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Inhibitory activity of salicylic acid on lipoxygenase-dependent lipid peroxidation

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ABSTRACT

Background: Since iron is essential for lipoxygenase activity and salicylic acid (SA) can interact with the metal, possible lipoxygenase inhibition by SA was investigated.
Methods: Kinetic spectrophotometric evaluation of enzymatic lipid peroxidation catalyzed by soybean lipoxygenase (SLO), rabbit reticulocyte 15-lipoxygenase (RR15-LOX), porcine leukocyte 12-lipoxygenase (PL12-LOX) and human recombinant 5-lipoxygenase (HR5-LOX) with and without SA.
Results: SA inhibited linoleic, arachidonic and docosahexaenoic acid or human lipoprotein peroxidation catalyzed by SLO with IC_{50} of, respectively, 107, 153, 47 and 108 μM. Using the same substrates, SA inhibited RR15-LOX with IC_{50} of, respectively, 49, 63, 27 and 51 μM. Further, arachidonic acid peroxidation catalyzed by PL12-LOX and HR5-LOX was inhibited by SA with IC_{50} of 101 and 168 μM, respectively. Enzymatic inhibition was complete, reversible and non-competitive. Conceivably due to its lower hydrophobicity, aspirin was less effective, indicating acetylation-independent enzyme inhibition. SA and aspirin were ineffective peroxyl radical scavengers but readily reduced Fe^{3+}, i.e. $FeCl_3$, to Fe^{2+}, suggesting their capacity to reduce Fe^{3+} at the enzyme active site. Indeed, similar to the catecholic redox inhibitor nordihydroguaiaretic acid, SA inhibited with the same efficiency both ferric and the native ferrous SLO form, indicating that these compounds reduce the active ferric enzyme leading to its inactivation.
General significance: SA can inhibit lipoxygenase-catalyzed lipid peroxidation at therapeutic concentrations, suggesting its possible inhibitory activity against enzymatic lipid peroxidation in the clinical setting.

1. Introduction

Lipoxygenases (LOXs) are a family of non-heme iron-containing dioxygenases able to induce enzymatic peroxidation of polyunsaturated fatty acids [1–5]. In general, LOXs contain an essential iron atom, which is present as Fe^{2+} in the ground-state inactive enzyme form; enzymatic activation occurs through lipid hydroperoxide-driven oxidation of Fe^{2+} to Fe^{3+} [1–3]. LOXs are widely distributed among plants and animals [1–3]; in mammals, major enzyme types are 5-, 8-, 12- and 15-LOX, which insert dioxygen, respectively, at C5, C8, C12 and C15 positions of arachidonic acid [1–5]. Of peculiar interest appears 15-LOX, since it can oxidize also esterified fatty acids in biomembranes and lipoproteins, forming 15-hydroperoxy-eicosatetraenoic acid (15-HPETE) from arachidonic acid and 13-hydroperoxy-octadecadienoic acid (13-HPODE) from linoleic acid [1–5], as well as 5-LOX, which only metabolizes free arachidonic acid generating 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) and then the inflammatory compounds leukotrienes [1,6,7]. Remarkably, 5- and 12/15-LOX have been implicated among other things in the pathogenesis of relevant diseases such as atherosclerosis [1,3–7]. Soybean lipoxygenase-1 (SLO) is a plant-derived 15-LOX catalyzing efficiently the oxidation of linoleic acid to 13-HPODE; because of structural and functional similarities with mammalian LOXs, SLO is commonly used for both mechanistic and inhibition studies and is widely accepted as a model for LOXs from other sources [1–3,8–11]. In such a context, hydrophobic interaction with the enzyme iron and scavenging of peroxyl radicals generated from LOX-polyunsaturated fatty acid interaction, may be involved in the pharmacological inhibition of enzymatic lipid peroxidation [1–3,8–10].

Salicylates, such as acetylsalicylic acid (ASA) and salicylic acid (SA), are non-steroidal anti-inflammatory agents of major therapeutic relevance, which is also related to inhibition of platelet aggregation [12,13]. In this regard, cyclooxygenase-1 (COX-1) acetylation–inactivation is responsible for the anti-platelet properties of ASA, while LOXs are generally not considered target of salicylate pharmacodynamic action [12,13]. However, since salicylates are capable of interacting with iron ions [14–16], we have hypothesized that they could interact also with the LOX iron, eventually resulting in inhibition of enzymatic lipid peroxidation. The present study was therefore conducted to investigate possible

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salicylate inhibitory effects on LOX-dependent lipid peroxidation. Since ASA is rapidly deacetylated *in vivo* to SA which exerts analgesic–antipyretic and anti-inflammatory activity [12,13], experiments were basically performed with SA.

2. Materials and methods

2.1. Reagents and lipoprotein preparation

Reagents were generally from Sigma-Aldrich Corp. (St. Louis, MO, USA), including SLO (EC 1.13.11.12), which was used as purchased. The rabbit reticulocyte 15-LOX (RR15-LOX) was a product of Calbiochem, San Diego, CA, while human recombinant 5-LOX and porcine leukocyte 12-LOX (PL12-LOX) were from Cayman Chemical Co., Ann Arbor, Michigan, and they were also used as purchased. 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) was from Polyscience, Warrington, USA. Chelex 100-treated buffers were used to assess enzymatic activities. LOXs are usually in the ferrous state after isolation and purification procedures carried out to obtain the native enzyme in commercially available preparations. In some experiments, we also used ferric SLO, which was prepared by pre-incubating the native ferrous enzyme with 2–3-fold molar excess of 13-HPODE for 10 min [17], followed by 60 min dialysis against phosphate buffered saline (PBS), pH 7.4, to remove the lipid material. The non-high-density lipoprotein (non-HDL) fraction, namely LDL plus VLDL, was obtained from EDTA plasma of healthy adults as reported previously [8,18], using dextrane sulfate (mol. wt. 500,000) plus $MgCl_2$ to precipitate the fraction itself and remove EDTA. Non-HDL protein content was measured by Lowry method. Since SA is poorly water-soluble whilst is highly soluble in ethanol, and considering also the absolute solubility in ethanol of the well-known LOX inhibitor nordihydroguaiaretic acid (NDGA), which had to be comparatively evaluated, all the agents tested were pre-dissolved in ethanol, using the same alcohol aliquots in control experiments.

2.2. LOX-dependent lipid peroxidation

Drug effects were at first tested on SLO-dependent linoleic acid peroxidation. Albeit experiments were performed essentially with SA, for comparative purposes and to understand whether the ASA acetyl group has some specific pharmacodynamic properties, in the basic experiments of SLO-dependent linoleic acid peroxidation the effect of ASA was also evaluated. Always for comparative purposes, in such experiments, besides NDGA, the non-steroidal anti-inflammatory agent ibuprofen was further specifically tested. Reactions were carried out in quartz cuvettes containing, in a final volume of 1.0 ml, 0.1 μM SLO, the agents tested, and 50 μM linoleic acid in PBS, pH 7.4, plus 1% Tween 20; incubation was for 300 min at 37 °C. In some experiments performed with SA and ASA, linoleic acid was replaced by 50 μM arachidonic acid, using 0.2 μM SLO with 180 min incubation at 37 °C, or by 50 μM docosahexaenoic acid, using in this case 0.1 μM SLO with 120 min incubation at 37 °C. At the concentrations used, the agents tested did not affect reaction mixture pH. Enzymatic lipid peroxidation was assessed through continuous spectrophotometric monitoring of absorbance increase at 234 nm due to formation of conjugated diene hydroperoxides (CDH) during specific oxidative processes [8,10,11,18]. Reference cuvettes contained the lipid oxidizable substrates (linoleic, arachidonic or docosahexaenoic acid, or the non-HDL fraction when used as the substrate of enzymatic lipid peroxidation) with or without the agents tested, as appropriate. Unless otherwise indicated, molar extinction coefficient of CDH was considered to be 29,500 at 234 nm [8,18].

The effect of SA was further tested on SLO-dependent human lipoprotein peroxidation. Optimal operative conditions for continuous spectrophotometric monitoring at 234 nm of CDH formation were found using 0.1 μM SLO and 0.1 mg non-HDL protein/ml, in PBS, pH 7.4, plus 5 mM sodium deoxycholate, allowing 120 min incubation at 37 °C.

In additional experiments, we assessed possible drug inhibitory activity on mammalian 15-LOX-catalyzed lipid peroxidation. As for SLO, reactions were carried out in quartz cuvettes containing, in a final volume of 1.0 ml, 0.15 μM RR15-LOX, SA and 200 μM linoleic acid in PBS, pH 7.4, plus 1% Tween 20; incubation was for 60 min at 37 °C. Also in this case, in some experiments 200 μM arachidonic or docosahexaenoic acids were employed as substrates instead of linoleic acid, using with the former 0.25 μM RR15-LOX and 40 min incubation, and with the latter 0.2 μM RR15-LOX and 30 min incubation. Moreover, the effect of SA was assessed on the peroxidation of the non-HDL fraction (0.05 mg non-HDL protein/ml) induced by 0.3 μM RR15-LOX in PBS, pH 7.4, plus 5 mM sodium deoxycholate; CDH formation was monitored spectrophotometrically for 120 min at 37 °C as reported above.

Finally, the effects of SA were tested on the other major LOXs, namely 12- and 5-LOX. Since in humans exists only the platelet-type 12-LOX which, similar to 5-LOX, is active essentially with arachidonic acid [1], 12- and 5-LOX-based experiments were carried out using right arachidonic acid as substrate. For PL12-LOX, experimental conditions were similar to those of the method by Huang et al. [19]. Briefly, reaction mixtures contained 0.6 μM PL12-LOX, various SA concentrations and 100 μM arachidonic acid, in 50 mM potassium phosphate buffer, pH 7.4, plus 0.03% Tween 20; CDH formation was monitored spectrophotometrically over 6 min incubation at 25 °C, using reference quartz cuvettes containing arachidonic acid in the same buffer with or without SA, as appropriate.

Human 5-LOX activity was basically assessed as reported by Percival [20] with some modifications; reaction mixtures contained 2.5 μM of the human recombinant enzyme, various SA concentrations, 50 μM arachidonic acid, 24 μg/ml phosphatidylcholine, 0.2 mM ATP and 0.2 mM $CaCl_2$, in 50 mM potassium phosphate buffer, pH 7.4; reference quartz cuvettes contained arachidonic acid plus these enzymatic cofactors, with or without SA, as appropriate. Activity was determined from total absorbance change over 8 min of continuous spectrophotometric monitoring of absorbance increase at 238 and 25 °C due to enzymatic formation of 5-HPETE, whose molar extinction coefficient is reportedly 23,000 at 238 nm [20].

2.3. Iron reduction

Since Fe^{3+} reduction at the LOX active site is relevant to enzymatic inhibition [1,2,5,8,17,21], possible iron reductive capacity of SA (and ASA) was investigated. To avoid any interference of buffers and phosphates with simple ferric iron ions, drug capability to reduce Fe^{3+} to Fe^{2+} was evaluated in 0.15 M NaCl plus 1% Tween-20; reaction mixtures contained 2.5 μM $FeCl_3$, the agents tested, and the specific iron(II) colorimetric detector ferene S at 0.5 mM final concentration. $FeCl_3$, previously dissolved in bidistilled water, was immediately used for specific experimental purposes. Absorbance at 594 nm (A_{594}) due to the ferene S–Fe^{2+} complex formation was assessed spectrophotometrically at 594 nm after 60 min incubation at 37 °C, using for calculation a molar extinction coefficient of 35,500. Because of its well known interaction with Fe^{3+} at the LOX active site resulting in reductive enzyme inactivation [2,17], NDGA was also tested as $FeCl_3$ reductant.

2.4. Peroxyl radical-scavenging assessment

Peroxyl radical-scavenging activity was tested evaluating drug capability to counteract non-HDL peroxidation induced by peroxyl radicals generated thermally by the azo-initiator AAPH. Since very high SA (and ASA) concentrations interfere in the spectrophotometric monitoring of absorbance increase at 234 due to CDH formation, lipid peroxidation was here assessed through a thiobarbituric acid (TBA)-test [18]. Experimental tubes contained, in a final volume of 1.0 ml, 0.2 mg non-HDL protein/ml and 10 mM AAPH, with or without the agents tested, in PBS, pH 7.4, plus 5 mM sodium deoxycholate and

0.1 mM diethylenetriaminepentaacetic acid; incubation was for 120 incubation at 37 °C. Then, 1.0 ml of a mixture containing equal volumes of 0.375% TBA aqueous solution, 15% trichloroacetic acid and 0.25 N HCl, plus 0.1 ml 8.1% sodium dodecyl sulfate and 2 mM butylated hydroxytoluene, was added, followed by 30 min boiling at 95 °C and cooling. The pink chromogen was extracted with *n*-butanol and read spectrophotometrically at 532 nm against an appropriate blank. Results are expressed as nmol TBA-reactants (TBAR)/mg non-HDL protein, using a molar extinction coefficient of 154,000 [18]. The agents tested gave no interference in the TBA-test.

2.5. Data statistical analysis

Data were calculated as means ± SD. Dose-dependent drug effects were analyzed by one-way analysis of variance (ANOVA) plus Student–Newman–Keuls test, with $p<0.05$ being considered statistically significant [22]. In line with literature data, IC_{50} values, namely drug concentrations able to inhibit by 50% lipid peroxidation, were estimated by regression analysis plotting various inhibitor concentrations versus the corresponding percentage inhibitions of CDH formation in experiments performed at fixed enzyme and substrate concentrations. Since in general 5 different drug concentrations were tested and for each drug concentration 6–7 independent experiments were carried out, 6–7 percentage inhibitions of enzymatic lipid peroxidation were detected for each drug concentration; thus, so many as 30–35 percentage inhibitions of lipid peroxidation were considered in each specific experimental setting. Moreover, we determined minimal drug concentrations inhibiting in a statistically significant manner lipid peroxidation (IC_{min}), and, when detectable without interference problems in the continuous UV spectrophotometric monitoring of CDH formation, drug concentrations inhibiting totally, i.e. by 100% (IC_{100}), lipid peroxidation [8]. It is of note that in the aforementioned statistical approach used to determine IC_{50}, similar results were observed considering at least 3 inhibitor concentrations, namely the IC_{min}, the IC_{100} and that concentration leading repeatedly (experiments were generally highly reproducible) to about 50% inhibition of enzymatic lipid peroxidation; considering this aspect and for simplicity, results relative to SA concentrations corresponding to IC_{min}, IC_{50} and IC_{100} are illustrated.

3. Results

3.1. Drug effects on 15-, 12- and 5-LOX-dependent lipid peroxidation

Unless otherwise indicated, the following results are referred to experiments carried out with the native ferrous enzyme forms.

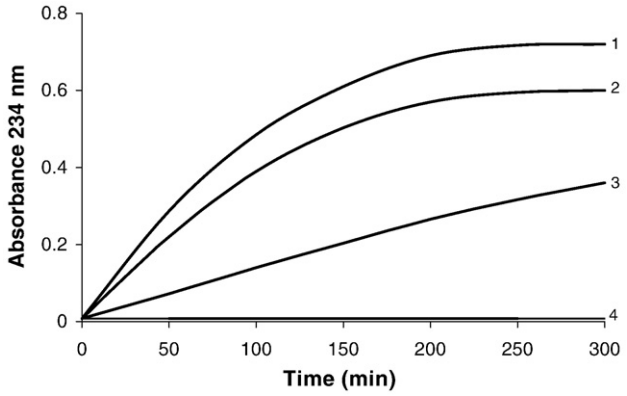

Fig. 1. The inhibitory effect of SA on SLO-dependent linoleic acid peroxidation evaluated by continuous spectrophotometric monitoring of absorbance increase at 234 nm due to CDH formation. Trace 1: control; traces 2, 3, and 4: 30, 107, and 200 μM SA, respectively. The results are representative of 7 similar experiments carried out with 0.1 μM SLO and 50 μM linoleic acid. See Materials and methods section for further explanations.

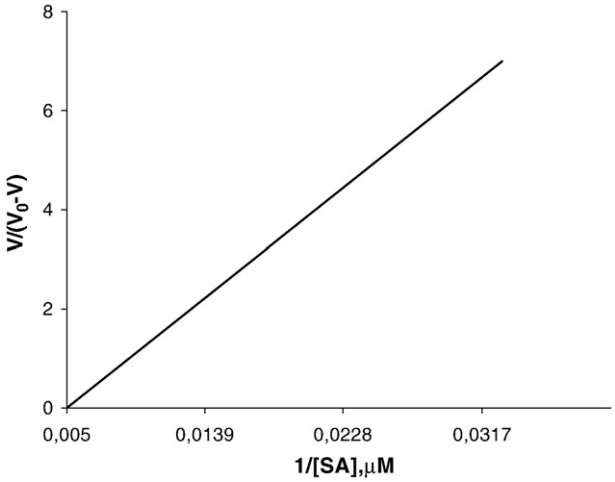

Fig. 2. Yoshino's fractional velocity plot versus reciprocal SA concentration, namely 1/[SA]. Concentrations of SA were those corresponding to its values of IC_{min} (30 μM), IC_{50} (107 μM) and IC_{100} (200 μM) detected with 0.1 μM SLO and 50 μM linoleic acid, which was here used at the various concentrations of 25, 50 and 100 μM. The yield of linoleic acid peroxidation catalyzed by 0.1 μM SLO in the absence (V_0) and presence (V) of SA was assessed spectrophotometrically at 234 nm as CDH formation and calculated as nmol CDH/min. See Materials and methods and Results sections further explanations.

As shown in Fig. 1, SA inhibited in a dose-related fashion SLO-dependent linoleic acid peroxidation with IC_{min}, IC_{50} and IC_{100} values of, respectively, 30, 107 and 200 μM. In this experimental setting, inhibition of lipid peroxidation began indeed to be statistically significant with 30 μM SA, which decreased CDH formation by about 18% (0.067 ± 0.0069 vs. 0.082 ± 0.0067 nmol CDH/min of control experiments, $p<0.05$; $n=7$), while no significant CDH formation was detectable with 200 μM SA, which totally inhibited SLO-catalyzed linoleic acid peroxidation.

From experiments performed with various linoleic acid and SA concentrations and represented graphically as Yoshino's fractional velocity plot [23], inhibition of SLO activity by SA appeared to be of complete, non-competitive type (Fig. 2). In fact, as shown in Fig. 2, the straight line obtained in this plot goes through the origin [23]; according to Yoshino [23], non-competitive inhibition is confirmed by the constant slope of the plot (single straight line) irrespective of the change in linoleic acid concentration (Fig. 2).

Using SLO and linoleic acid as the substrate, a reversible inhibitory effect of SA was observed, suggesting noncovalent drug interaction with the enzyme. In this regard, SLO regained its oxidative activity after 30 min dialysis against 100 ml PBS, pH 7.4, of 1.0 ml mixtures containing 0.1 μM SLO and a concentration of SA corresponding to its IC_{100} value (200 μM). Accordingly, after such dialytic procedure, SLO oxidized 50 μM linoleic acid at a rate of 0.077 ± 0.0056 nmol CDH/min in comparison with 0.079 ± 0.0065 nmol CDH/min of specific control experiments (p=NS; $n=5$).

As expected, NDGA strongly counteracted linoleic acid peroxidation induced by SLO, inhibiting CDH formation with an IC_{50} of 2.8 μM. ASA inhibited SLO-dependent linoleic acid peroxidation less efficiently than SA, showing indeed an IC_{50} value of 300 μM; thus, the acetyl group of ASA, which is crucial in COX inhibition by acetylating the critical enzyme serine residue 530, is not involved in 15-LOX inhibition-inactivation. Ibuprofen, up to 300 μM, was instead ineffective on SLO-catalyzed linoleic acid peroxidation (not shown).

Using arachidonic acid as substrate and 0.2 μM SLO, SA showed an IC_{min} of 60 μM, which resulted in 0.09 ± 0.0078 vs. 0.11 ± 0.01 nmol CDH/min of control experiments ($p<0.05$; $n=6$), while its IC_{50} and IC_{100} values were, respectively, 153 and 310 μM. Using instead docosahexaenoic acid as substrate and 0.1 μM SLO, the IC_{min} of SA

was 18 μM (0.116±0.011 vs. 0.14±0.013 nmol CDH/min of control experiments, $p<0.05$; $n=6$), with IC_{50} and IC_{100} values of, respectively, 47 and 120 μM. Once again, ASA was less effective than SA also using as substrate arachidonic acid ($IC_{50}=457$ μM) or docosahexaenoic acid ($IC_{50}=288$ μM).

In the experiments performed with the human non-HDL fraction, SA also inhibited SLO-catalyzed lipid peroxidation in a dose-related fashion with IC_{min}, IC_{50} and IC_{100} values of, respectively, 30, 108 and 200 μM. Indeed, inhibition of enzymatic lipid peroxidation began to be statistically significant with 30 μM SA, which lowered CDH formation from 0.64±0.08 nmol CDH/min/mg non-HDL protein of control experiments to 0.5±0.05 nmol CDH/min/mg non-HDL protein ($p<0.05$; $n=6$), while total inhibition of lipoprotein peroxidation was evident with 200 μM SA.

Under the experimental conditions used, 0.15 μM RR15-LOX oxidized linoleic acid yielding 0.053±0.005 nmol CDH/min in control experiments ($n=6$). The IC_{min} of SA was 23 μM, which resulted in 0.042±0.004 nmol CDH/min ($p<0.05$ vs. controls; $n=6$), with IC_{50} and IC_{100} values of, respectively, 49 and 90 μM. Using arachidonic acid as substrate and 0.25 μM RR15-LOX, the IC_{min} of SA was 32 μM, which decreased CDH formation from 0.072±0.0053 to 0.058±0.0065 nmol CDH/min ($p<0.05$ vs. controls; $n=6$), while its IC_{50} and IC_{100} values were, respectively, 63 and 110 μM. With docosahexaenoic acid as substrate and 0.2 μM RR15-LOX, SA showed an IC_{min} of 12 μM, which gave 0.067±0.0055 nmol CDH/min as compared to 0.085±0.006 nmol CDH/min of control experiments ($p<0.05$; $n=6$); drug IC_{50} and IC_{100} were, respectively, 27 and 60 μM. Moreover, as depicted in Fig. 3, SA inhibited RR15-LOX-dependent peroxidation of the non-HDL fraction, showing an IC_{50} of 51 μM; the IC_{min} of SA was 23 μM (0.42±0.045 vs. 0.52±0.06 nmol CDH/min/mg non-HDL protein of control experiments, $p<0.05$; $n=6$), and total inhibition of lipoprotein peroxidation was observed with 90 μM SA (Fig. 3).

The peroxidation of 50 μM linoleic acid catalyzed by 0.1 μM ferric SLO gave 0.075±0.0065 nmol CDH/min ($n=6$), and it was inhibited by SA with the same efficiency observed with the native ferrous enzyme form ($IC_{50}=107$ μM). Relevantly, also the well known redox inhibitor NDGA counteracted ferric SLO-catalyzed linoleic acid peroxidation with the same IC_{50} observed with the ferrous enzyme form, i.e. 2.8 μM, indicating that reductive inactivation is involved in NDGA- and salicylate-dependent enzymatic inhibition.

SA could inhibit dose-dependently arachidonic acid peroxidation catalyzed by PL12-LOX with IC_{min}, IC_{50} and IC_{100} values of, respectively, 45, 101 and 200 μM (Fig. 4). Indeed, inhibition of enzymatic lipid peroxidation was statistically significant with the lowest SA concentration of 45 μM (1±0.1 vs. 1.25±0.13 nmol CDH/min of control experiments, $p<0.05$; $n=6$), and was total with 200 μM SA (Fig. 4).

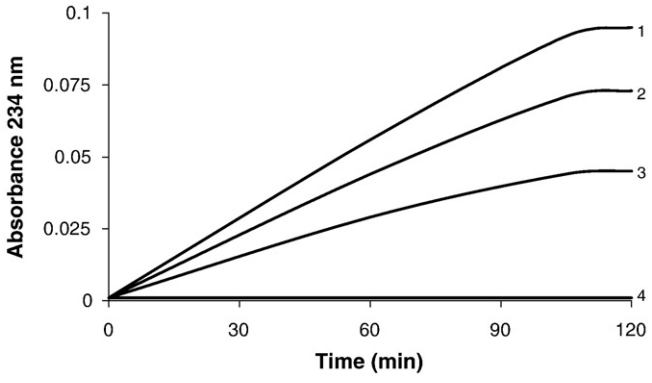

Fig. 3. The inhibitory effect of SA on RR15-LOX-dependent human non-HDL peroxidation evaluated by continuous spectrophotometric monitoring of absorbance increase at 234 nm due to CDH formation. Trace 1: control; traces 2, 3, and 4: 23, 51, and 90 μM SA, respectively. The results are representative of 6 similar experiments carried out with 0.3 μM RR15-LOX and 0.05 mg non-HDL protein/ml. See Materials and methods section for further explanations.

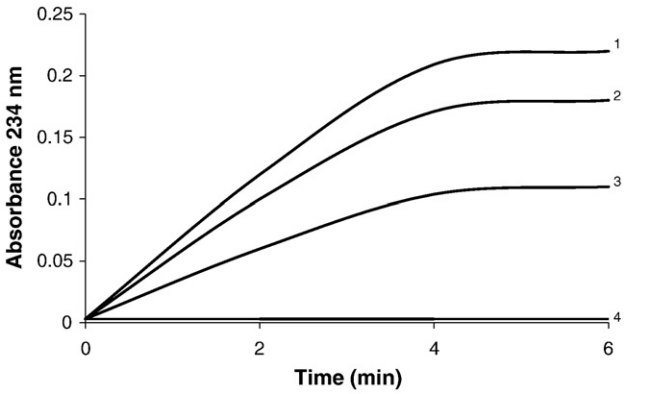

Fig. 4. The inhibitory effect of SA on PL12-LOX-dependent arachidonic acid peroxidation evaluated by continuous spectrophotometric monitoring of absorbance increase at 234 nm due to CDH formation. Trace 1: control; traces 2, 3, and 4: 45, 101, and 200 μM SA, respectively. The results are representative of 6 similar experiments carried out with 0.6 μM PL12-LOX and 100 μM arachidonic acid. See Materials and methods section for further explanations.

Finally, human recombinant 5-LOX oxidized arachidonic acid generating 0.86±0.078 nmol 5-HPETE/min in control experiments ($n=6$). In this experimental setting, the IC_{min} of SA was 120 μM, which decreased 5-HPETE formation to 0.7±0.05 nmol/min ($p<0.05$ vs. controls), while its IC_{50} and IC_{100} values were 168 and 240 μM, respectively (Fig. 5).

3.2. Iron reduction

SA, at 1.4 μM, could totally reduce Fe^{3+} (0.092±0.004 A_{594} units, $n=5$). Moreover, total Fe^{3+} reduction was observed with 1.4 ASA and 0.04 μM NDGA, which was therefore the most potent iron(III) reductant.

3.3. Peroxyl radical-scavenging

After 120 min incubation at 37 °C with 10 mM AAPH, 4±0.35 nmol TBAR mg/non-HDL protein was generated in control experiments

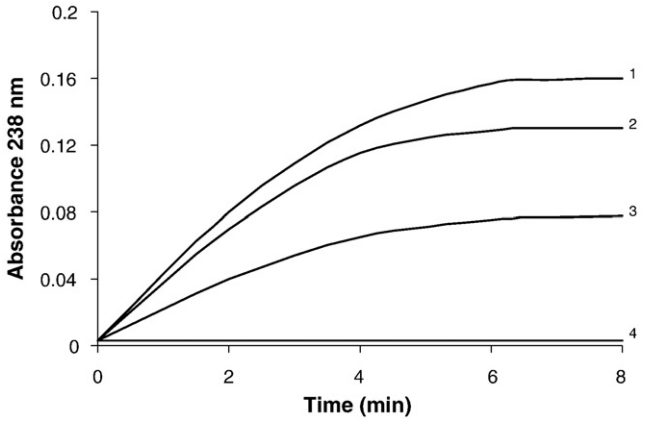

Fig. 5. The inhibitory effect of SA on human recombinant 5-LOX-dependent arachidonic acid peroxidation evaluated by continuous spectrophotometric monitoring of absorbance increase at 238 nm due to CDH, i.e. 5-HPETE, formation. Trace 1: control; traces 2, 3, and 4: 120, 168, and 240 μM SA, respectively. The results are representative of 6 similar experiments carried out with 2.5 μM 5-LOX and 50 μM arachidonic acid in the presence of the enzyme cofactors phosphatidylcholine, ATP and $CaCl_2$. See Materials and methods section for further explanations.

(n=6); SA, as well as ASA, inhibited AAPH-mediated, peroxyl radical-induced non-HDL peroxidation with an IC_{50} as high as 7 mM, while NDGA was very effective showing an IC_{50} of 25 μM.

4. Discussion

The present study shows that SA can inhibit LOX-catalyzed lipid peroxidation. As far as the mechanistic aspect of such inhibition is concerned, our data demonstrate that SA (and ASA), as well as NDGA, can readily reduce ferric iron ions, suggesting that they may operate a similar reductive phenomenon with the ferric iron of the LOX active form leading to enzymatic inactivation. Consistently, it has been reported that the behaviour of simple ferric ions in aqueous solution is similar to that of ferric LOX toward possible inhibitors such as catechols [17]. Moreover, it is known that various non-competitive LOX inhibitors, including the catecholic compound NDGA, act right through Fe^{3+} reduction at the active site (enzyme reductive inactivation) and may so be regarded as redox inhibitors [1,2,5,8,17,21,24]. In this context, it is noteworthy that, similar to the redox inhibitor NDGA, SA inhibits with the same efficiency both ferric SLO and the native ferrous enzyme form. Inhibition of ferric enzyme can occur only through two mechanisms, namely pure complexation or reduction. Inhibitors acting through the former mechanism counteract solely lipid peroxidation catalyzed by the ferric enzyme, but are notably ineffective against that catalyzed by the native ferrous one [24]. Hence, inhibition with the same efficiency by SA of both ferric and ferrous SLO-dependent lipid peroxidation is indicative of enzyme reductive inactivation. In fact, NDGA, which is known to reductively inactivate LOXs, also inhibits with the same efficiency both the ferric and ferrous SLO form.

Despite the same capability of SA and ASA to reduce iron ions, the former is more potent than the latter as enzymatic inhibitor. It is possible that the higher hydrophobicity of SA, whose log P value is 2.4 compared to 1.4 of ASA [25], could be relevant to the degree of enzymatic inhibition. As a matter of fact, the LOX channels or cavities conducting to iron at the active site are hydrophobic in nature [3,9], thus allowing proper interactions with hydrophobic compounds; accordingly, enhancing the hydrophobicity of LOX inhibitors makes them more effective [11]. In this regard, the powerful inhibitor NDGA is not only the most effective iron reductant but is also highly hydrophobic, having a log P value of 5.8 [25]. On the other hand, the acidic non-steroidal anti-inflammatory agent ibuprofen, albeit more hydrophobic than SA [25], is ineffective, indicating that the phenolic-type structure of SA is crucial in 15-LOX inhibition.

Enzymatic lipid peroxidation could be favored by peroxyl radicals generated from 15-LOX-unsaturated lipid interaction [1–3,26], raising the possibility that the SA (and ASA) could have exerted specific antilipoperoxidative effects acting as peroxyl radical scavengers. However, similar to a previous report [27], we have shown that these salicylates are not good peroxyl radical scavengers, showing in our test an IC_{50} as high as 7 mM; on the other hand, especially SA can inhibit enzymatic lipid peroxidation at far lower concentrations, indicating that reductive interaction with enzyme iron is primarily involved in such antilipoperoxidant activity. In this context, it is worth mentioning that NDGA has been reported to inhibit SLO not by trapping peroxyl radicals but instead through iron reduction at the active site [17]. However, it is also of note that enzyme-generated radical intermediates are not really free, so that their effective trapping could be difficult even for reactive radical scavengers (e.g., α-tocopherol).

The aforementioned considerations are based also on experiments performed with SLO. As in other investigations, SLO has been used for its large availability and relative inexpensiveness; more importantly, SLO may represent a suitable model for mammalian and human 15-LOX activity. Indeed, given also the polyunsaturated fatty acid oxygenation sites, SLO has been reported to be most like the mammalian 15-LOX [1]. Further, although SLO is not totally identical to human 15-LOX, nonetheless there is a 39% identity between these enzymes based on amino acid sequences, with maximal alignment in two conserved histidine regions thought to be the substrate binding and the catalytic sites [1–3,28,29]. Indeed, it is remarkable that a well-conserved histidine-rich sequence is present in all LOXs at the iron-containing catalytic site [1]. Thus, inhibitors of SLO may be expected to inhibit also other LOXs including human 15-LOX. It has in fact been shown the capacity of several enzymatic inhibitors to inactivate both SLO and human 15-LOX, sometimes acting even more efficiently on the latter than on the former enzyme [28]. As already noted, SLO may be therefore accepted as a model for mammalian and human 12/15-LOX [1–3,8–11,28]. In such a context, SA can also inhibit lipid peroxidation catalyzed by RR15-LOX; this enzyme shares an 81% overall sequence identity with the corresponding human form [29], further suggesting possible inhibitory effects of SA against lipid peroxidation induced by human 15-LOX. Human 12-LOX-catalyzed lipid peroxidation could also be counteracted by SA, considering that the human enzyme also shares a high overall sequence identity, i.e. near 70%, with the corresponding porcine leukocyte form [1], while it is worth emphasizing the capacity of SA to inhibit directly human 5-LOX.

In anti-inflammatory therapy, plasma salicylate concentrations are 1–2 mM with accumulation during repeated drug administration [12,13], while they may reach about 150 μM when taking a single dose of 325 mg ASA as anti-platelet agent [30], or 460 μM after administration of 1200 mg ASA for analgesic purposes [31]; taking into account a mean plasma protein binding of about 20% [32], these concentrations correspond to free salicylate levels of, respectively, 200–400, 30 and 90 μM. It is therefore remarkable that SA counteracts significantly LOX-dependent lipid peroxidation at therapeutically achievable concentrations especially when used as anti-inflammatory agent. Moreover, salicylates, which accumulate in inflamed tissue [13], are present not only in the extra-but also in the intracellular environment, where can also accumulate potentially reaching localized high concentrations [12,33]. Since LOXs, including 12/15-LOX, are intracellular enzymes, SA could inhibit enzymatic lipid peroxidation within the cell, preventing for example the transferring across plasma membranes of lipoperoxides able to "seed" extracellular lipoproteins [4]. Considering the salicylate biomembrane-binding capacity [33,34], peculiar drug antilipoperoxidant effects could also be exerted right at biomembrane level. In this regard, active 5-LOX is notably membrane-associated [7]; moreover, lipoprotein oxidation by 12/15-LOX seems to occur in plasma membranes, where the enzyme translocate from cytoplasm and cholesterol ester is transferred from the lipoprotein particle after a signaling cascade resulting from low density lipoprotein receptor stimulation [35]. Drug inhibition of enzymatic lipid peroxidation could finally occur in the extracellular space, where LOXs, such as 12/15-LOX, may be released from disrupted cells in inflammatory sites or in growing atherosclerotic lesions. Collectively, LOX inhibition by salicylates appears possible *in vivo*, eventually resulting in relevant pharmacodynamic effects such as pain and inflammation modulation or antagonism of lipid peroxidation and atherogenic processes. As a matter of fact, 12/15-LOX lipid products have hyperalgesic and pro-inflammatory properties [4,6,36–39], while cell 12/15 LOX deletion can decrease systemic lipid peroxidation and prevent the development of atherosclerotic lesions in apolipoprotein E-deficient mice [5,40]; pharmacological enzymatic inhibition exerts similar antiatherogenic effects in cholesterol-fed rabbits [4,5]. Also 12- and, especially, 5-LOX have relevant inflammatory and atherogenic properties. For example, the 12-LOX product 12(*S*)HETE, which is a strong growth-promoting factor, induces monocyte adhesion to the vascular endothelium and migration of arterial smooth muscle cells [1,6]; moreover, the 5-LOX metabolite leukotriene B_4 is a potent chemotactic stimulus for monocytes and favors foam cell formation [7]. Interestingly, 5-LOX,

which is present in human atherosclerotic vessels mostly at macrophage level [7], is considered expression of active inflammation with tissue breakdown [7]; in fact, the 5-LOX-(cysteinyl) leukotriene pathway, which is selectively activated in the vascular adventitia leading to recruitment-activation of macrophages with metalloproteinase formation, is implicated in aneurysm formation and atherosclerotic plaque rupture [7]. Further regarding 12- and 5-LOX, they are generally considered pro-carcinogenic [41], suggesting that enzymatic inhibitors such as salicylates may modulate neoplastic processes. Consistently, there is clinical evidence of reduced incidence of colorectal cancer by ASA, especially when used at elevated dose [42] potentially resulting in localized higher SA concentrations. Finally, it is of note that 12/15-LOX activation occurs in diabetes, hypercholesterolemia, arterial hypertension and smoke [39,43–45], namely the major risk factors for atherosclerosis, further indicating appropriate ASA administration in these conditions. Also owing to its COX-1 inhibitory activity, ASA has an established efficacy especially in the prevention and treatment of cardiovascular diseases, such as ischemic heart diseases and stroke. Whether salicylate-dependent inhibition of enzymatic lipid peroxidation may contribute to the beneficial clinical effects of ASA warrants further investigation.

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