Enhancement by Glucose of Low Density Lipoprotein-Oxidation by Peroxynitrite[§]

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Oxidation of low density lipoprotein (LDL) by copper ions is not influenced by glucose in a concentration range between 1 and 20 mM. LDL-oxidation by peroxynitrite or the simultaneous action of nitrogen monoxide and superoxide, produced by morpholino-sydnonimine (SIN-1) is considerably enhanced by typical hydroxyl-radical scavengers such as formate or mannitol and by glucose. Since both free radicals, nitrogen monoxide and superoxide, are produced by activated leukocytes and endothelial cells the presented reaction might represent a simple model for the cooperative function of reactive oxygen species (ROS) and glucose in certain diabetic blood vessel diseases such as atherogenesis and retinopathy.

Introduction

Uptake of oxidized LDL by the so-called "scavenger receptor" of macrophages seems to represent one initial step in atherosclerosis (Brown and Goldstein, 1979; 1985). LDL-oxidation seems to be driven by diverse oxidants such as copper-ions or chelated iron-ions, enzymes such as lipoxygenase or ROS produced by activated neutrophils or stimulated endothelial cells. On the other hand endogenous antioxidants such as vitamin E delay the LDL-oxidation (Esterbauer *et al.*, 1988, 1990; Jürgens *et al.*, 1987; Steinbrecher *et al.*, 1989; Kuzuya *et al.*, 1991; Jessup *et al.*, 1990). Recently peroxynitrite, $ONOO^-$, formed from nitrogen monoxide and superoxide, has been shown to significantly accelerate LDL-oxidation (Darley-Usmar *et al.*, 1992; Graham *et al.*, 1993; Hogg *et al.*, 1993; Moore *et al.*, 1995). Nitrogen monoxide is formed in all types of leukocytes and in endothelial cells from L-arginine catalysed by the enzyme nitrogen monoxide-synthase (NOS). The product of the extremely fast interaction between nitrogen monoxide and superoxide ($k = 6.7$ x 10^9), ONOOH, may be considered as a "solvent cage" {HO-NOO} (Pryor and Squadrito, 1995), decaying either heterolytically into OH^- and NO_2^+ or homolytically into $OH^.$ and $NO_2^.$. The strong oxidant ONOOH which is produced in slightly acidic milieu ($pK_{a,app}$ for ONOOH = 6.8) may correspond with the "free" $OH^.$ radical (Pryor and Squadrito, 1995) formed according to:

$$ONOOH \rightarrow \{ONO^. \ ^.OH\} \rightarrow NO_2^. + OH^. \qquad (I)$$
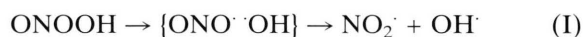
For this type of oxidants the term "crypto-OH" has been introduced (Youngman and Elstner, 1981). The strong oxidant, ONOOH is also formed during the decay of SIN-1, which primarily produces nitrogen monoxide and superoxide and thus finally ONOOH (Hogg *et al.*, 1992). According to the update of knowledge, ROS are produced by all types of leukocytes and endothelial cells and are undoubtedly involved in microbial killing, cytotoxicity and blood vessel damage (Esterbauer *et al.*, 1991). Damage of endothelial cells and induction of atherogenesis is a very complex interaction of several metabolic and/or pathological processes possibly involving peroxynitrite (Marin and Rodriguez-Martinez, 1995). If OH-type oxidants are involved in atherogenesis (Darley-Usmar *et al.*, 1992; Graham *et al.*, 1993; Hogg *et al.*, 1993; Moore *et al.*, 1995), however, efficient OH-scavengers should have inhibitory effects on LDL-oxidation.

It is well known that sugars like glucose and other polyalcohols such as mannitol, are fairly good OH-scavengers (Stocker and Frei, 1991). Hence, under diabetic conditions where glucose concentrations in the blood are found between 8 and 20 mM, LDL-oxidation by OH-type oxidants should be diminished. This is contradicted by experimental data: increased physiological glucose concentrations (10 mM) promote LDL-oxidation by peripheral blood mononuclear cells and isolated macrophages in an SOD-inhibitable reaction (Rifici *et al.*, 1994; Kawamura *et al.*, 1994). These results indicate an initiating process for LDL-oxidation involving superoxide but which is apparently not the typical pathway via Fenton-type chemistry (including iron catalysis) where polyoles are well-known inhibitors. Our investigation concerned possible interactions between ONOOH and glucose on LDL-oxidation. An interaction of ONOOH with glucose has been reported to yield a stable, NO-releasing product (Moro *et al.*, 1995). Surprisingly typical OH-scavengers such as formate, mannitol and also glucose enhance LDL-oxidation by SIN-1 and synthetic ONOOH dependent on concentration. Glucose-concentrations measured in blood plasma from diabetic patients exhibit maximal effects in our model system.

Materials and Methods

Materials

SIN-1 has been given free of charge by Dr. R. Grewe, Fa. Hoechst AG, Frankfurt; all other chemicals were either purchased from SIGMA, Boehringer-Mannheim or Merck-Darmstadt.

Methods

LDL-preparation

LDL was isolated from human blood serum (donor: healthy male, 58j., 88 kg) by ultracentrifugation as recently described (Kögel *et al.*, 1994; Schlüssel and Elstner, 1995). LDL was stored (4 °C, in the dark) no longer than two weeks before use. LDL concentration (µM) was estimated on the basis of protein content of the solution (determined by means of the Coomassie Brilliant Blue microassay of BioRad). The α-tocopherol content of each LDL preparation was determined by organic extraction with n-hexane and HPLC analysis (Nucleosil 300, methanol/isopropanol 92.5/7.5 v/v, UV detection at 280 nm). The α-tocopherol content was essential the same for each LDL-preparation (5.1 mol/mol LDL).

Oxidation of LDL

LDL oxidation was followed via diene conjugation ("Esterbauer"-method as modified by Schlüssel and Elstner, 1995) or by electrophoretic mobility i.e. agarose gel chromatography (Kögel *et al.*, 1994).

Formation of conjugated dienes was measured spectrophotometrically by monitoring the increase in absorbance at 234 nm (37 °C) using 0.05 µM LDL and 1.67 µM Cu^{2+}-ions or the indicated amounts of SIN-1 or ONOOH.

Electrophoretic mobility after incubation of 0.73 µM LDL with 3 µM Cu^{2+} or the indicated amounts of SIN-1 or ONOOH for 24 h (37 °C) was determined by electrophoresis on 0.8 % agarose gels. Lipoprotein staining was performed by incubation of the reaction mixture (30 min, room temperature) with sudan black (200 mg in 5 ml ethanol, 3 ml glycerol 87 %, 2 ml aq. bidest.).

Synthesis of peroxynitrite

Peroxynitrite (ONOOH) was synthezised according to Beckmann *et al.* (Beckmann *et al.*, 1994) as follows: 0.7 M hydrogen peroxide solution in 5 ml 0.6 M HCl was mixed with 5 ml 0.6 M KNO_2 on ice for one second. The reaction was quenched with 5 ml ice cold 1.2 M NaOH. This mixture was then frozen over night (−20 °C). The top layer was collected and stored as stock solution. The concentration was determined before each experiment by measuring the absorbance at 302 nm based on an absorption coefficient of $E_{302} = 1670\ M^{-1}cm^{-1}$. A comparison of the oxidative capabilities measuring ethene release from keto-methylthiobutyrate (Hippeli *et al.*, 1997) indicated that SIN-1 on a "molar basis" had about double the oxidative power as compared to synthetic ONOOH probably due to nitrate impurities in ONOOH also absorbing at 302 nm. Further experimental details are given in the individual figures. All experiments were repeated at least three times.

Results

Diene conjugation (DC)

Treatment of isolated LDL by low concentrations of copper-ions is a frequently used method for testing the oxidizability of LDL or substances delaying it such as α-tocopherol (Esterbauer *et al.*, 1990; Kuzuya *et al.*, 1991). The increase of both,

light-absorbance at 234 nm (DC; "diene conjugation") and electrophoretic mobility (EM, as indication of increase in negative charge of the apolipoprotein) have been proven as most useful for routine measurements for monitoring the oxidation of LDL (Kögel *et al.*, 1994; Schlüssel and Elstner, 1995). As shown in Figure 1 A, during incubation of LDL with either 1.67 μM Cu²⁺-ions,
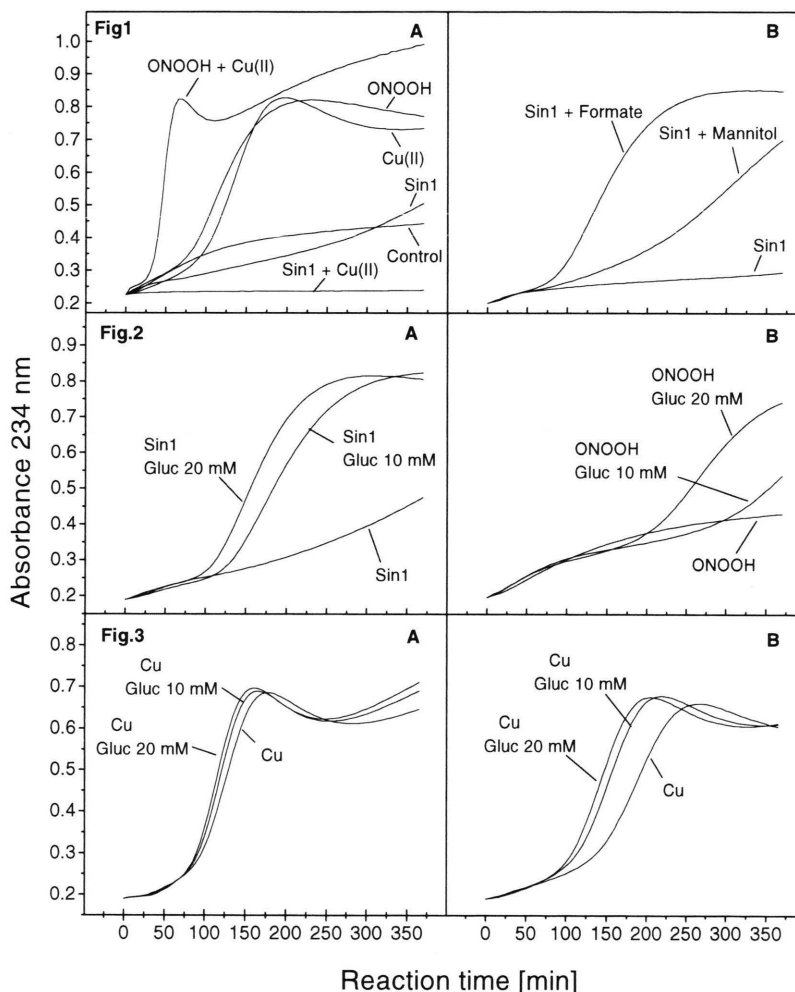
Fig. 1. (**A**) Differences between copper, ONOOH and SIN-1 driven LDL-oxidation. (**B**) Enhancement by mannitol and formate of Sin1 initiated LDL-oxidation. Time dependent photometric monitoring of absorbance increase at 234 nm (37 °C) of isolated LDL (0.05 μM).
Added oxidants: Cu²⁺ (1.67 μM), Sin1 (10 μM), ONOOH (20 μM).
Fig. 2. Enhancement by glucose of Sin1 (**A**) and ONOOH (**B**) initiated LDL-oxidation. The same LDL sample as was used in Fig. 1 was oxidized, and the conjugated diene increase was measured.
Added oxidants: Cu²⁺ (1.67 μM), Sin1 (10 μM), ONOOH (5 μM).
Fig. 3. Enhancement by glucose of copper induced LDL-oxidation. The same LDL sample as was used in Figure 1 was oxidized, and the conjugated diene increase was measured.
Added oxidants: (**A**) Cu²⁺ (1.67 μM), (**B**) Cu²⁺ (0.21 μM).

20 μM ONOOH or 10 μM SIN-1, diene conjugation is increased in a charateristic manner: Where ONOOH and Cu-ions exhibit similar diene conjugation-kinetics, the SIN-1 induced reaction shows a characteristic lag phase proceeding below the control in the first four to five hours and is accelerated after five hours of incubation. We used the indicated concentrations of SIN-1 and synthetic ONOOH since ONOOH exhibits only half the potential to drive ethene release from keto-methylthiobutyrate (KMB, as an indicator for the production of a strong oxidant of the OH-type) (Hippeli *et al.*, 1997). Combination of Cu^{2+} with SIN-1 or ONOOH has striking effects: while the ONOOH- or Cu^{2+}-reactions are strongly enhanced in presence of both oxidants, the SIN-1 and Cu-reactions annihilate each other to virtually zero. The "classical" OH-radical scavengers, mannitol and formate (5 mM) inhibit KMB oxidation (Hippeli *et al.*, 1997) but cause a strong stimulation of diene conjugation (Fig. 1 B). Likewise, addition of increasing concentrations of glucose (0.5 to 20 mM) to SIN-1 strongly accelerate diene conjugation: A concentration of 10 mM glucose + SIN-1 exhibits an almost identical initial velocity as 1.67 μM copper ions (Fig. 2A). If ONOOH is compared to SIN-1, 5 μM ONOOH alone has no effect on diene conjugation but stimulation by 10 and 20 mM glucose is clearly visible (Fig. 2B). At 1.67 μM Cu^{2+}, 10 and 20 mM glucose have almost no effect (Fig. 3A). At much lower Cu^{2+}-concentration (0.21 μM) a stimulation by these glucose concentrations is visible (Fig. 3B). This effect is much weaker as compared to the effects in the presence of SIN-1 or ONOOH, however.

Electrophoretic mobility (EM)

Cu^{2+}-oxidation of LDL strongly accelerates its electrophoretic mobility. Addition of increasing amounts of SIN-1 (2.5; 5; 10 μM) cause a increase of diene conjugation (data not shown). On the other hand such low concentration of SIN-1 have no influence on the electrophoretic mobility of LDL (Table I). Significant effects of SIN-1 on electrophoretic mobility are observable above 750 μM and effects of 20 mM glucose are clear with 750 μM and 1 mM SIN-1. Acceleration of electrophoretic mobility by ONOOH are determined at 50 μM and increase up to 750 μM. Glucose effects (20 mM) in the presence of 50 μM ONOOH yield acceleration up to 10 % of electrophoretic mobility and are gradually lost with increasing ONOOH-concentrations up to 750 μM (Table I).

Discussion

The lag-time of the appearance of absorbance-increase at 234 nm as an indicator for LDL-diene conjugation after oxidation by copper ions is a common and well known method for determining the intrinsic antioxidative potential of LDL or functions of external antioxidants. We used this method in comparison to electrophoretic mobility in order to study LDL-oxidation by SIN-1 and peroxynitrite. As shown under results, DC- and EM-changes of LDL induced by identical concentrations of oxidant can only be measured by Cu^{2+}-oxidation but not by oxidation with SIN-1. Very high and thus unphysiological concentrations of either SIN-1 (750 μM) or synthetic ONOOH (500 μM), respectively, yield approximately 70 % and 100 % of the maximal electrophoretic mobil-

Table I. Electrophoretic mobility of LDL (0.73 μM) after 24–h incubation with Cu^{2+}, Sin–1 or ONOOH.

Oxidants added [μM]	Electrophoretic mobility [%] (3 μM Cu^{2+} = 100%, 0 μM Cu^{2+} = 63.6%)			
	Sin–1		ONOOH	
	without Glucose	with Glucose	without Glucose	with Glucose
10	63.4 +/− 0.95	63.9 +/− 0.58	64.2 +/− 2.54	65.4 +/− 3.26
50	67.3 +/− 0.85	65.0 +/− 1.22	74.8 +/− 2.89	83.5 +/− 1.98
100	67.9 +/− 0.83	65.4 +/− 1.91	87.4 +/− 0.14	92.5 +/− 2.69
250	68.2 +/− 0.35	69.4 +/−1.20	93.8 +/− 1.21	96.3 +/− 0.71
500	69.1 +/− 1.63	70.3 +/− 0.14	99.1 +/− 1.27	101.0 +/− 1.34
750	71.7 +/− 0.36	74.7 +/− 0.99	101.8 +/− 0.12	103.8 +/− 2.62
1000	75.2 +/− 2.8	79.3 +/− 2.05	102.7 +/− 1.20	104.7 +/− 3.89

ity obtained with Cu^{2+} (Table I). Changes of electrophoretic mobility by SIN-1 are not significant up to 500 μM while 50 μM ONOOH already prove significant (ONOOH-LDL: EM = 75 %; control: EM = 63.6 %). Interactions between Cu^{2+} and SIN-1 or Cu^{2+} and ONOOH during LDL-oxidation, are controversial: while the presence of both Cu^{2+} and ONOOH cooperate in LDL-oxidation, the presence of both Cu^{2+} and SIN-1 annihilate each other (Fig. 1A). This might be due to an interaction between copper ions and superoxide produced by SIN-1 in an SOD-like manner. We are currently studying the mechanism of this interaction. Our result might have a physiological consequence, however: if we assume that the interaction between superoxide and nitrogen monoxide is a basic physiological event in atherogenesis, then Cu^{2+} ions cannot have a dominant physiological function in LDL-oxidation.

Oxidation of LDL is thought to represent one most critical step during the initiation of atherosclerosis (Brown and Goldstein, 1979; 1985; Esterbauer et al., 1988, 1990; Jürgens et al., 1987; Steinbrecher et al., 1989; Kuzuya et al., 1991; Jessup et al., 1990; Darley-Usmar et al., 1992; Graham et al., 1993; Hogg et al., 1993; Moore et al., 1995) and peroxynitrite has recently been discussed as one physiological oxidant with high relevance (Darley-Usmar et al., 1992; Hogg et al., 1993; Moore et al., 1995). Likewise, there are several reports on LDL-oxidation referring to the diabetic situation: Insulin- and glucose-enhanced LDL-oxidation by white blood cells (Rifici et al., 1994; Kawamura et al., 1994) seems to proceed via reactions probably including superoxide. Peroxynitrite in turn reacts similar to the strongly oxidizing OH-radical; thus polyoles and sugars such as glucose, acting as OH-scavengers, should provide at least some protection against ONOOH-mediated oxidative damage. This can actually be shown by measuring ethene release from KMB as a very common test system for the production of strong oxidants of the OH-radical type (v. Kruedener et al., 1995). Mannitol and formate at 10 mM concentrations are inhibitory in these reactions (v. Kruedener et al., 1995; and ref. therein). Similarly, OH-radicals produced by autoxidation of the diabetes-inducing molecule, dialuric acid, can be detected as ethene release from methional in a reaction which is inhibited by 20 mM glucose (Miwa and Okuda, 1982) in analogy

to KMB oxidation. Likewise, ESR signals indicating free radicals produced by coupling of anti-cancer drugs with microsomal NAD(P)H oxidases, are quenched in the presence of glucose (Sato et al., 1977). Thus, in addition to the production of free radicals following the Maillard reaction (Thornally and Stern, 1984; Ookawara et al., 1992) and glycation of proteins (Jennings, 1994), the reaction of glucose with free radicals such as OH· or nitroxyl-radicals has to be envisaged where reaction constants in the diffusion-limited range of approximately 10^8 M^{-1} sec^{-1} have been reported (Asmus and Nigam, 1976).

During LDL-oxidation driven by SIN-1 or ONOOH, OH-scavengers such as mannitol, formate or glucose stimulate diene conjugation, however. This is, compared to the above mentioned data an unexpected result. On the basis of some other reports, our results may be explained by the following hypothesis:

Interactions of glucose and ONOOH have been described to yield stable products (Moro et al., 1995). Glucose, on the other side, prevents ONOOH-dependent inhibition of respiration by brain mitochondrial particles with an IC_{50} of 8 mM but does not influence nitrogen monoxide-dependent inhibition of respiration at 100 mM (Lizasoain et al., 1996). We have to assume, therefore, that glucose reacts with ONOOH and not with NO. Since the reactivity of superoxide is some orders of magnitude slower as compared to the OH-radical, a reaction with glucose can also be neglected. Thus, on the basis of the "cage"-hypothesis (Pryor and Squadrito, 1995), the following sequence of reactions might be feasible: Release of NO_2· or OH· (II) from the cage is very slow,

$$\{ONO· OH\} \rightarrow NO_2· + OH· \qquad (II)$$

but glucose (glu-OH) may act as electron donor for cage-OH· (III) yielding the products NO_2·, glucose-radical and water

$$\{ONO· OH\} + glu-OH \rightarrow ONO· + HOH + glu-O· . \qquad (III)$$
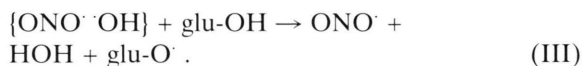
In other words: glucose may "liberate" NO_2· from the cage:

That this is really the case has recently been shown by Zhu et al. (1992) who demonstrated that the excellent OH-scavenger, dimethylsulfoxide,

releases substantial amounts of $NO_2^{\cdot}$ from peroxynitrite under physiological conditions.

Either the glucose-radical, which might also be produced during the Cu^{2+}-catalyzed reaction, or $NO_2^{\cdot}$, which is a very reactive radical species, interact with LDL initiating its oxidation. That $NO_2^{\cdot}$ might be the proper candidate can be deduced from the following reports:

Pulse radiolysis data (Prutz *et al.*, 1985) yield rate constants for the reactions of $NO_2^{\cdot}$ with linoleate of approximately 2×10^5 and inhalation of gaseous $NO_2^{\cdot}$ has been shown to initiate lipid peroxidation measurable as ethane exhalation (Sagai *et al.*, 1982) or as peroxidized red blood cell lipids

(Posin *et al.*, 1978). A very recent report by Kikugawa *et al.* (1995) strongly supports our assumption: these authors exposed an LDL-solution at pH 7.5 to a 70 ppm $NO_2^{\cdot}$-atmosphere at 37° C for several hours and detected lipid- and tryptophan-oxidation. LDL oxidized by $NO_2^{\cdot}$ also resulted in increased binding to mouse peritoneal macrophages. The authors discuss the possibility that $NO_2^{\cdot}$ *in vivo* might participate in the formation of atherosclerotic leasons. All together there is good reason to assume that the cooperation of superoxide, nitrogen monoxide and glucose produces an oxidant for LDL which might be operating in both diabetic persons and smokers: nitrogen dioxide.

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