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Salicylate inhibits LDL oxidation initiated by superoxide/nitric oxide radicals

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Abstract Simultaneously produced superoxide/nitric oxide radicals ($O_2^{\bullet-}$/NO$^\bullet$) could form peroxynitrite ($OONO^-$) which has been found to cause atherogenic, i.e. oxidative modification of LDL. Aromatic hydroxylation and nitration of the aspirin metabolite salicylate by $OONO^-$ has been reported. Therefore we tested if salicylate may be able to protect LDL from oxidation by $O_2^{\bullet-}$/NO$^\bullet$ by scavenging the $OONO^-$ reactive decomposition products. When LDL was exposed to simultaneously produced $O_2^{\bullet-}$/NO$^\bullet$ using the sydnonimine SIN-1, salicylate exerted an inhibitory effect on LDL oxidation as measured by TBARS and lipid hydroperoxide formation and alteration in electrophoretic mobility of LDL. The cytotoxic effect of SIN-1 pre-oxidised LDL to endothelial cells was also diminished when salicylate was present during SIN-1 treatment of LDL. Spectrophotometric analysis revealed that salicylate was converted to dihydroxybenzoic acid (DHBA) derivatives in the presence of SIN-1. 2,3- and 2,5-DHBA were even more effective to protect LDL from oxidation by $O_2^{\bullet-}$/NO$^\bullet$. Because $O_2^{\bullet-}$/NO$^\bullet$ can occur in vivo, the results may indicate that salicylate could act as an efficacious inhibitor of $O_2^{\bullet-}$/NO$^\bullet$ initiated atherogenic LDL modification, thus further supporting the rationale of aspirin medication regarding cardiovascular diseases.

Key words: LDL oxidation; Superoxide; Nitric oxide; Antioxidant; Aspirin; Salicylate; SIN-1

1. Introduction

There is some experimental evidence that the oxidative modification of LDL plays a pathophysiological role in the onset of atherogenesis [1]. This observation has led to studies dealing with the inhibition of LDL oxidation by drugs or naturally occurring compounds [2–8]. Lipid peroxidation can be initiated by e.g. copper ions, organic peroxyl radicals, hypochlorite, tyrosyl radicals, tocopheryl radicals and $O_2^{\bullet-}$/NO$^\bullet$ [9–13].

When $O_2^{\bullet-}$/NO$^\bullet$ are formed simultaneously, peroxynitrite ($OONO^-$) could be formed [14]. The highly reactive decomposition products of $OONO^-$ have been found to oxidise lipoproteins and membrane lipids [11,14–18]. In addition $OONO^-$ has been found to nitrate and hydroxylate amino acids (i.e. tyrosine) [19–21] and nitro-tyrosine formation in proteins has been suggested to be an indicator – although not specific – for in vivo formation of $OONO^-$ [22]. The pathophysiological consequences of tyrosine nitration have been recently outlined by Ischiropoulos [23]. $OONO^-$ induced aromatic hydroxylation (and nitration) of salicylate has been reported [24]. Taking these observations into account, one could assume that salicylate may be able to protect LDL from oxidative modification by scavenging the $OONO^-$ reactive decomposition products leading to lipid oxidation.

We report that salicylate inhibited the atherogenic modification of LDL initiated by $O_2^{\bullet-}$/NO$^\bullet$. Spectroscopic analysis revealed that in presence of SIN-1, a compound which generates simultaneously both radical species, salicylate is converted to dihydroxybenzoic acid derivatives which were even more effective in inhibiting $O_2^{\bullet-}$/NO$^\bullet$ induced LDL oxidation.

2. Materials and methods

Salicylic acid (2-hydroxybenzoic acid), 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA) and SIN-1 (3-morpholinosydnonimine) were from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals used were of analytical grade.

2.1. LDL isolation

The isolation of LDL from human plasma followed procedures reported previously [25]. The final preparation was filter sterilised and stored in 0.15 mol/l NaCl containing 0.1 mmol/l EDTA.

2.2. Lipoprotein oxidation

LDL (1 to 2 mg protein/ml) was incubated in 0.15 mol/l NaCl, 0.025 mol/l phosphate, pH 7.4 with or without SIN-1 for up to 18 h at 37°C [11,26].

2.3. Measurement of lipid oxidation

LDL oxidation products were measured as TBARS and total lipid hydroperoxides. Briefly, to 250 μl sample 0.5 ml of TBARS reagent (15% trichloroacetic acid; 0.375% thiobarbituric acid; 0.25 N hydrochloric acid) [27] was added and incubated at 100°C for 45 min. After cooling and centrifugation at $1000\times g$ for 10 min the absorbance was determined at 535 nm. Malondialdehyde concentration was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. Salicylate, 2,3- and 2,5-DHBA did not interfere with TBARS formation. As 2,3- and 2,5-DHBA (but not salicylate) were found to interfere with the lipid hydroperoxide (LPO) assay reported by El Sadaany [28], samples (100 μl) were applied to 0.9×2.0 cm Sephadex G-50 columns (NICK Column, Pharmacia Biotech) equilibrated with 0.15 M NaCl and eluted according to the manufacturer's protocol. 0.4 ml of sample was mixed with 1.0 ml LPO reagent and incubated for 60 min at 37°C (Wallin and Camejo [29]). Absorbance was read at 365 nm and LPO concentration was calculated using an extinction coefficient of 1.73×10^4 M^{-1} cm^{-1} [28].

2.4. Electrophoresis

20 μg of treated or untreated LDL were analysed on cellulose acetate sheets. Electrophoresis was run in veronal buffer pH 8.6 at 250 V for 60 min. Lipoproteins were stained with Ponceau Red S.

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Measurement of relative electrophoretic mobility (REM) was taken as an indicator of LDL oxidation [1,9], setting the electrophoretic mobility of untreated LDL arbitrarily as 1.

2.5. Endothelial cells

Bovine arterial endothelial cells (BAEC) were prepared and cultured as previously reported [30]. Cells were seeded in 6 well culture plates. After cells had reached confluency the cells were washed with Hank's balanced salt solution (HBSS) and further cultured for 18 h in the absence or presence of the respective LDL preparation (0.4 mg/ml HBSS) as indicated in the figure legends.

2.6. Cytotoxicity

The release of lactate dehydrogenase activity (LDH) into the cell culture medium was taken as an indicator of cytotoxicity. LDH activity was measured by a commercial test kit (Boehringer Mannheim Automated Analysis for BM/Hitachi 717, Germany).

2.7. Analysis of salicylate hydroxylation products

Hydroxylation products of salicylate were analysed spectrophotometrically exactly as described in [31] using 2,3-DHBA as a standard. Salicylate (2 mmol/l) was incubated in 0.15 mol/l NaCl, 0.025 mol/l phosphate, pH 7.4 (final volume 2.0 ml) in the presence or absence of SIN-1 (up to 5 mmol/l) at 37°C for 18 h and subsequently the hydroxylation products estimated.

3. Results and discussion

3.1. LDL oxidation

LDL was subjected to $O_2^{\bullet-}$/NO$^{\bullet}$ induced lipid oxidation using the sydnonimine SIN-1, which generates simultaneously both radical species in solution at 37°C [11]. LDL was incubated for 15 h at 37°C with SIN-1 (1 mmol/l) in the absence or presence of salicylate and subsequently lipoprotein oxidation was monitored by TBARS (MDA) and LPO formation. Salicylate from 0.125 to 1 mmol/l exerted a concentration dependent decrease in MDA concentration when present in SIN-1/LDL incubations (see Fig. 1). 1 mmol/l salicylate suppressed MDA concentration from 0.96 μmol/l (= 100%) to 0.48 μmol/l. Lipid hydroperoxides were also reduced to about 50% (see inset Fig. 1). It should be mentioned that plasma levels up to 2 mmol/l of salicylate can be reached during aspirin therapy [32]. In addition, electrophoretic analysis revealed that the SIN-1 (1 mmol/l) induced increase in REM, which is a further indicator of LDL oxidative modification, was substantially decreased in the presence of 1 mmol/l salicylate (see Fig. 2).

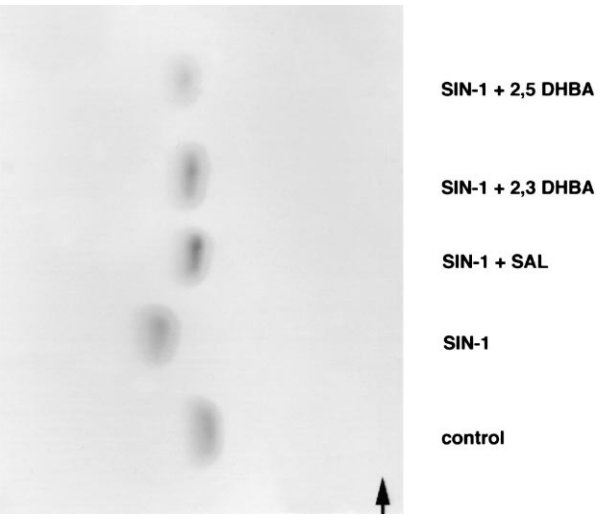

Fig. 2. Effect of salicylate, 2,3- and 2,5-DHBA (1 mmol/l each) on SIN-1 induced changes of LDL electrophoretic mobility. LDL (2 mg/ml) was incubated in the presence or absence of SIN-1 (1 mmol/l) for 18 h at 37°C. Lipoproteins were separated as given in Section 2.

Oxidised LDL is cytotoxic to a variety of cells [33,34]. Fig. 3 shows the cytotoxic effect of $O_2^{\bullet-}$/NO$^{\bullet}$ pretreated LDL on cultured endothelial cells. SIN-1 oxidised LDL preparations added to BAECs caused an about 50-fold increase in LDH release into the culture medium. This was not due to cytotoxic undecomposed SIN-1 in the culture medium, as it was shown in a control experiment, that 18 h preincubated SIN-1 (1 mmol/l) did not cause any cytotoxicity (SIN-1: 2.5 ± 0.5 U/l vs. control: 4 ± 2 U/l). Salicylate when present during

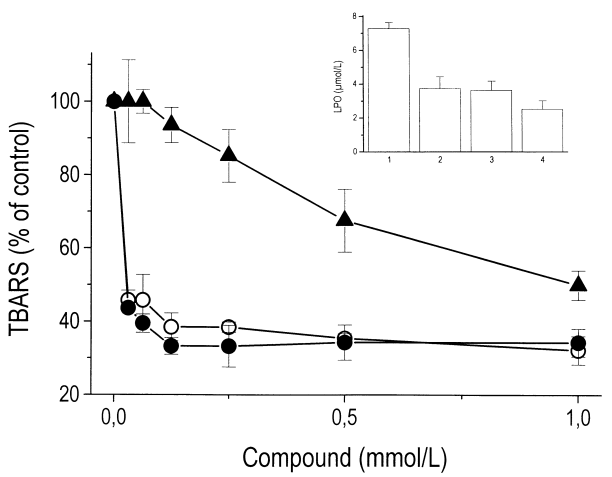

Fig. 1. Effect of salicylate, 2,3- and 2,5-DHBA on SIN-1 induced LDL oxidation. LDL (1 mg/ml) was incubated in the presence or absence of the respective compound (0.031 to 1 mmol/l) with SIN-1 (1 mmol/l) for 15 h at 37°C. LDL oxidation was monitored by TBARS formation as given in Section 2. 100% represents 0.96 μmol/l MDA. Salicylate (▲), 2,3-DHBA (○), 2,5-DHBA (●). Inset: Lipid hydroperoxides (LPO) in SIN-1 (1 mmol/l) treated LDL in absence (1) or presence of salicylate (2), 2,3-DHBA (3) or 2,5-DHBA (4). All compounds 1 mmol/l.

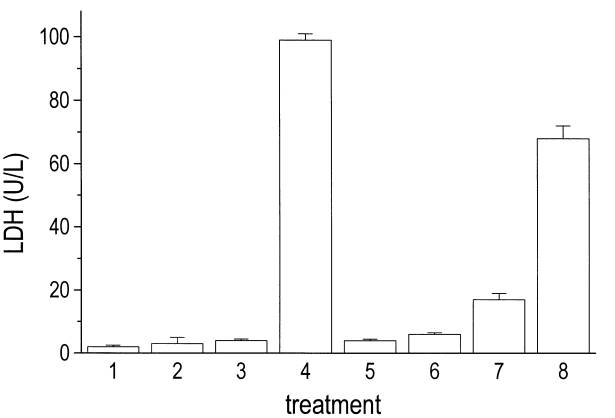

Fig. 3. Effect of salicylate on endothelial cell cytotoxicity of SIN-1 pretreated LDL. LDL was preincubated with SIN-1 (1 mmol/l) in the absence or presence of salicylate for 18 h at 37°C. Subsequently endothelial cells were treated with the respective LDL preparations (0.4 mg/ml) for 18 h and cytotoxicity was monitored by LDH release as given in Section 2. 1: untreated cells; 2: cells+LDL; 3: cells+LDL/salicylate (1 mmol/l); 4: cells+LDL/SIN-1; 5: cells+LDL/SIN-1+1 mmol/l salicylate, 6: cells+LDL/SIN-1/0.5 mmol/l salicylate; 7: cells+LDL/SIN-1/0.25 mmol/l salicylate; 8: cells+LDL/SIN-1/0.125 mmol/l salicylate.

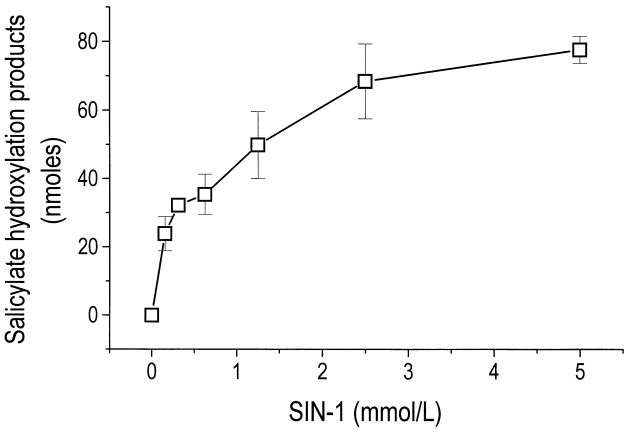

Fig. 4. Hydroxylation of salicylate by SIN-1. Salicylate (2 mmol/l) was incubated in the presence or absence of SIN-1 up to 5 mmol/l at 37°C for 18 h and the reaction products were estimated as described in Section 2.

the SIN-1/LDL oxidation reaction effectively counteracted the cytotoxic effect.

Thus one may assume that salicylate may have scavenged the radical species resulting from SIN-1 or the reactive peroxynitrite decomposition products that is $O_2^{\bullet-}$/NO$^\bullet$ and OH$^\bullet$/$NO_2^\bullet$, respectively. However, it has to be explicitly stressed that there is still a considerable debate regarding OH$^\bullet$ formation from $OONO^-$ [23,35]. Nevertheless, hydroxylation (and nitration) of salicylate may occur during SIN-1/salicylate incubation, thus protecting LDL from reactive radical species. The hydroxylation derivatives generated from salicylate by OH$^\bullet$ attack had been identified as mainly 2,3-DHBA (49%) and 2,5-DHBA (40%) and to a minor extent catechol (11%) [30]. As can be seen in Fig. 4 when salicylate (2 mmol/l) was incubated in presence of SIN-1 (up to 5 mmol/l) at 37°C for 18 h, salicylate hydroxylation products were formed. At the highest concentration tested, 2% of the salicylate was hydroxylated by SIN-1 treatment. However, it has to be mentioned that the spectrophotometric method used detects about 50–70% of reaction products as outlined in [31], but is very suitable to indicate salicylate hydroxylation [31]. 2,3-DHBA and 2,5-DHBA may have good radical scavenging (antioxidant) properties due to their diphenolic nature. In SIN-1/LDL incubation mixtures both compounds showed strong LDL protective action compared to salicylate as measured by MDA formation (Fig. 1). The alteration in REM of LDL by SIN-1 was also counteracted by 2,3- and 2,5-DHBA (see Fig. 2). The LPO concentrations in SIN-1 treated LDL were decreased from 7.28 μmol/l (SIN-1; 1 mmol/l) to 3.64 and 2.42 μmol/l by 2,3-DHBA and 2,5-DHBA, respectively when present at 1 mmol/l during LDL oxidation reaction (see inset Fig. 1).

In summary, the present study suggests that salicylate is an efficacious antioxidant in the $O_2^{\bullet-}$/NO$^\bullet$ induced oxidation of LDL. This was obviously due to the ability of salicylate to scavenge the reactive decomposition products of peroxynitrite and salicylate-hydroxylation (nitration) products are formed. These products, i.e. 2,3- and 2,5-DHBA (the latter represents the pharmacological salicylate metabolite) are still potent antioxidants, thus supporting the salicylate effect. Salicylate (aspirin) has been favoured as a preventive for cardiovascular disease [36] due to its antithrombotic potential, but this medication may also have a beneficial effect regarding LDL oxidation caused by $O_2^{\bullet-}$/NO$^\bullet$ and $OONO^-$, respectively.

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