The salicylate metabolite gentisic acid, but not the parent drug, inhibits glucose autoxidation-mediated atherogenic modification of low density lipoprotein

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Abstract Oxidation of low density lipoprotein (LDL) by glucose-derived radicals may play a role in the aetiology of atherosclerosis in diabetes. Salicylate was shown to scavenge certain radicals. In the present study, aspirin, salicylate and its metabolites 2,5- and 2,3-dihydroxybenzoic acid (DHBA) were tested for their ability to impair LDL oxidation by glucose. Only the DHBA derivatives, when present during LDL modification, inhibited LDL oxidation and the increase in endothelial tissue factor synthesis induced by glucose oxidised LDL. The LDL glycation reaction was not affected by DHBA. The antioxidative action of DHBA may be attributed to free radical scavenging and/or chelation of transition metal ions catalysing glucose autoxidation.

Key words: Glucose autoxidation; Low density lipoprotein oxidation; Aspirin; Salicylate; Gentisic acid; 2,3-Dihydroxybenzoic acid; 2,5-Dihydroxybenzoic acid

1. Introduction

It has been assumed that oxidative stress contributes to the development of the diabetic complications [1]. In this respect, lower levels of antioxidants like ascorbic acid and vitamin E have been reported to occur in diabetes [2,3]. Elevated levels of glycoxidation and lipoxidation products have been found in plasma and tissue proteins in diabetes [4–7]. Insulin treatment of type 1 diabetes patients reduced plasma hydroperoxides and, thus, reactive oxygen species [8].

The onset and progression of atherosclerosis is accelerated in diabetes [9]. Low density lipoprotein (LDL) oxidative (atherogenic) modification has been found to play a pivotal role in early stage atherogenesis [10–12]. As an in vitro model of protein alterations in diabetes, glucose treatment of LDL causes peroxidation (and glycation) of the lipoprotein [13–15]. Glucose autoxidation results in the generation of several radical species [16,17]. Salicylate has been shown to scavenge hydroxyl radicals [18,19] and we have recently shown that salicylate and gentisic acid (2,5-DHBA) the first and second line aspirin metabolites impaired superoxide/nitric oxide radical-initiated LDL oxidation [20].

Thus the aim of this study was to assess the potential of aspirin and its metabolites to act as inhibitors against the battery of glucose-derived radicals mediating atherogenic modification of LDL, a property that might have implications in preventing diabetic complications.

2. Materials and methods

Aspirin (acetylsalicylic acid), salicylic acid (2-hydroxybenzoic acid), 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA, gentisic acid), bovine serum albumin and lipopolysaccharide (*Escherichia coli* 055:B5) were from Sigma Chem. Co. and D-glucose monohydrate from Merck. All other chemicals used were of analytical grade.

2.1. LDL isolation

LDL was isolated from EDTA–plasma of healthy male volunteers by ultracentrifugation as reported previously [21]. The final preparation was dialysed against 150 mmol/l NaCl containing 0.1 mmol/l EDTA and filter-sterilised.

2.2. LDL oxidation by glucose

Prior to LDL oxidation, the lipoprotein was equilibrated in 0.1 mol/l phosphate buffer pH 7.4 using Sephadex G-25 chromatography (PD-10 columns, Pharmacia). LDL (1 mg/ml) was incubated in the presence of 200 mmol/l D-glucose [7,15] up to 9 days at 37°C under sterile conditions.

2.3. LDL glycation

LDL (1 mg/ml) was incubated in 0.1 mol/l phosphate buffer, pH 7.4, in the presence of 20 mmol/l D-glucose and 10 μCi/ml [U-^{14}C]glucose (ICN Biomedicals, specific activity 316 mCi/mmol) at 37°C for 6 days. Lipoproteins were precipitated by the addition of TCA to a final concentration of 5%. The pellet was washed in 5% TCA and finally dissolved in formic acid before scintillation counting [14].

2.4. Cell culture

Endothelial cells (HUVEC) were isolated from human umbilical veins and maintained in culture as reported previously [22].

2.5. Analysis of LDL oxidation

2.5.1. Conjugated dienes (CD). Prior to the estimation of CD, 100 μl of the respective LDL sample was applied to small Sephadex columns (Nick columns, Pharmacia) equilibrated in 0.025 mol/l phosphate buffer pH 7.4 to get rid of low molecular weight compounds. Absorbance of the samples was read at 234 nm. The CD concentra-

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tion was calculated using a molar extinction coefficient of $\varepsilon = 2.95 \times 10^4$ M^{-1} cm^{-1} [23].

2.5.2. Thiobarbituric acid assay. LDL oxidation products were assayed as TBARS as reported [21].

2.5.3. Lipid hydroperoxide assay. Lipid hydroperoxides were assayed based on the method of El Saadani et al. [24] as modified by Wallin and Camejo [25]. The concentration of hydroperoxides was calculated from the molar absorption coefficient of $\varepsilon = 2.46 \times 10^4$ M^{-1} cm^{-1} at 365 nm. Prior to the estimation of lipid hydroperoxides, the LDL samples were applied to small Sephadex columns (Nick columns, Pharmacia) equilibrated in 0.025 mol/l phosphate buffer pH 7.4 to get rid of interfering low molecular weight compounds [20].

2.6. Lipid electrophoresis

Aliquots (10 μl) of treated or untreated LDL were applied to agarose gels (1% in veronal buffer) and run for 90 min, and lipoproteins were detected according to the supplier of the analytical system (Lipidophor All In, Immuno-Baxter AG). Measurement of relative electrophoretic mobility (REM) was taken as an indicator of LDL oxidation [23], setting the electrophoretic mobility of native (untreated) LDL arbitrarily as one.

2.7. Tissue factor (TF), clotting assay

Confluent HUVEC cultures grown in 6-well plates were washed with RPMI 1640 and treated for 4 h with the respective LDL preparations (100 μg/ml medium) to stimulate TF production [26]. Prior to the addition of the treated LDL to the cells, low molecular weight compounds were removed by gel filtration on small Sephadex columns. At the end of incubation, cells were washed with phosphate-buffered saline (PBS). HUVEC were scrape-harvested into 1 ml of PBS and sonicated by a cell disrupter for 1 min at 4°C. The lysate was assayed in a one-stage clotting assay: 100 μl citrated normal donor platelet poor plasma was incubated for 1 min with 100 μl cell lysate at 37°C in prewarmed plastic tubes of a KC-10 coagulometer (Amelung, Germany); 100 μl $CaCl_2$ (30 mmol/l) was then added and coagulation time was measured. Control experiments with factor VII, IX and X deficient plasma (Behring, Germany) characterised the procoagulant activity as TF [22].

2.8. Spectroscopy

Stock solutions of salicylate, 2,3-DHBA or 2,5-DHBA and ions (Fe^{2+} as $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, Fe^{3+} as $FeCl_3 \cdot 6H_2O$) were mixed to give a final concentration of 200 μmol/l in 0.15 mol/l sodium chloride with or without 10 mmol/l phosphate buffer (or Tris–HCl) pH 7.4. Spectra were recorded between 700 and 220 nm.

3. Results

To examine the potential of aspirin and its pharmacological metabolites, salicylate and gentisic acid to prevent the oxidative (atherogenic) modification of LDL by the various reactive oxygen species generated by glucose autoxidation [16,17], LDL (1 mg/ml) was incubated in the presence of glucose (200 mmol/l) [7,15] with or without the respective compound. As can be seen in Fig. 1A, when LDL was incubated with glucose for 4 days at 37°C, both 2,3- and 2,5-DHBA reduced CD formation in a concentration dependent manner. Neither the parent drug aspirin nor the first line metabolite salicylate had any influence on LDL oxidation. Parallel estimation of other parameters of lipid peroxidation (lipid hydroperoxides (Fig. 1B) and TBARS formation (Fig. 1C)) showed similar results. 20–50 μmol/l of 2,3- or 2,5-DHBA suppressed lipid hydroperoxide production and TBARS formation.

TF can be induced in endothelial cells by oxidised LDL [26,27]. When LDL (1 mg/ml) was incubated in the presence of glucose (200 mmol/l) for 9 days at 37°C and subsequently HUVEC cultures were treated with this LDL preparation (100 μg/ml medium) for 4 h, TF was induced as assessed by a clotting assay (see Section 2). TF induction could be prevented if 2,3- or 2,5-DHBA were present during glucose-mediated LDL oxidation. Aspirin and salicylate again had no influence in this system.

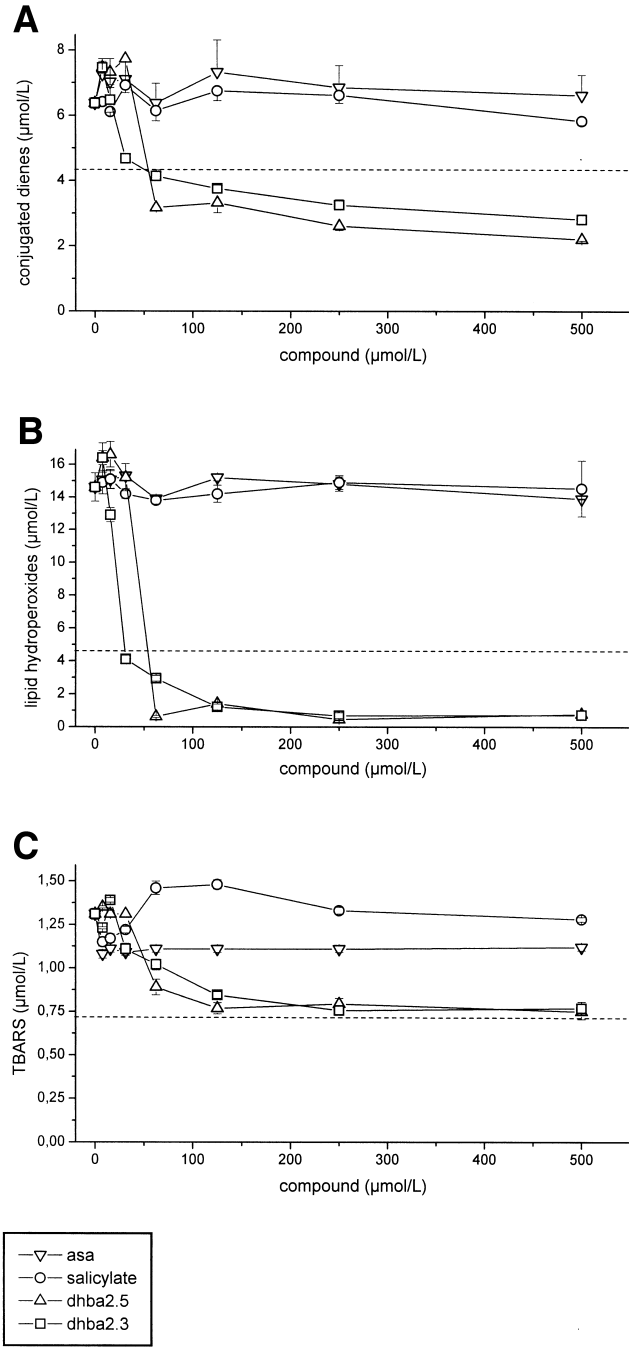

Fig. 1. Influence of aspirin, salicylate, 2,5-DHBA and 2,3-DHBA on glucose-mediated LDL oxidative modification. LDL (1 mg/ml) was incubated in 0.1 mol/l phosphate buffer pH 7.4 for 4 days at 37°C in the presence of glucose (200 mmol/l) and the respective compound. At the end of incubation, 100 μl sample was applied to Sephadex columns to get rid of low molecular weight compounds and subsequently CD (A) and lipid hydroperoxides (B) were estimated in aliquots of the eluted lipoprotein fraction. TBARS (C) were estimated in an aliquot of the incubation mixtures. The dotted line represents levels of CD, lipid hydroperoxides and TBARS in LDL preparations without glucose.

The very different ability of the compounds to overcome the effect of glucose treatment on LDL in respect to TF induction in HUVEC cultures was also reflected in their antioxidant

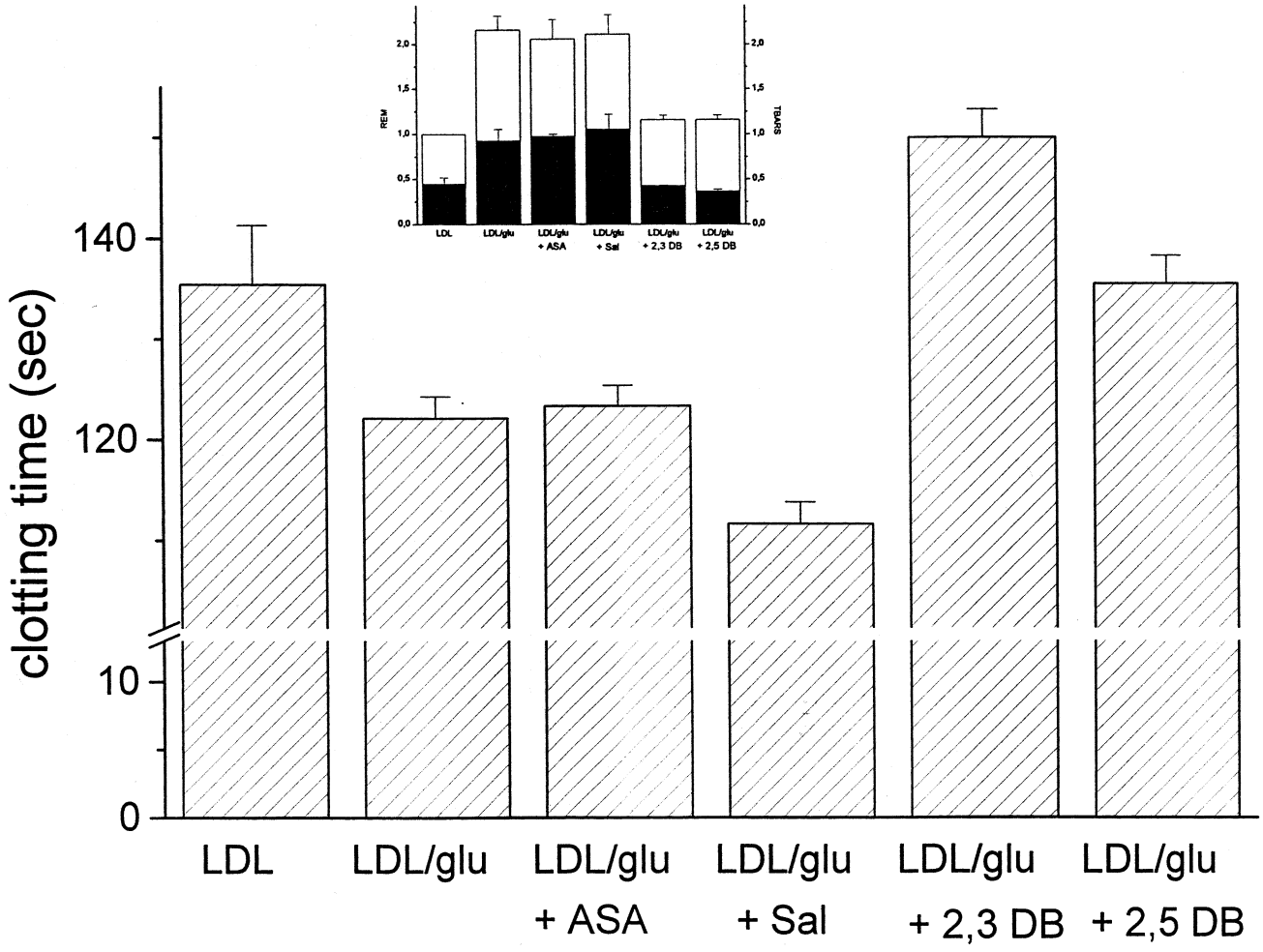

Fig. 2. Influence of glucose-oxidised LDL on TF coagulant activity in human endothelial cells. LDL (1 mg/ml) was preincubated in 0.1 mol/l phosphate buffer pH 7.4 for 9 days at 37°C in absence or presence of glucose (200 mmol/l) and the respective compound (500 μmol/l). At the end of incubation, 100 μl sample was applied to Sephadex columns to get rid of low molecular weight compounds and subsequently, HUVEC cultures were exposed for 4 h to the respective LDL preparations (100 μg/ml medium). Cells were washed with PBS and the TF coagulant activity was estimated in aliquots of HUVEC protein extracts as given under Section 2. Inset: TBARS (right axis/black columns) and REM (left axis/empty columns) in the respective LDL preincubations.

potential under these LDL/glucose preincubation conditions (9 days at 37°C, see Fig. 2, inset).

Glycated LDL has been shown to be more prone to oxidation than unmodified LDL [15]. Thus, the DHBA derivatives may inhibit LDL glycation resulting in less LDL oxidation by glucose. Incubation of LDL with [^{14}C]glucose (see Section 2) resulted in the binding of 13.7 ± 0.87 nmol glucose/mg LDL (n = 2). Neither 2,3- nor 2,5-DHBA (molar ratio glucose/compound = 400) inhibited LDL glycation (14.1 ± 1.51 and 14.0 ± 1.32 nmol glucose/mg LDL, respectively) (n = 2). Aspirin and salicylate were also ineffective.

Metal ions can catalyse glucose oxidation [14–17]. Binding of such ions may result in inhibition of LDL oxidation. Spectral analysis revealed that salicylate, 2,3- and 2,5-DHBA formed violet complexes (absorption maxima 526, 575 and 612 nm, respectively) with Fe^{3+} but not Fe^{2+} in unbuffered sodium chloride solution. However, in the presence of phosphate ions, the Fe^{3+} complexes were not formed. In contrast to salicylate and 2,5-DHBA in phosphate (or Tris–HCl)-buffered (pH 7.4) solution, 2,3-DHBA formed a coloured chelate with Fe^{2+}. Thus under the conditions of the LDL oxidation system (phosphate buffer), metal chelation may be limited to 2,3-DHBA only.

4. Discussion

The autoxidation of glucose generates a battery of reactive products like superoxide, hydroxyl, hydroxyalkyl and peroxyl radicals and hydrogen peroxide [16,17,28]. LDL exposed to glucose in vitro is known to undergo glycation and peroxidation of the apoprotein and lipid moiety, respectively [13–15]. These modifications result in a lipoprotein particle known to initiate and support atherogenic events [29–31].

Taken all these observations into account, antioxidative compounds [32,33] may have beneficial effects regarding diabetic complications like atherosclerosis. Salicylate has been shown to scavenge particular radical species and salicylate hydroxylation resulting in 2,3-DHBA formation has been implicated as an indicator of hydroxyl radicals produced and involved in certain diseases as e.g. arthritis [18,19]. We now show that the second line aspirin metabolite gentisic acid effectively counteracted the oxidative modification of LDL by

the battery of glucose autoxidation-derived radicals. However, the parent drug salicylate as well as aspirin were not able to prevent glucose-mediated LDL oxidation. In contrast, salicylate has been found to protect LDL from oxidation by superoxide/nitric oxide radicals [20] and aspirin inhibited LDL oxidation by UV irradiation initiated radical reactions [34].

Our results show that both DHBA compounds do not act via reduced LDL glycation which may result in lower susceptibility of LDL to oxidative modification. Another possible mechanism of inhibiting glucose-mediated LDL oxidation by 2,3- or 2,5-DHBA may be the ability to complex transition metal ions. For some phenolics, it has been proposed and demonstrated that they scavenge catalytically active metal ions [35]. Our results show that 2,3-DHBA was able to complex Fe^{2+} and thus the compound may exert its antioxidative potential, at least partially, by this mechanism. On the other hand, 2,5-DHBA mainly acts as a free radical scavenger under these conditions. It should be mentioned that salicylate was not found to inhibit the transition metal ion-promoted LDL oxidation [36].

The present results were obtained at glucose/gentisic acid ratios (mol/mol) of 8000 and 400. In humans plasma, levels of 2 mmol/l salicylate, which can be reached under aspirin therapy, lead to levels of about 20 μmol/l gentisic acid [37]. Thus in normoglycemic individuals assuming a plasma glucose concentration of 4–6 mmol/l, the molar ratio of glucose/gentisic acid may be 160–240. In the present in vitro system, gentisic acid at molar ratios of 400–8000 inhibited the oxidative modification of LDL by glucose. Thus one may speculate that in vivo, the compound may also be able to protect LDL from glucose oxidative reaction products generated in hyperglycaemia.

In summary, our results show that the pharmacological (2,5-DHBA) and pathophysiological (2,3-DHBA) salicylate metabolite but not the parent drug was an efficacious antioxidant in respect to LDL modification by glucose autoxidative radical reaction products, potently inhibiting the formation of atherogenic LDL particles at glucose/gentisic acid molar ratios which may also be found in vivo, further supporting the rationale for prophylactic aspirin medication [38,39] against vascular complications.

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