 100.

ological range. When micromolar concentrations of SNP or SNAP were added to the LOX-2 assay system, a linear release of NO in the nanomolar range could be observed (Fig. 1, upper panel). Under the same experimental conditions, dioxygenation of linoleic acid catalyzed by LOX-2 was inhibited by SNP and SNAP, in a dose-dependent manner (Fig. 1, lower panel). Kinetic analysis showed that both NO-donors acted on the LOX-2 reaction as complete, competitive inhibitors, with inhibition constants (Ki) of 525 μM and 710 μM, for SNP and SNAP respectively. It is reasonable to assume that the inhibition of lipoxygenase is due to the binding of NO to iron. In fact, it has been shown by EPR that NO binds to the ferrous form of type I LOX [31]. It should be taken into account that NO may also reduce ferric LOX to ferrous. If it is the case, from Fig. 1 it is possible to calculate a Ki of NO for LOX of approximately 400 nM. However, it is impossible to rule out a further direct effect of SN(A)P on enzyme activity. In order to get a better insight into the mechanism of NO inhibition, the progress curves of the LOX-2-catalyzed dioxygenation of linoleic acid were analysed. Fig. 2 shows that both NO-donors prolonged the lag phase of the reaction, which may indicate a reduction of Fe(III) to inactive Fe(II) in the LOX-2 catalytic site [32]. On the other hand, physiologically attainable H_2O_2 concentrations were able to stimulate LOX-2, the largest activation being obtained at a H_2O_2:LOX-2 molar ratio of 2:1 (Fig. 3). This finding is similar to the reported H_2O_2 stimulation of type I lipoxygenase activity [22], and is attributable to the oxidation of Fe(II) to Fe(III) by peroxides [13, 22]. Neither NO-releasing agent affected oxodiene formation during the LOX-2-catalyzed dioxygenation of linoleic acid (data not shown), suggesting that NO did not interact with free radicals escaping from the active site during the enzymatic reaction [20, 21]. SNP and SNAP also inhibited the co-oxidative and peroxidative activities of LOX-2 (Table 2). Such LOX activities can play a key-role both in xenobiotic oxidation, especially in those tissues that lack or possess low levels of catalase and glutathione peroxi-



SCHEME 1. The iron oxidation state of LOX-2 during the dioxygenation reaction in the presence of NO or peroxides (ROOH). LH, linoleate; LOOH, linoleate hydroperoxide.

dase, and in glutathione oxidation [29]. Moreover, the peroxidase activity of LOX can contribute to the control of radical reactions and, more in general, to the antioxidant defence of cells [29, 33]. NO cytotoxicity/cytoprotection occurs indeed through the alteration of the cellular antioxidant balance [1–3].

Altogether, these results may be explained by the reaction mechanism reported in scheme I, showing that peroxides and NO have opposite effects on the iron oxidation state, and that NO competes with the substrate (linoleate) for the binding site. This scheme is consistent with EPR spectroscopic data showing that NO forms nitrosyl complexes with ferrous iron [31].

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