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S-nitrosothiols do not induce oxidative stress, contrary to other nitric oxide donors, in cultures of vascular endothelial or smooth muscle cells

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Abstract

Nitric oxide (NO) has been described to exert various anti-atherogenic actions. However, NO, in some cases, has been shown to stimulate the oxidation of low-density lipoprotein (LDL), which constitute an important triggering event in atherosclerosis. Thus, some NO donors, despite their advantages, might also induce oxidative stress. Therefore, the purpose of this study is to examine the effect of three different NO donors on LDL oxidation, in acellular system as well as in cultures of normal endothelial cells or smooth muscle cells, which constitute the two major cellular components of the arterial wall. Sodium nitroprusside oxidized strongly LDL in medium alone as well as in endothelial or smooth muscle cell cultures. Sydnonimine-1 (SIN-1) oxidized LDL already in the absence of cells and enhanced clearly the LDL oxidation in the cultures. *S*-nitroso-*N*-acetylpenicillamine was unable to oxidize LDL in synthetic medium alone as well as in the presence of cells, showing that the amount of superoxide and other reactive oxygen species released by these cells did not suffice, contrary to those liberated by macrophages, to combine to NO providing oxidant activity.

Keywords: Atherosclerosis; Nitric oxide (NO); Low-density lipoprotein (LDL) oxidation; Superoxide; Endothelial cell; Smooth muscle cell

1. Introduction

Nitric oxide (NO) is known to exert various anti-atherogenic effects such as inhibition of vasoconstriction, monocyte adhesion, smooth muscle cell migration and proliferation, platelet adhesion and aggregation (for reviews: Radomski and Salas, 1995; Liu et al., 1998; Jeremy et al., 1999). Therefore, NO-releasing compounds might be of benefit in a variety of cardiovascular disorders including atherogenesis. Some NO-donor drugs are already in widespread clinical use, in particular the organic nitrates (e.g., nitroglycerin, isosorbide dinitrate or isosorbide-5-mononitrate), the organic nitrites (e.g., amyl nitrite), the ferrous nitro complexes (e.g., sodium nitroprusside) and the sydnonimines (e.g., molsidomine) (reviewed in Tullett and Rees, 1999). Nitroglycerin and amyl nitrite are widely used in both prophylaxis and treatment of angina pectoris. Sodium nitroprusside is used in treating hypertensive emergencies as well as severe cardiac failure and never for a chronic treatment (reviewed in Al-sa'doni and Ferro, 2000). Other commercialized NO-donor drugs contain as active agent molsidomine, which is degraded in the liver to 3-morpholinosydnonimine (SIN-1) and liberates NO. Although these drugs are effective, they may however present some drawbacks. Indeed, continuous organic nitrate infusions can rapidly develop tolerance in patients, with reduced therapeutic effect of the drugs with time. Moreover, administration of sodium nitroprusside may be toxic since this drug is converted to cyanide and thiocyanate (Yamamoto, 1992; Johanning et al., 1995). Toxic accumulation of cyanide may lead to severe lactic acidosis, arrhythmia and excessive hypotension. SIN-1 decomposition can give concomitant large amounts of NO and superoxide leading to the formation of peroxynitrite, which has been associated to cytotoxic effects through oxidation and nitration reactions (Hogg et al., 1992; Ischiropoulos and Al-Mehdi, 1995; Szabo and Ohshima, 1997).

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Recently, *S*-nitrosothiols have been recognized as novel NO-donor drugs (Wang et al., 2000). Some of these occur naturally in vivo such as *S*-nitroso-albumin, *S*-nitroso-L-cysteine or *S*-nitroso-gluthatione. Others have been synthesized chemically like *S*-nitroso-*N*-acetylpenicillamine, *S*-nitroso-captopril or *S*-nitroso-mercaptoethylamine (reviewed in Upchurch et al., 1996; Sogo et al., 2000). *S*-nitrosothiols offer advantages over the existing drugs since they do not appear to engender nitrate tolerance or cyanide poisoning (Al-sa'doni and Ferro, 2000). In contrast to the other NO donors described above, only initial small clinical studies have been reported for *S*-nitrosothiols, suggesting that they may be valuable therapeutic agents in a variety of cardiovascular disorders.

However, NO by itself (Chang et al., 1994; Wang et al., 1994) or in combination with superoxide anions (Darley-Usmar et al., 1992) has been described to stimulate the oxidation of LDL, which constitute a critical triggering event in atherogenesis (for reviews: Holvoet and Collen, 1994; Berliner and Heinecke, 1996). Thus, *S*-nitrosothiols in spite of their advantages could also contribute to oxidative stress. Therefore, the purpose of this work is (1) to examine the effect of *S*-nitroso-*N*-acetylpenicillamine, known as an *S*-nitrosothiol on native LDL oxidation, and (2) to compare the effect of *S*-nitroso-*N*-acetylpenicillamine with those of two other known NO donors: SIN-1 and sodium nitroprusside on LDL oxidation either in acellular condition or in the presence of normal endothelial cells or smooth muscle cells, which constitute the two major cellular components of the artery wall. The cells were derived from porcine pulmonary arteries.

2. Materials and methods

2.1. Isolation of endothelial and smooth muscle cells

Cell cultures have been performed according to the method described by Kinard et al. (1997). Pulmonary arteries were dissected from Largewhite pigs weighing 80–100 kg, age about 6 months, freshly slaughtered. Arteries were transported in cold phosphate-buffered saline (PBS) containing glucose 11 mM, streptomycin 500 μg/ml, penicillin 500 U/ml and nystatin 40 U/ml. Endothelial and smooth muscle cells were collected about 2 h after removal of the arteries. Arteries were first rinsed with PBS and then perfused with collagenase (0.5 mg/ml, 186 U/mg, CLS type I, Whortington, Freehold, NJ) in a mixture (1:1) of Dulbecco's modified Eagle's medium (DME, Gibco, Life Technologies Paisley, UK) and Ham's F12 (Gibco), supplemented with penicillin 200 U/ml, streptomycin 200 μg/ml and nystatin 40 U/ml (Gibco), during 10 min, to detach the endothelial cells. The same arteries were afterwards perfused again with collagenase (2 mg/ml) for 1 h, providing a mixture of endothelial cells and smooth muscle cells that were discarded. Finally, a third digestion in similar conditions was carried out and provided smooth muscle cells.

2.2. Culture of endothelial and smooth muscle cells

At the end of the first digestion, arteries were rinsed with DME/F12 medium containing 10% (v/v) fetal bovine serum (Gibco) and antibiotics (penicillin 200 U/ml, streptomycin 200 μg/ml and nystatin 40 U/ml), and the cell suspension was centrifuged for 10 min at 1000 rpm. The pellet containing the endothelial cells was harvested in the same medium and inoculated at a ratio of one artery equivalent per 35-mm Petri dish (3001 Falcon, Becton Dickinson Labware Plymouth, UK), precoated with type I collagen at 30 μg/ml (collagen S type I, Boehringer Mannheim, Germany). The cell suspension obtained from the third digestion was also centrifuged and the harvested smooth muscle cells were inoculated in 75-cm^2 flasks (3084 Falcon), precoated with collagen S type I (Boehringer).

Cells were grown at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 in air in DME/F12 medium supplemented with 10% fetal bovine serum and antibiotics. For endothelial cells, 100 ng/ml of fibroblast growth factor (b-FGF, Bachem Feinchemikalien, Bubendorf, Switzerland) were added to the medium. The culture media were changed the next day (at that time, the nystatin was omitted) and then every other day.

After 1 week, endothelial and smooth muscle cells were detached with trypsin 2 mg/ml (Difco Laboratories, Detroit, MI, USA) and EDTA 5.3 mM (Gibco) in Earle's solution without calcium and magnesium, for 3–5 min at 37 °C. They were transferred and cultivated for 1 week in 75-cm^2 flasks (one Petri dish into one flask for endothelial cells and one flask into five flasks for smooth muscle cells, respectively) precoated with type I collagen.

At the second transfer, smooth muscle cells were transferred at a density of 35,000 cells/cm^2 and endothelial cells at a density of 50,000 cells/cm^2 on microporous poly-(ethylene terephtalate) (PET) membranes (Whatman, Louvain-La Neuve, Belgium) with 1.6×10^6 pores of 1-μM diameter/cm^2 for 1 week. Before cell inoculation, the surface of the filter was irradiated at 254 nm for 1 h at 30 °C. Membranes were assembled in culture inserts, consisting of two concentric rings, made of polycarbonate, giving an inner surface of 5 cm^2 with a height of 13 mm. Thereafter, membranes were sterilized by autoclaving 15 min at 120 °C and were coated overnight at 37 °C with 30 μg/ml of type I collagen.

After 1 week, cells were transferred in serum-free, chemically defined medium for 24 or 48 h. This medium was prepared on a customary basis by Gibco and consisted of a 5:5:1 (v/v/v) mixture of Iscove's Dulbecco's modified Eagle's, Ham's F12 and NCTC 135 media. It contains 25 mM glucose, 6 mM glutamine, 50 μM ethanolamine,

36 nM $NaHCO_3$, 0.16 nM insulin (Gibco), 164 nM epidermal growth factor (Boehringer), trace elements (Mn, Mo, Ni, Si, Sn and Va, as a mixture from Gibco), 150 nM of albumin complexed to linoleic acid (molar ratio 5:1; Sigma St. Louis, MO), 2 nM tri-iodothyronine (Sigma), 100 nM hydrocortisone (Sigma) and 39 nM b-FGF (Bachem).

2.3. Isolation and oxidation of LDL

LDL were isolated from plasma of human healthy donors by sequential preparative ultracentrifugation in a Vti 50 Beckman rotor (Beckman L8-70 ultracentrifuge, Fullerton, CA, USA). Briefly, two successive runs were performed at 47,000 rpm for 16 and 3 h, respectively. Lipoproteins were then dialyzed against a Tris 10 mM–NaCl 0.15 M buffer containing 0.1 g/l EDTA at 4 °C. After a last dialysis against PBS without EDTA, the LDL preparations were sterilized by filtration, using a 0.22-μm filter (Millipore, Brussels, Belgium).

LDL were moderately oxidized by incubation with 10 μM $CuSO_4$ for 20 h at 4 °C (Ox-LDL). Oxidation was stopped by dialyzing LDL against PBS containing EDTA at 50 μM. Malondialdehyde-modified LDL (MDA-LDL) was prepared by incubating native LDL with 0.1 M phosphate buffer pH 6.4 (1:1) containing 0.2 M tetramethoxypropane and 4% (v/v) 12 M HCl, for 3 h at 37 °C (Haberland et al., 1982). The preparation was dialyzed twice against PBS containing EDTA 16 h at 4 °C and 2 h at 4 °C, respectively. The protein content of the different LDL preparations was determined by the method of Lowry et al. (1951).

2.4. Characterization of LDL

Electrophoretic mobility of LDL preparations was determined on agarose gel (Paragon, SPE Gel, Beckman), at 100 V, for 25 min, in barbital buffer. The gel was then fixed in 10% acetic acid–methanol for 3 min and dried. LDL proteins were stained with Coomassie Blue (Brillant Blue, ACROS, NJ, USA). Electrophoresis of LDL was also performed after cellular contact with or without NO donors. The supernatants were collected and centrifuged at 1000 rpm for 8 min. Their protein contents were determined by the micro-Bradford assay with bovine serum albumin as standard. Thereafter, the samples were diluted in order to apply the same protein concentration in the different wells of gel.

Malondialdehyde content of LDL was determined by analysing thiobarbituric Acid-Reactive Substances (TBARS) expressed as nanomoles of malondialdehyde per milligram protein (Maseki et al., 1981). Fifty microliters of LDL, diluted in 500 μl H_2O, were mixed with 1 ml of 20% (w/v) trichloroacetic acid and 1 ml of 1% (w/v) thiobarbituric acid in 0.3 N NaOH. The samples were heated in a water bath at 95 °C for 30 min, cooled and then centrifuged at 2800 rpm for 10 min. Optical density of the supernatants was read at 532 nm. 1,1,3,3-Tetramethoxypropane (malonaldehyde bis (dimethyl acetal)) (Sigma) that produces malondialdehyde by acid hydrolysis was used as a standard. For measuring TBARS in culture supernatants, 2 ml of butanol were added to each sample. Tubes were mixed and centrifuged at 2500 rpm for 10 min. Then, supernatants were collected and fluorescent emission was measured after excitation at 515 nm in a kontron fluorimeter (Zürich, Switzerland).

Conjugated dienes have been measured at 234 nm in a Beckman DU 8 spectrophotometer (Esterbauer et al., 1989).

Lipid hydroperoxides formed during oxidation of LDL have been measured according to the method of El-Saadani et al. (1989). Briefly, 100 μl of a lipoprotein solution at 0.1 mg protein/ml were mixed with 900 μl of the color reagent containing 0.2 M potassium phosphate, pH 6.2, 0.12 M potassium iodide, 0.15 mM sodium azide, 2 g/l mono[*p*-(1,1′,3,3′-tetramethyl-butyl)-phenyl]ether, 0.1 g/l alkylbenzyl-dimethylammonium chloride and 10 μM ammonium molybdate (all products from Sigma) for 1 h at 30 °C.

The absorbance was measured at 365 nm against the color reagent as blank, with a detection limit of 10–12 nmol hydroperoxides/mg protein.

Lysophosphatidylcholine levels were measured in a total lipid extract of LDL by thin layer chromatography on Silica 60G plates (Merck; 20 × 20 cm; 0.25 mm) in chloroform/methanol/acetic acid/0.9% (w/v) NaCl (100:50:16:5, v/v). After visualization with iodine vapor, lysophosphatidylcholine spots were scraped in Pyrex tubes, wet ashened, complexed with ammonium molybdate and extracted into *n*-butanol for reading at 820 nm (Van Veldhoven and Bell, 1988).

LDL, freshly prepared, was injected at a concentration of 100 μg protein/ml in the upper compartment of inserts either in BDM alone or in the presence of endothelial cells and smooth muscle cells, with or without NO donors at a concentration of 100 μM, and with or without superoxide dismutase, a superoxide scavenger at 500 u/ml (Boehringer) or desferrioxamine, an iron chelator at 120 μM. After 24 h, the supernatants were collected to quantify the LDL oxidation (TBARS assay) and to measure the corresponding NO production (diaminonaphtalene assay).

2.5. LDH assay

The lactate dehydrogenase release into the cell culture medium was used to assess the cytotoxic effect of LDL and/or NO donors. It was measured using a commercially available kit (LDH-L20, Sigma). Cytotoxicity was expressed as percentage LDH activity obtained from the cell-free medium compared to total LDH activity present in corresponding cells (incubated with 0.1% Triton X-100, Sigma) plus medium.

Table 1
Compounds released by the three NO donors which may be involved in LDL oxidation and experimental treatments

NO donors	Compounds released		Additional controls
SNAP	$^{\cdot}NO$		
SIN-1	$^{\cdot}NO$		
	$OO^{\cdot -}$	$\xleftarrow{-}$	SOD
			DFO
SNP	$^{\cdot}NO$		
	Fe^{2+}	$\xleftarrow{-}$	DFO
			SOD

SOD: superoxide dismutase, DFO: desferrioxamine.

2.6. Nitrite assay

Nitrite, a stable end product of NO oxidation, was measured by a fluorometric procedure, based upon the reaction of nitrite with the 2,3-diaminonaphtalene (Molecular Probes, Eugene, OR, USA) to form the fluorescent product, 1-(*H*)-naphthotriazole. This method allows measurement of nitrite at levels as low as 10 nM (Misko et al., 1993). In order to measure total NO production in the culture media, nitrate was converted to nitrite by the action of nitrate reductase from *Aspergillus* species (Sigma). Samples (100 μl) were incubated with 100 μl of 20 mM Tris buffer, pH 7.6 containing in final concentration 80 μM NADPH (to initiate the reaction) and 56 mU of enzyme. The reaction was stopped after 5 min at room temperature by dilution with 1800 μl ultrapure water, followed by the addition of the diaminonaphtalene reagent (200 μl of a 0.05 mg/ml solution in 0.62 M HCl). Finally, 100 μl of 2.8 M NaOH were added to each sample. Nitrite concentration was determined by using sodium nitrite (Sigma) as standard. The fluorescence was measured in a kontron fluorimeter. The excitation and emission wavelengths were 365 and 450 nm, respectively.

2.7. Statistical analysis

All values were expressed as means ± S.E.M. Statistically significant differences between means were assessed using the Scheffe post hoc test after one- or two-way variance analysis (ANOVA) when appropriate; differences were considered significant when $P < 0.05$.

3. Results

3.1. NO release and cytotoxicity of NO donors

Three NO donors have been used (Table 1). They release either NO alone (*S*-nitroso-*N*-acetylpenicillamine) or NO in combination with superoxide anion (SIN-1) or with iron (sodium nitroprusside). The three NO donors have been tested in acellular condition but also in the presence of cells, which are supposed to release reactive oxygen species.

The serum-free BDM medium was incubated with or without cells for 24 or 48 h. Thereafter, supernatants were collected to quantify NO production by the diaminonaphtalene method (see Materials and methods). Both endothelial cells and smooth muscle cells were able to produce small amounts of NO (Table 2). The three NO donors were incubated in BDM medium or in the presence of endothelial cells or smooth muscle cells, cultivated in the same medium. NO production as well as potential cytotoxicity of NO donors were evaluated. SIN-1 and *S*-nitroso-*N*-acetylpenicillamine released large quantities of NO rapidly, whereas sodium nitroprusside released smaller amounts of NO when used at the same concentration (Table 2). LDH activity in smooth muscle cell supernatants was, respectively, 12 ± 0.2% and 31 ± 1.3% ($n = 3$) of the total activity, when sodium nitroprusside was added for 24 and 48 h

Table 2
Nitrite content in culture media with or without endothelial cells (EC) and smooth muscles (SMC), in the presence of NO donors

	Medium 24 h	Medium 48 h	EC 24 h	EC 48 h	SMC 24 h	SMC 48 h
Control	0	0	7.5 ± 1.1	11.6 ± 1.1	6.6 ± 0.7	12.1 ± 1.4
SNAP 10 μM	27.5 ± 0.1	31.6 ± 5.1	33.1 ± 0.8	31.3 ± 0.1	27 ± 1.3	30.4 ± 0.8
SNAP 100 μM	124.4 ± 3.1	115.8 ± 0.2	176.3 ± 1.6	179.7 ± 5.8	160.7 ± 4.3	164.8 ± 3.5
SNAP 1 mM	1200.9 ± 39	1303.5 ± 8.2	970.5 ± 11.4	1032 ± 4	943.7 ± 19.8	942.4 ± 9.4
Control	0	0	3.3 ± 1.1	4.9 ± 1.5		
SIN-1 10 μM	26.5 ± 0.6	27.5 ± 0.3	34.6 ± 2.5	34 ± 0.6		
SIN-1 100 μM	297.9 ± 3.7	294.1 ± 4.5	266.1 ± 6.6	269.5 ± 10.8		
SIN-1 1 mM	2744.3 ± 15.4	2754.5 ± 2	2727.3 ± 45	2776.2 ± 49.4		
Control	0	0	17.7 ± 0.1	20.6 ± 0.8	3.5 ± 0.6	4.5 ± 0.6
SNP 10 μM	31.2 ± 1.8	42.7 ± 0.1	24.5 ± 0.6	38.6 ± 0.8	32 ± 0.6	32.9 ± 1.3
SNP 100 μM	59.3 ± 0.6	91.3 ± 0.1	58 ± 0.7	74.6 ± 1.2	65.8 ± 1.5	114.5 ± 3.6
SNP 1 mM	119.5 ± 13	182.9 ± 2.4	91.3 ± 11.1	143.6 ± 10.6	220.3 ± 5.2	251.4 ± 6.7

Nitrite content was expressed as nanomoles/insert (means ± SEM); $n = 3$–4 independent assays. Medium: basal defined medium (BDM); SNAP: *S*-nitroso-*N*-acetylpenicillamine; SIN-1: 3-morpholinosydnonimine; SNP: sodium nitroprusside.

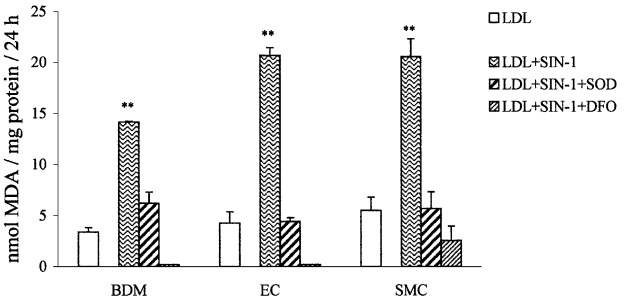

Fig. 1. Effect of 3-morpholinosydnonimine (SIN-1) at 100 μM on LDL oxidation quantified by TBARS assay after 24 h in BDM medium, in the absence of cells (BDM) or in the culture medium of endothelial cells (EC) or smooth muscle cells (SMC); SOD: superoxide dismutase at 500 u/ml, DFO: desferrioxamine at 120 μM; means ± S.E.M., $n = 3–12$. Data were submitted to analysis of variance, followed by Scheffe's test; $^{**}P < 0.01$.

at 1 mM. No significant cytotoxicity was observed when it was added at 10 and 100 μM for 24 or 48 h in endothelial cells and smooth muscle cell cultures. Moreover, no significant apoptosis was detected by the terminal deoxy-uridine triphosphate nick end labeling method in endothelial cell cultures in the presence of sodium nitroprusside at 100 μM. On the basis of these cytotoxicity data, we have chosen to use a concentration of sodium nitroprusside of 100 μM for further experiments. *S*-nitroso-*N*-acetylpenicillamine and SIN-1 at 100 μM did not induce cytotoxicity in our cellular cultures and were therefore also used at the same concentration as sodium nitroprusside.

3.2. LDL oxidation in the absence of NO donors

Native LDL before addition to the culture medium contained 1.2 ± 0.2 nmol TBARS/mg protein ($n = 4$), 16 ± 0.5 nmol lipid hydroperoxides/mg protein ($n = 3$), 36 ± 10 nmol lysophosphatidylcholine/mg protein ($n = 4$) and showed a diene-absorption at 234 nm of 0.18. After 24-h incubation in BDM alone, LDL exhibited a small increase in TBARS to 3.4 ± 0.4 nmol TBARS/mg protein ($n = 11$). The value was slightly higher in the presence of endothelial cells or smooth muscle cells with, respectively, 4.3 ± 1.1 nmol TBARS/mg protein ($n = 11$) or 5.5 ± 1.3 nmol TBARS/mg protein ($n = 12$). LDL oxidation in BDM was completely inhibited in the presence of superoxide dismutase (at 100 μg/ml) or desferrioxamine (at 120 μM) with undetectable TBARS values.

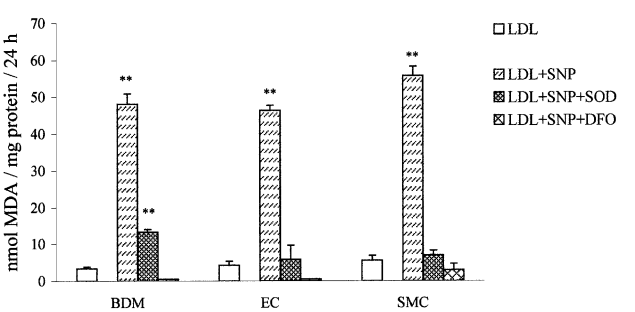

Fig. 2. Effect of sodium nitroprusside (SNP) at 100 μM on LDL oxidation quantified by TBARS assay after 24 h in BDM medium, in the absence of cells (BDM) or in the culture medium of endothelial cells (EC) or smooth muscle cells (SMC); SOD: superoxide dismutase at 500 u/ml, DFO: desferrioxamine at 120 μM; means ± S.E.M., $n = 3–12$. Data were submitted to analysis of variance, followed by Scheffe's test; $^{**}P < 0.01$.

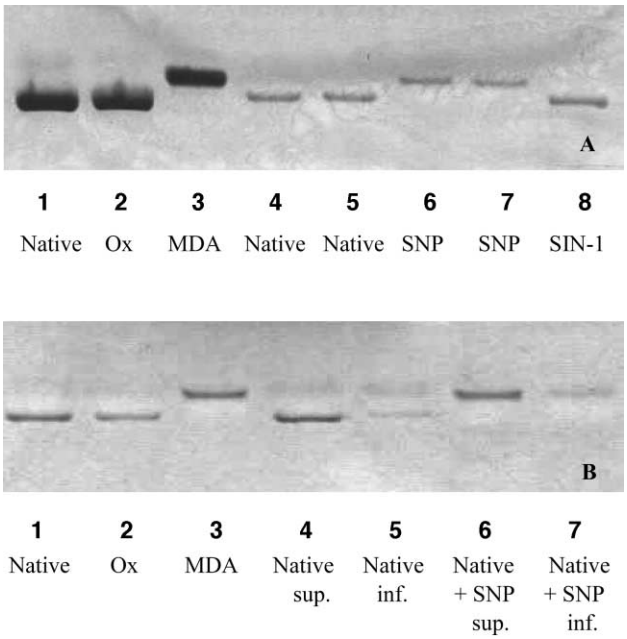

Fig. 3. (A) Effect of sodium nitroprusside (SNP, 6–7) and sydnonimine 1 (SIN-1,8) on native LDL oxidation detected by agarose gel electrophoresis, in cell-free medium compared to the control: LDL in culture medium without NO donors (4–5). References: native LDL (1), Ox-LDL (2), MDA-LDL (3) before incubation in the culture medium. (B) Effect of sodium nitroprusside (SNP) on native LDL oxidation in the presence of smooth muscle cells, detected by agarose gel electrophoresis, in the upper (sup. 6) or the lower (inf. 7) compartment of the insert compared to the control: LDL in BDM without NO donor, in the upper (sup. 4) or the lower (inf. 5) compartment of the insert. References: native LDL (1), Ox-LDL (2), MDA-LDL (3).

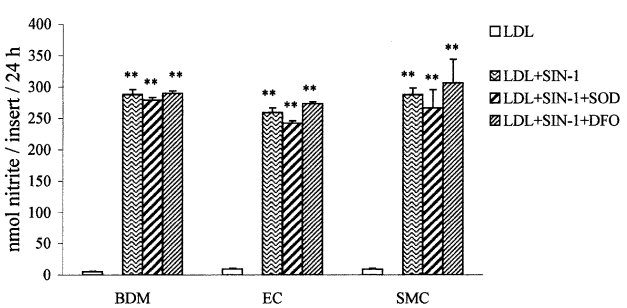

Fig. 4. NO production in the presence of LDL with or without 3-morpholino-sydnonimine (SIN-1) after 24 h in BDM medium, in the absence of cells or in the culture medium of endothelial cells (EC) or smooth muscle cells (SMC); means ± S.E.M., $n = 3–12$. Data were submitted to analysis of variance, followed by Scheffe's test; $^{**}P < 0.01$.

3.3. LDL oxidation in the presence of NO donors

S-nitroso-*N*-acetylpenicillamine was unable to oxidize LDL in BDM alone (5.9 ± 1.5 nmol TBARS/mg protein, $n = 5$) vs. control LDL of 3.4 ± 0.4 nmol TBARS/mg protein ($n = 11$). *S*-nitroso-*N*-acetylpenicillamine had no more activity in cellular cultures, showing TBARS values expressed in nmol/mg protein of 5.3 ± 1.9 ($n = 6$) vs. control LDL of 4.3 ± 1.1 ($n = 11$) in endothelial cell supernatants and 8.5 ± 0.2 ($n = 5$) vs. control LDL of 5.5 ± 1.3 ($n = 12$) in smooth muscle cell supernatants. SIN-1 stimulated already LDL oxidation in BDM alone and, to a larger extent, in endothelial cells and smooth muscle cells cultures (Fig. 1). Sodium nitroprusside was the NO donor, which exhibited the strongest pro-oxidant effect, which resulted in a dramatic stimulation of TBARS production in acellular system as well as in the presence of cells (Fig. 2). The stimulatory effect of sodium nitroprusside on LDL oxidation was also detected and confirmed by electrophoresis on agarose gel (Fig. 3). Electrophoretic mobility of malondialdehyde-modified LDL which contained 117 ± 3.2 nmol TBARS/mg protein ($n = 7$), 15 ± 5.4 nmol lipid hydroperoxides/mg protein ($n = 3$), and 63 ± 13 nmol lysophosphatidylcholine/mg protein ($n = 3$) was used as positive control and compared to that of native LDL or copper-oxidized LDL which contained 8 ± 2 nmol TBARS/mg protein ($n = 4$), 149 ± 4 nmol lipid hydroperoxides/mg protein ($n = 3$), and 65 ± 13 nmol lysophosphatidylcholine/mg protein ($n = 3$). No change in the electronegativity was observed when native LDL was inoculated in medium alone or in the presence of smooth muscle cells, whereas a significant increase was detected in the presence of sodium nitroprusside at 100 μM in these two conditions (Fig. 3A,B). In order to examine if superoxide anions or iron were needed to induce LDL oxidation in the presence of, respectively, SIN-1 and sodium nitroprusside, superoxide dismutase at 100 μg/ml or desferrioxamine at 120 μM were added to the media. SIN-1 and sodium nitroprusside-stimulated oxidation of LDL in the medium alone or in the presence of cells were decreased by superoxide dismutase and inhibited by desferrioxamine (Figs. 1 and 2). As shown in Figs. 4 and 5, desferrioxamine did not reduce NO production by SIN-1 or sodium nitroprusside in the absence or in the presence of cells. NO production by SIN-1 was not affected by superoxide dismutase (Fig. 4), whereas NO production by sodium nitroprusside was twofold diminished by superoxide dismutase both in the absence or in the presence of cells (Fig. 5).

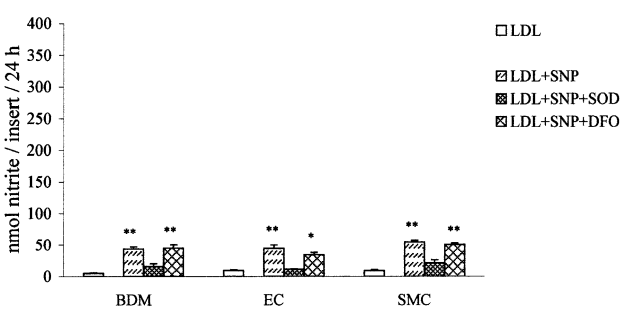

Fig. 5. NO production in the presence of LDL with or without sodium nitroprusside (SNP) after 24 h in BDM medium, in the absence of cells or in the culture medium of endothelial cells (EC) or smooth muscle cells (SMC); means ± S.E.M., $n = 3$–12. Data were submitted to analysis of variance, followed by Scheffe's test; $^{*}P < 0.05$, $^{**}P < 0.01$.

4. Discussion

Relatively little is known about the effect of *S*-nitrosothiols on native LDL oxidation in normal endothelial cells and smooth muscle cells. Here, we demonstrate that *S*-nitroso-*N*-acetylpenicillamine is not able to modify LDL either in acellular system or in the presence of cells. It has been shown that other *S*-nitrosothiols such as *S*-nitrosogluthatione could protect endothelial cells from the toxic effect of oxidized LDL (Struck et al., 1995). In addition, *S*-nitroso-*N*-acetylpenicillamine was reported to be useful in the treatment of heart failure, reducing myocardial oxygen consumption (Mital et al., 1999), inducing vasodilation in rat femoral arteries (Megson et al., 1997) and inhibiting platelet activation and aggregation through cGMP accumulation (Salas et al., 1994; Gordge et al., 1998). Taken together, these observations suggest that *S*-nitrosothiols could limit the progression of vascular disorders such as atherogenesis, without increasing oxidative stress.

In contrast, sodium nitroprusside, another NO donor which releases NO and iron, stimulated strongly LDL oxidation in cell-free medium in our experiments and at a similar rate in the presence of vascular cells. This contrasts with the results obtained in mouse peritoneal macrophages and in mouse macrophage cell lines (RAW 264.7 cells) where the oxidation of LDL in the presence of sodium nitroprusside at 100 μM was, respectively, fourfold and twofold increased compared to the cell-free system (Hogg et al., 1995). The toxicity mediated by sodium nitroprusside, which is actually used in clinical studies, has been principally reported to be due to cyanide and thiocyanate accumulation in the body. Nevertheless, some studies reported that coadministration of sodium thiosulfate with sodium nitroprusside effectively and safely prevents cyanide toxicity (Yamamoto, 1992; Johanning et al., 1995) and then encouraged its clinical application, at least under controlled infusions. Here, we demonstrate that sodium nitroprusside can also induce a significant oxidative stress in the presence of normal endothelial cells or smooth muscle cells, in accordance with some studies which reported the same effect of sodium nitroprusside but in nigrostriatal dopamine neurons (Rauhala et al., 1998), hepatocytes (Niknahad and O'Brien, 1996) or macrophages (Hogg et al., 1995). Our result shows that sodium nitro-

prusside would have to be used carefully in therapeutic vascular treatments, especially under conditions of high concentration and prolonged administration. In order to elucidate the mechanism of oxidation by sodium nitroprusside, superoxide dismutase as superoxide scavenger or desferrioxamine as iron chelator was added to the culture medium. Superoxide dismutase diminished the pro-oxidant effect of sodium nitroprusside. Since superoxide has been reported to increase iron release from sodium nitroprusside and therefore its pro-oxidant activity (Hogg et al., 1995), the inhibitory effect of superoxide dismutase on sodium nitroprusside-induced LDL oxidation could be due to its scavenging activity on superoxide. This might be true in the presence of cells, but the reduction by superoxide dismutase of the sodium nitroprusside-induced LDL oxidation is observed also in acellular system and thus in principle in absence of superoxide. This suggests that superoxide dismutase reduces the pro-oxidant activity of sodium nitroprusside by another mechanism than scavenging superoxide, at least in our experimental conditions. It is more likely that ferric ion rather than NO mediates the pro-oxidative properties of sodium nitroprusside. Indeed, by measuring the formation of the ferrozine–iron complex, Hogg et al. (1995) observed that superoxide dismutase partially inhibited both cell-dependent and cell-independent iron releases from SNP. Moreover, we observed that desferrioxamine did not alter NO release from sodium nitroprusside but significantly inhibited the sodium nitroprusside-stimulated LDL oxidation. Taken together, these findings suggest that ferric ion rather than NO are implicated in the pro-oxidant activity of SNP. This result is in accordance with some reports recently described (Rauhala et al., 1998) and in contrast with older studies showing that toxicity of sodium nitroprusside could be mediated by its NO moiety (Chen et al., 1991; Dawson et al., 1991). As suggested previously (Niknahad and O'Brien, 1996), the coadministration of sodium nitroprusside with antioxidant enzymes like superoxide dismutase or iron chelator like desferrioxamine could prevent lipid peroxidation induced by sodium nitroprusside.

As a matter of fact, the other NO donor SIN-1 stimulated also the LDL oxidation in the absence of cells in agreement with other studies (Darley-Usmar et al., 1992; Hogg et al., 1992). SIN-1-stimulated oxidation of LDL was inhibited by superoxide dismutase suggesting that superoxide ions play an important role in the mechanism of oxidation by SIN-1 and that NO moiety derived from SIN-1 was not able to stimulate LDL oxidation alone. Since SIN-1 liberates superoxide and NO (Hogg et al., 1992) simultaneously, the pro-oxidant action of SIN-1 may depend on the formation of peroxynitrite. Indeed, some studies suggest that peroxynitrite is the primary oxidant formed from SIN-1 decomposition and that it could be the main molecule responsible for the pro-oxidant activity of SIN-1 (Castro et al., 1996; Crow, 1997). The LDL oxidation by SIN-1 was considerably decreased in the presence of superoxide dismutase, as was previously reported (Darley-Usmar et al., 1992). Therefore, NO probably in the presence of appreciable amounts of H_2O_2 (due to the action of superoxide dismutase) was no longer able to stimulate LDL oxidation. In contrast with our results, recent studies demonstrated that under conditions with elevated levels of NO and hydrogen peroxide, superoxide dismutase caused production of the peroxynitrite which could therefore enhance LDL oxidation (McBride et al., 1999). The authors proposed as plausible mechanism, a partial reversal of the dismutation reaction in the presence of superoxide dismutase and hydrogen peroxide, causing formation of superoxide through the reduction of the copper ion in Cu/Zn–superoxide dismutase. The superoxide so produced could then rapidly react with added NO to form peroxynitrite and the copper in Cu/Zn–superoxide dismutase would have to be reoxidized probably by NO itself, in order for the reaction to be catalytic. The LDL oxidation by SIN-1 was also inhibited in the presence of desferrioxamine. Indeed, desferrioxamine has been reported to inhibit peroxynitrite-dependent oxidation (Castro et al., 1996). A possible explanation is the inhibition of the oxidative chemistry of peroxynitrite by reaction of the hydroxamic acid moieties with *trans*-peroxynitrous acid (Denicola et al., 1995). Importantly, SIN-1 stimulated significantly in a more efficient manner the LDL oxidation in the presence of vascular cells than in cell-free medium. The additional oxidative effect was similar for endothelial cells and smooth muscle cells, at equivalent cell density. This effect might be induced by the superoxide generated both from cells and by SIN-1, but also by other reactive oxygen species released from cells. Another possible explanation could be the pro-oxidant action of peroxynitrite generated from SIN-1 not only on LDL exogeneously added to the culture medium but also on lipids in cell membranes. Peroxynitrite anion and peroxynitrous acid have been indeed reported to diffuse in cell membranes, respectively, through ion channel and passive mechanisms (Denicola et al., 1998).

Endothelial cells or smooth muscle cells in the absence of SIN-1 did not enhance the light LDL oxidation observed in the medium alone containing only small amounts of transition metal ions (1.3 μM of Fe^{2+} and 4.4 nM of Cu^{2+}). Thus, the amount of superoxide and other reactive oxygen species released by endothelial cells or smooth muscle cells did not suffice, contrary to those liberated by macrophages, to combine to NO providing oxidant activity.

In conclusion, we demonstrated that the *S*-nitroso-*N*-acetylpenicillamine SNAP does not induce LDL oxidation in the medium alone as well as in the presence of normal endothelial cells or smooth muscle cells, while sodium nitroprusside and SIN-1 stimulated significantly LDL oxidation either in the absence or presence of vascular cells. This result shows that *S*-nitrosothiols did not induce oxidative damage contrary to the two other NO donors and thus

encourage their clinical applications in the therapeutic treatment of atherogenesis. Further investigations are required for better understanding of the biological relevance and pharmacological potential of this very interesting family of NO donors.

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