Glyc-oxidized LDL impair endothelial function more potently than oxidized LDL: role of enhanced oxidative stress

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Abstract

Hypercholesterolemia is associated with impairment of endothelial function due to increased levels of LDL. In diabetic patients, however, attenuation of endothelial function occurs even under normocholesterolemic conditions. Here we assessed whether glycation of LDL potentiates their influence on endothelial function, with particular emphasis on the oxidizability of LDL and the role of O$_2^-$.

Human LDL was glycated by dialyzation for 7 days against buffer containing 200 mmol/l glucose, or sham-treated without glucose, and oxidized by incubation with Cu$^{2+}$. Glycation significantly enhanced the oxidizability of LDL, as detected by diene formation and by electrophoretic mobility (27.5 mm for oxidized LDL vs. 34 mm for oxidized glycated LDL at 20 h of oxidation). Isolated rings of rabbit aorta were superfused with physiological salt solution, and isometric tension was recorded. Incubation of the aortic rings with sham-treated or with glycated LDL, not oxidized, had no influence on acetylcholine-induced, endothelium-dependent relaxation. Exposure of the aortic rings to oxidized non-glycated LDL caused a significant inhibition (30% at 1 $\mu$M acetylcholine) of the endothelium-dependent relaxation only in the presence of diethyl-dithiocarbamate (DDC), an inhibitor of the endogenous superoxide dismutase (SOD). Incubation of aortic rings with oxidized glycated LDL attenuated endothelium-dependent relaxation even in the absence of DDC (by 31% at 1 $\mu$M acetylcholine). The presence of DDC potentiated the inhibition of relaxation (65% inhibition at 1 $\mu$M acetylcholine), and co-incubation with exogenous SOD and catalase prevented the inhibition of relaxation, indicating a mediator role of O$_2^-$. Endothelium-independent relaxation induced by forskolin was unaffected by any of the lipoproteins. Using a chemiluminescence assay, significantly increased O$_2^-$ production of aortic rings pretreated with oxidized glycated LDL (4101 ± 360 counts/s) in comparison to control rings (753 ± 81 counts/s) or arteries pretreated with oxidized non-glycated LDL (2358 ± 169 counts/s) could be detected, suggesting that enhanced NO-inactivation by O$_2^-$ could be the underlying mechanism for the stronger impairment of endothelium-dependent dilations by oxidized glycated LDL. Glycation increases the oxidizability of LDL and potentiates its endothelium-damaging influence. The likely mechanism for attenuation of endothelium-dependent dilations is increased formation of O$_2^-$, resulting in inactivation of nitric oxide. This mechanism may play an important role in diabetic patients and may contribute to disturbed organ perfusion. © 1998 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Nitric oxide released from the arterial endothelium is an important modulator of vascular tone [1,2]. Attenuation of endothelial function may lead to severely impaired blood supply to organ tissue [3].
Pathophysiological conditions associated with impaired endothelial function include, among others, hypercholesterolemia and diabetes mellitus. Attenuation of endothelium-dependent dilations has been observed, for example, in coronary arteries of hypercholesterolemic patients [4,5], and has been related to high blood levels of low density lipoprotein (LDL). In diabetic patients, however, impairment of endothelial function occurs even under normocholesterolic conditions with normal LDL levels [6]. The reason for this difference has not been fully elucidated yet.

In order to analyze the effects of LDL on endothelial function, various in vitro studies have been undertaken [7–11]. Suggested mechanisms underlying the effects of LDL have in common that oxidative modification of the lipoprotein is considered to be a prerequisite for damaging the endothelium. In vivo, oxidized LDL accumulates in the wall of atherosclerotic arteries [12], thus in close vicinity to the endothelium and the vasculature. Oxidized LDL may than interfere with formation of nitric oxide [9], may directly inactivate nitric oxide [8,13], or may interact with vasomotor activity at the smooth muscle level [14,15]. An important feature of oxidized LDL which may explain many of its biological effects may be its capacity to stimulate formation of superoxide anion [7,16], resulting in inactivation of nitric oxide [17] and potential endothelial and smooth muscle cell damage. Indeed, arteries of hypercholesterolemic rabbits have been shown to produce large amounts of superoxide anion [18].

Reactive oxygen species are also considered to play an important pathophysiological role in diabetes mellitus [19–21]. Furthermore, it has been suggested that glycation of LDL, as it occurs under hyperglycemic conditions in a time and glucose concentration-dependent way, favors the oxidation of LDL [22–25]. However, as yet it has not been determined whether glycation of LDL alters or potentiates its negative influence on endothelial function. Therefore, it was the aim of this study to investigate the impact of LDL-glycation on its oxidizability, and to compare the potency of glycated and oxidized (glyc-oxidized) LDL with control LDL to attenuate endothelium-dependent dilations. We hypothesized that glycation leads to a stronger oxidative modification of LDL, and that such a glyc-oxidized LDL is a more potent inhibitor of endothelium-dependent dilations, compared to LDL oxidized under control conditions. To evaluate this hypothesis, we studied the influence of native and oxidized LDL on endothelium-dependent dilation of isolated rabbit arteries, and compared its effect with the potency of glycated and glyc-oxidized LDL. Furthermore, we investigated whether the effect of glyc-oxidized LDL on endothelium-dependent dilations involves formation of reactive oxygen species, since evidence has been provided for a linkage between endothelial dysfunction in diabetes mellitus and oxidative stress [19–21,26,27].

2. Methods

2.1. Chemicals

Phenylephrine was from Hoechst (Frankfurt, Germany), sodium nitroprusside, indomethacin, acetylcholine, forskolin, superoxide dismutase (SOD), catalase, ethylene-diamine-tetra-acetic acid (EDTA), diethyldithiocarbamate (DDC), 4,5-dihydroxy-1,3-benzene disulfonic acid salt (TIRON), \(N^G\)-nitro-L-arginine, lucigenin, and butylated-hydroxy-toluene (BHT) were from Sigma (Munich, Germany). Forskolin was dissolved in DMSO and, as with all other drugs if not indicated otherwise, further diluted in physiological salt solution of the following composition in mmol/l: 118.3 NaCl, 4.0 KCl, 2.5 CaCl₂, 1.0 MgSO₄, 1.2 KH₂PO₄, 24.0 NaHCO₃, 0.026 calcium sodium EDTA, and 11.1 glucose). Indomethacin was dissolved in ethanol—0.1 mol/l NaHCO₃ (1:3) vol/vol.

2.2. Isolation and glycation of LDL

Human LDL was isolated as described recently by ultracentrifugation [7]. Protein content of lipoproteins was measured using a commercially available kit (Sigma protein kit) which bases on a modification of the method as initially described by Lowry [28]. Lipoprotein concentrations are always given as µg protein per ml solution. Homogeneity of lipoproteins was tested by agarose gel electrophoresis (REP–HDL-plus cholesterol electrophoresis, Helena Diagnostika, Hartheim, Germany). Lipoproteins were prepared fresh every two weeks. During this period apolipoprotein B remained intact and not degraded.

To induce non-enzymatic glycation of LDL, LDL was filled in dialysis tubes (Spectra Por™, Spectrum, pore size 50,000 dalton) under sterile conditions, and dialyzed against phosphate buffered saline (PBS) containing 200 mmol/l glucose for seven days at 37°C in the dark. The dialysis buffer (3 l) contained 200 µmol/l EDTA to prevent autoxidation of LDL and was changed every 48 h. Control LDL was sham treated by dialysis against PBS without glucose under otherwise identical conditions. The dialysis tubes contained 20 ml of LDL at a concentration of 1000 µg/ml.

2.3. Detection of glycation of LDL

The relative degree of LDL-glycation was detected by the increase in relative mobility on agarose gel (lipidophor electrophoresis kits, IMMUNO, Heidelberg, Germany), and by affinity gel chromatography with...
2.5. Oxidative modification of native and glycated LDL

The resistance of native and glycated LDL against oxidative modification was compared photometrically by measuring the lag phase of conjugated diene formation at 234 nm wavelength as described by Esterbauer et al. [33]. For this purpose, either glycated or sham treated LDL (50 μg) were filled in a quartz cuvette containing 1.66 μmol/l CuSO₄ in 1 ml PBS and placed in a spectrophotometer (Perkin Elmer Spectrometer Lambda 12). The lag phase was defined as the duration until the antioxidative defense of LDL was consumed, as detected by the rapid increase in absorption at 234 nm.

Preparation of oxidized LDL was carried out as described recently [7]. Briefly, antioxidant-free LDL (300 μg protein/ml) was incubated with CuSO₄ (5 μmol/l) in PBS, or, in case of glycated LDL, in CuSO₄ containing PBS supplemented with 50 mmol/l glucose. The degree of oxidation was quantified by the increase in relative mobility on agarose gel (lipidophor electrophoresis kits, IMMUNO, Heidelberg, Germany), indicating an enhanced negative charge of oxidized LDL [34]. In order to study whether the time-dependent degree of oxidation varies between glycated and control LDL, we determined electrophoretic mobility at different time points after initiation of the LDL oxidation. For this purpose, glycated and non-glycated control LDL of the same charge were oxidized in parallel by incubation with CuSO₄ (5 μmol/l) in PBS. At various time points between start and end of the oxidation process, samples were taken from both preparations, and electrophoretic mobility was determined (Fig. 1). According to these data, the difference in the degree of oxidation between native and glycated LDL was most pronounced at approximately 20 h of oxidation. Therefore, we used LDL preparations oxidized for 20 h to study the effect on endothelium-dependent dilator responses unless indicated otherwise.

2.6. Determination of the ratio of lysophosphatidylcholine to phosphatidylcholine (LPC/PC) in LDLs

Lipids were extracted from the lipoproteins (native LDL, oxidized LDL, and glyc-oxidized LDL) by the method of Folch et al. [35]: to 100 μl of the lipoprotein solution (protein concentration approx. 1 mg/ml) were added 133 μl methanol, then 267 μl chloroform. The mixtures were shaken vigorously and then centrifuged to separate the phases. The upper phases were carefully withdrawn and washed with 300 μl of synthetic lower phase. The combined lower phases were taken to dryness under a stream of N₂, then re dissolved in 100 μl chloroform/methanol (2:1, v/v). Aliquots of 25 μl were applied to an HPTLC plate (Merck Kieselgel 60, 10 ×
10 cm) in 1 cm wide streaks. The plate was developed with chloroform/methanol/water (70/30/5 by vol.). After drying thoroughly, the plates were immersed in a solution of 2 g ammonium molybdate, 1 g cerium-IV sulfate and 5 ml conc. sulfuric acid in 90.5 ml water. Excess reagent was allowed to drip off, the plates were heated at 150°C until the substances appeared as blue spots on yellow background, then after short cooling, were covered with a clean glass plate. The amounts of individual substances were quantified by scanning the plates (LKB 5301 densitometer, transmission mode with yellow filter) and comparing the areas under each peak with those of standards (LPC, PC, and sphingomyelin) with known concentrations run in parallel on the same plate. The results obtained for LPC and PC were set in relation to sphingomyelin as an internal standard, because the sphingomyelin content was not altered by oxidative modification of LDL.

2.7. Vessel preparation and diameter determination

The experimental set up for detection of force development of isolated rabbit aortas superfused with physiological salt solution under isometric conditions has been described recently [36]. Briefly, the thoracic aortas were removed from rabbits of either sex (New Zealand rabbits, 4–5 months old, 2.5–3.5 kg, killed by decapitation). All procedures were carried out in accordance with the guidelines of the German Ministry of Agriculture for the use and care of laboratory animals. The blood vessels were placed in a physiological salt solution (composition in mM: 118.3 NaCl, 4.0 KCl, 2.5 CaCl₂, 1.0 MgSO₄, 1.2 KH₂PO₄, 24.0 NaHCO₃, 0.026 calcium sodium EDTA, and 11.1 glucose). The arteries were cleaned of fat and connective tissue and cut into rings (3–4-mm long). The rings were suspended between two stirrups, one anchored to a steel plate and the other connected to a strain gauge transducer (Hugo Sachs, Germany) to record changes in isometric tension, and placed in a small organ bath (volume 2 ml). The aortic rings were superfused at constant flow (3 ml/min) with physiological salt solution (37°C, bubbled with a 95% O₂–5% CO₂ gas mixture). At the beginning of the experiments, the rings were prestretched progressively by increments of 0.5 g until the optimal point of their length-active tension curve (2.5 g; determined in preliminary experiments with 1 μmol/l phenylephrine) was reached. The aortic rings were then contracted with phenylephrine (1 μM). Once the contractions reached a plateau, the endothelial integrity of the preparations was tested by adding 1 μmol/l acetylcholine to the superfusate. Only arteries with a dilator response of > 70% (calculated as the % inhibition of preconstriction) were further investigated. The preparations were then washed with physiological salt solution for 45 min before being contracted a second time with phenylephrine. When a stable preconstriction of 5–7 g was reached, a full dose response curve for acetylcholine (1 nmol/l–3 μmol/l) was performed. In each experiment, two aortic rings obtained from the same animal were studied simultaneously, with one ring serving as time-matched control for the lipoprotein-treated artery. To confirm that the acetylcholine-induced dilation was mediated by endothelium-derived nitric oxide, we studied the inhibitory effect of N⁶-nitro-L-arginine on the dilator responses as described recently [7]. Following determination of the first complete acetylcholine dose-response, the influence of the various lipoprotein-preparations on dilator responses was studied. Acetylcholine was washed out, and one aortic ring was incubated for 90 min with native or oxidized LDL, glycated (but not oxidized) LDL, or with glyc-oxidized LDL (300 μg/ml each). The other aortic ring was incubated with the respective buffer as time matched control prior to addition of acetylcholine. After washout of the lipoproteins, endothelium-dependent dilations were elicited by adding cumulative doses of acetylcholine (1 nmol/l–3 μmol/l) to the superfusion. To investigate the influence of glyc-oxidized LDL on endothelium-independent dilator capacities, forskolin (0.01–100 μmol/l) was used instead of acetylcholine, according to the otherwise unchanged protocol. The influence of superoxide dismutase (SOD, 10 U/ml), of catalase (100 U/ml), and of the SOD-inhibitor diethyl-dithio-carbamate (DDC, 10 mmol/l) on acetylcholine-induced dilations in the presence and absence of ox-
Table 1. Some text is missing from the image.
with 50 mmol/l glucose, the duration of the lag phase was significantly further reduced to 156 ± 9 min. Thus, both glycation and the presence of glucose enhance the oxidizability of LDL. Data are obtained from the means of three repeated measurements of four individual LDL preparations.

In another set of experiments, we studied the time course of LDL oxidation in glycated and in control preparations of the same charge by determination of the electrophoretic mobility on agarose gel at various time points after initiation of the oxidative process with Cu\(^{2+}\). As shown in Figs. 1 and 3, the glycated LDL preparations showed a slight increase in electrophoretic mobility on agarose gel compared to controls already before the initiation of the oxidative process, presumably resulting from Schiff-base formation on lysine groups during glycation [37]. However, after initiation of the oxidative process, electrophoretic mobility was significantly enhanced in glycated LDL preparations compared to controls (Figs. 1 and 3), reflecting a higher degree of oxidation [34]. It should be noted that in this set of experiments, oxidation of glycated LDL was carried out in PBS without glucose. Thus, the increased electrophoretic mobility of glyc-oxidized LDL was not due to a further glycation of the lipoprotein. In control experiments, we confirmed by boronate gel chromatography that the degree of glycation remained stable during the oxidative modification in the absence of glucose. Since the difference in the degree of oxidation was most pronounced at approximately 20 h of oxidation, we used these preparations to study the effect on

Fig. 3. Representative agarose gel electrophoresis demonstrating electrophoretic mobility of native, glycated, oxidized, and of glyc-oxidized LDL (nLDL, glyLDL, oxLDL, glyoxLDL). Electrophoretic mobility of glycated LDL was slightly increased compared to native LDL. Electrophoretic mobility of oxidized LDL was increased by a factor of 2.5 as parameter for the extent of Cu\(^{2+}\)-induced lipid-peroxidation, and electrophoretic mobility of glyc-oxidized LDL was increased by a factor of 3.3.

Fig. 4. Effect of native LDL and of oxidized LDL in the absence and in the presence of DDC (= diethyl-dithio-carbamate, inhibitor of the endogenous superoxide-dismutase; 10 mmol/l) on the acetylcholine-induced, endothelium-dependent dilation of isolated rabbit aortic rings. Incubation of phenylephrine-precontracted aortic rings with 300 µg/ml native LDL or oxidized LDL in the absence of DDC had no influence on the dilator responses. However, in the presence of DCC, endothelium-dependent dilator responses were significantly attenuated. Dilator responses are expressed as percent of precontraction. n = 9; * p < 0.05.

Fig. 5. Effect of glycated LDL and of glyc-oxidized LDL on the acetylcholine-induced, endothelium-dependent dilation of isolated rabbit aortic rings. Incubation of phenylephrine-precontracted aortic rings with 300 µg/ml glycated LDL or oxidized LDL in the absence of DDC had no influence on the dilator responses. After incubation of the segments with 300 µg/ml glyc-oxidized LDL, dilator responses were significantly attenuated. Dilator responses are expressed as percent of precontraction. n = 10; * p < 0.05.
endothelium-dependent dilator responses unless indicated otherwise.

3.3. Advanced glycation end products (AGEs) and modification of lysine residues in LDLS

Fig. 2 shows a representative fluorescence spectrum of samples of LDLS (native LDL, glycated LDL, oxidized LDL, and glyc-oxidized LDL). AGEs enhance emission between 440 and 450 nm, as determined by use of bovine serum albumin-advanced glycation endproduct as internal standard [30]. There was no difference between native and glycated LDL, indicating that there was no formation of AGEs in glycated LDL. Glyc-oxidized LDL elicited the highest emission between 440 and 450 nm. However, the test is not specific for AGEs, since oxidized amino acids on proteins also contribute to fluorescence [31]. Thus, the difference of the fluorescence emission between oxidized LDL and glyc-oxidized LDL is not clearly attributable to enhanced formation of AGEs in glyc-oxidized LDL, but could also be caused by more advanced oxidation of the lipoprotein. Therefore, we also used a competitive ELISA method for determination of AGEs. This ELISA measures the ability of the probe (containing 65 μg LDL per well, respectively) to compete with an immobilized standard AGE (5 ng AGE-BSA per well, prepared as described [30]) on the microtiter plate surface for binding to the first AGE antibody. The degree of displacement induced by each probe is expressed in percent. Thus, 50% displacement theoretically corresponds to 5 ng AGE-equivalent in the respective probe. With this test, however, only minimal concentrations of AGEs were detectable in LDLS: binding of native LDL accounted to 94 ± 7%, binding of glycated LDL accounted to 82 ± 3%, binding of oxidized LDL accounted to 80 ± 11%, and binding of glyc-oxidized LDL accounted to 78 ± 2% (assays were done in triplicate). Thus, displacement was < 7% for native LDL (which is below the detection limit), and was < 22% for glycated, for oxidized LDL, and for glyc-oxidized LDL, indicating that formation of AGEs was minimal during our preparation procedures.
Modification of lysine residues in LDLs was estimated with the trinitrobenzenesulfonic acid assay. Free lysine groups accounted to 142 ± 7 mol/mol ApoB100 for native LDL, to 133 ± 10 mol/mol ApoB100 for glycated LDL, to 67 ± 11 mol/mol ApoB100 for oxidized LDL, and to 57 ± 12 mol/mol ApoB100 for glycoxidized LDL (data obtained from three independent measurements).

3.4. Formation of lysophosphatidylcholine in modified lipoproteins

Formation of LPC through the activity of phospholipase A2 on phosphatidylcholine (PC) is a well known reaction during oxidative LDL modification [38]. LPC may be responsible for part of the effects of oxidized LDLs on endothelial function [39]. We therefore determined the ratio LPC/PC in native, oxidized, and glycoxidized LDL. As expected, the non-oxidized LDLs contained only minimal amounts of LPC (ratio LPC/PC = 0.003). However, during oxidative modification of LDL, significant amounts of LPC were formed: the ratio LPC/PC was 0.181 for oxidized LDL, and increased to 0.295 in glycoxidized LDL (results obtained from three independent measurements). Thus, glycoxidized LDL contained a higher amount of LPC compared to oxidized LDL, indicating a more advanced oxidative modification.

3.5. Endothelium-dependent dilations

Acetylcholine dose-dependently elicited endothelium-dependent dilations in isolated rabbit aortic rings pre-constricted with phenylephrine. N^G-nitro-L-arginine (1 mmol/l) inhibited the acetylcholine-induced vasomotor response by 87 ± 4% (n = 7 experiments), identifying the dilation—as in previous studies—as mediated by endothelium-derived nitric oxide.

3.6. Influence of native and oxidized LDL on endothelium-dependent dilations in rabbit aorta

Acetylcholine-induced dilations were not impaired in aortic rings following 90 min treatment with 300 µg/ml native or oxidized LDL (Fig. 4). However, when the arteries were incubated with DDC, an inhibitor of the CuZn form of superoxide dismutase, treatment with oxidized LDL resulted in a significant inhibition of the endothelium-dependent dilations (Fig. 4), indicating a role for superoxide anion in the inhibitory process. DDC alone or together with native LDL had no effect on endothelium-dependent dilations (data not shown).

3.7. Effect of glycated and glycoxidized LDL on endothelium-dependent dilations in rabbit aorta

Treatment of the aortic rings with glycated, not oxidized LDL (90 min, 300 µg/ml) was without effect on the acetylcholine-induced endothelium-dependent dilations (Fig. 5). Glycoxidized LDL, however, significantly inhibited endothelium-dependent dilations. This effect was observed already in the absence of DDC, but was further increased by the presence of DDC, as shown in Fig. 6. Thus, glycoxidized LDL was a more potent inhibitor of endothelium-dependent dilations than non-glycoxidized oxidized LDL. As mentioned above, DDC alone had no effect on endothelium-dependent dilations (data not shown).

Since glycation enhanced the degree of LDL oxidation (Fig. 1), we investigated whether a non-glycated LDL oxidized for a longer period of time (35 h) would elicit the same effect on endothelium-dependent dilations as glyccoxidized LDL. However, although oxidation of non-glycated LDL for 35 h resulted in a similar degree of oxidation as oxidation of glyccoxidized LDL for 20 h as determined by electrophoretic mobility on agarose gel electrophoresis, the effect of this strongly oxidized LDL on endothelium-dependent dilations was not different from the effect of LDL oxidized under reference conditions (n = 10 experiments, data not shown).

3.8. Impact of SOD and catalase on the effect of glyccoxidized LDL

In order to further investigate the role of reactive oxygen species for the inhibitory effect of glyccoxidized LDL, we incubated the aortic rings additionally with SOD (10 U/ml) and with catalase (100 U/ml), enzymes catabolizing O_2^- and H_2O_2. In control experiments, we established that SOD and catalase had no influence on dilator responses in the absence of lipoproteins (n = 7 experiments each, data not different from untreated controls). We then studied the influence of SOD and catalase on dilator responses of arteries treated with 300 µg/ml glyccoxidized LDL. We incubated the arteries with SOD and catalase together because preliminary experiments revealed that SOD treatment alone—like in a previous study using the same experimental set up [7]—did not restore endothelium-dependent dilations, but rather increased the effect of glyccoxidized LDL (data not shown). Catalase alone was without significant influence on the effect of glyccoxidized LDL (n = 6 experiments, data not shown). However, as shown in Fig. 7, the presence of SOD and catalase together completely prevented the endothelium-damaging effect of glyccoxidized LDL, indicating that formation of superoxide anion was involved.
3.9. Detection of $O_2^-$ generation

To provide direct evidence for the hypothesis that enhanced superoxide anion formation was the underlying mechanism for the inhibition of endothelium-dependent dilations, we attempted to directly detect $O_2^-$ formation in the isolated arteries. $O_2^-$ generation detected by chemiluminescence of lucigenin in arteries treated with oxidized LDL and with glyc-oxidized LDL is shown in Fig. 8. Treatment of the arteries with 300 μg/ml oxidized LDL induced a significant increase in the chemiluminescence signal. Formation of $O_2^-$ was significantly further enhanced when the arteries were incubated with glyc-oxidized LDL in the same concentration. The $O_2^-$-scavenger TIRON, which easily enters the intracellular space, completely blunted the chemiluminescence signal (not shown). Treatment of arterial segments with native or glycated LDL had no influence on the chemiluminescence signal ($n=4$ each).

3.10. Endothelium-independent dilation induced by forskolin after treatment with glyc-oxidized LDL

To determine whether the smooth muscle dilator capacity was altered after treatment of the arteries with glyc-oxidized LDL, we studied in an additional series of experiments the influence of 300 μg/ml glyc-oxidized LDL on dilator responses induced by forskolin (0.1–100 μmol/l), a stimulator of adenylate cyclase in smooth muscle cells. Forskolin-induced dilator responses were not impaired by treatment with glyc-oxidized LDL, indicating that smooth muscle dilator functions were fully preserved (Fig. 9).

![Fig. 9. Effect of glyc-oxidized LDL on the forskolin-induced, endothelium-independent dilation of isolated rabbit aortic rings. Incubation of phenylephrine-precontracted aortic rings with 300 μg/ml glyc-oxidized LDL had no effect on the endothelium-independent dilator responses. Dilator responses are expressed as percent of precontraction. $n=8$.](image)

4. Discussion

In this study, we investigated the impact of LDL glycation 1) on its susceptibility to oxidative modification, and 2) on its effect on endothelium dependent dilation, in comparison to non-glycated LDL. The data presented demonstrate that glycation of LDL facilitates its oxidizability. Native control and glycated, but not oxidized LDL were without influence on the endothelium-dependent dilation in isolated aortic rings. Oxidized LDL inhibited dilator responses in the presence of DDC, an inhibitor of the endogenous superoxide dismutase, indicating a mechanism involving $O_2^-$ formation. The salient finding of this report is that glyc-oxidized LDL was more potent than LDL oxidized under control conditions. It significantly inhibited endothelium-dependent dilations even in the absence of DDC, and its effect was further increased by DDC. The prevention of the effect of glyc-oxidized LDL on endothelial function by additional treatment with SOD and catalase provides additional evidence for the involvement of oxygen derived radicals. This hypothesis is further strengthened by the direct detection of enhanced $O_2^-$ formation in arteries treated with glyc-oxidized LDL.

In the past years, it has become clear that the vascular endothelium is an important modulator of arterial tone and organ perfusion through the continuous release of vasoactive compounds [2], in particular through its formation of nitric oxide [1,40]. Attenuation of endothelial NO formation may lead to impaired blood supply to organ tissue and favours thrombus formation [3,41]. It has also become evident that endothelial function is disturbed in association with various systemic diseases, such as arteriosclerosis [42] and diabetes mellitus [6]. The observation that endothelial function is disturbed not only in severe arteriosclerosis but also during the early phase of the disease related to hypercholesterolemia [4] provided the rationale for many investigators to study the influence of atherogenic lipoproteins on the vasculature. These studies showed that atherogenic lipoproteins, in particular oxidatively modified LDL and Lp(a), interact with NO effects at different levels of the NO-signalling cascade [8–10,14,15,43]. Albeit through different mechanisms, the various in vitro effects of oxidized LDL and oxidized Lp(a) finally lead to an increase in vascular tone and can well explain the observations made with patients suffering from hypercholesterolemia and arteriosclerosis.

Recently, evidence has been provided that oxidized lipoproteins elicit part of their damaging effects on endothelial function through stimulation of reactive oxygen species formation. Increased rates of $O_2^-$ generation have been observed in arteries obtained from hypercholesterolemic rabbits [18], and we could directly...
demonstrate that atherogenic lipoproteins stimulate \( \text{O}_2^- \) formation in isolated rabbit arteries [7] and cultured mouse and rat renal cells [16]. Formation of reactive oxygen species such as \( \text{O}_2^- \) or consequently formed metabolites such as hydroxyl radical or peroxynitrite could account for the influence of \( \text{LDL} \) on endothelial NO formation [44,45] or on NO half-life [17,46]. Thus, oxidative stress involving or induced by atherogenic lipoproteins may be an important pathophysiologic modulator of vascular tone.

Endothelial function is disturbed also in diabetes mellitus [6,47,48]. Attenuation of endothelium-dependent dilations occurs not only in the late stage of the disease, but also during its early course, or—in vitro—even after short term hyperglycemia [49]. Another similarity to the effects of hypercholesterolemia on endothelial function relates to the potential influence of reactive oxygen species; evidence has been provided for a linkage between endothelial dysfunction in diabetes mellitus and oxidative stress [19–21,26,27]. Thus, the effects of hypercholesterolemia and diabetes mellitus have some features in common. In addition, it has recently been suggested that impaired vascular reactivity in diabetes mellitus is related to \( \text{LDL} \) levels [50].

An important metabolic effect of diabetes mellitus is glycation of lipoproteins: \( \text{LDL} \), like other proteins, are non-enzymatically glycated depending on the glucose concentration, a reaction which is considered to contribute to the severity of arteriosclerosis in diabetic patients [51,52]. It has, however, not yet been studied whether glycation and/or glycoxidation of \( \text{LDL} \) increases its negative influence on the function of vascular endothelium. Therefore, we were interested to study the impact of glycation on \( \text{LDL} \) properties with regard to its oxidizability and to its effects on endothelial dysfunction.

Dialysis of isolated \( \text{LDL} \) against glucose-enriched medium resulted in non-enzymatic glycation of approximately 40%, and in an increased negative charge. Glycation rendered \( \text{LDL} \) more susceptible to oxidative modification, as shown by the significantly shorter lag phase in comparison to control \( \text{LDL} \). Since the presence of glucose further reduced the lag phase duration, we oxidized glycated \( \text{LDL} \) in the presence of glucose for use in the vasomotor experiments. The difference in the degree of oxidation between glycated and control \( \text{LDL} \) was most pronounced at 20 h. Therefore, we used \( \text{LDL} \) preparations oxidized for 20 h for comparison of control and glycated \( \text{LDL} \). The effect of dialysis against glucose on glycation of \( \text{LDL} \) and on its oxidizability in vitro confirms previously published papers [23,24,29,53]. The study of in vitro glycated \( \text{LDL} \) may be considered artificial; however, our in vitro glycated \( \text{LDL} \) has many features in common with \( \text{LDL} \) isolated from diabetic patients [25]. In addition, the in vitro preparation enabled us to investigate the impact of glycation on biochemical and biological features of \( \text{LDL} \) under controlled conditions, using identical preparations from which control and glycated \( \text{LDL} \) were prepared. Furthermore, our results obtained with in vitro glycated \( \text{LDL} \) correlate well with studies on oxidizability of \( \text{LDL} \) performed with preparations isolated from diabetic patients [25,54]. At this point, it should be noted that non-enzymatic glycation of \( \text{LDL} \) is not identical with formation of advanced glycosylation end products (AGEs), which also produce significant effects on vascular integrity [37]. AGEs arise from glucose-derived Amadori products at a much later stage during the glycosylation process. Thus, experiments performed with \( \text{LDL} \) glycated for seven days refer rather to early pathophysiological changes. This view is supported by the results of the competitive ELISA, testing for the presence of AGEs. Our data indicate that formation of AGEs was only minimal during the preparation of glycated \( \text{LDL} \) and of glyc-oxidized \( \text{LDL} \). In this context, the fluorescence spectra of \( \text{LDL} \)s can also be interpreted as a more advanced oxidation of glyc-oxidized \( \text{LDL} \) in comparison to oxidized \( \text{LDL} \), since oxidized amino acids on proteins also contribute to fluorescence [31]. An additional support for more advanced oxidation of glyc-oxidized \( \text{LDL} \) is the increased LPC/PC ratio, in comparison to oxidized \( \text{LDL} \). The mechanism leading to more advanced oxidation of glycated \( \text{LDL} \) is likely to involve reactive oxygen species formation. The enhancement of oxidation of native \( \text{LDL} \) in the presence of pathophysiological concentrations of glucose can be prevented by SOD and butylated hydroxytoluene [24], scavengers of \( \text{O}_2^- \), and high glucose levels induce an increase in antioxidant enzyme levels in cultured human endothelial cells [20]. Furthermore, nonenzymatic protein glycation itself may also increase oxidative stress [19,55].

In the vasomotor experiments, glycated \( \text{LDL} \) was—like control \( \text{LDL} \)—without effect on endothelium-dependent dilations. Oxidized \( \text{LDL} \) significantly inhibited acetylcholine-induced dilator responses, confirming previous studies using lipoprotein concentrations within the same dose range [9–11]. The observation that oxidized \( \text{LDL} \) inhibited endothelium-dependent dilations only when the endogenous superoxide dismutase was inhibited by DDC points to a mediator role of reactive oxygen species in this process. This finding is in accordance with other studies from our laboratory [7,16] which demonstrated a stimulatory action of atherogenic lipoproteins on \( \text{O}_2^- \) generation in various tissues. \( \text{O}_2^- \) might lead to attenuation of endothelium-dependent dilations through augmented inactivation of NO and/or damage of endothelial cells [17,46]. Glyc-oxidized \( \text{LDL} \) inhibited endothelium-dependent dilations more potently than oxidized \( \text{LDL} \), since its effect took place even in the absence of DDC. The potentiation of its effect by inhibition of the endogenous superoxide dis-
mutase, and the prevention of its effect by co-incubation with SOD and catalase provide further evidence for the hypothesis that glyc-oxidized LDL attenuates endothelial function via production of \( \text{O}_2^- \). In line with this interpretation, we could directly show using a chemiluminescence assay that arteries treated with glyc-oxidized LDL produce \( \text{O}_2^- \) at a significantly higher rate compared to arteries treated with non-glycated, oxidized LDL. At a first glance, it seems contradictory that SOD alone did not restore endothelium-dependent dilations, but rather increased the effect of glyc-oxidized LDL. However, this effect of SOD has also been observed in a previous study using the same experimental set-up [7] and could be explained by the deleterious effects of too much SOD activity in relation to \( \text{H}_2\text{O}_2 \), removing enzymes. Indeed, it has been shown that SOD may favours formation of the biologically most active hydroxyl radical in the absence of catalase [45,56].

Thus, oxidative stress seems to be the mediator for both the enhanced modification of LDL as well as for its enhanced pathological effect on endothelial function. It should be noted that the more potent effect of glyc-oxidized LDL in comparison to control oxidized LDL is not solely due to the fact that it was oxidized to a higher degree, since non-glycated LDL oxidized for a longer period of time (35 h) elicited the same effect on endothelium-dependent dilations as LDL oxidized under reference conditions. We therefore suggest that glycation itself contributed to the oxidative stress. Endothelium-independent dilator responses induced by forskolin were not affected by glyc-oxidized LDL, indicating that vascular smooth muscle function was not attenuated.

The mechanism of the enhanced formation of reactive oxygen species has not been determined yet. The fact that only oxidized, but not native lipoproteins had a stimulatory capacity hints to the lipid peroxidation process itself. A potential pathway is the stimulatory influence of lipid peroxidation products on cellular \( \text{O}_2^- \) generating enzymes. During the lipoprotein oxidation, various more or less stable products are formed, including LPC, aldehydic lipid peroxidation products, and fatty acids produced by phospholipase A\(_2\) [57–59]. LPC has been shown to increase \( \text{O}_2^- \) production in vascular smooth muscle cells via stimulation of protein kinase C [60]. Our finding that the LPC/PC ratio was more increased in glyc-oxidized LDL, in comparison to oxidized LDL, could explain why glyc-oxidized LDL stimulated formation of reactive oxygen species more potently. Alternatively, fatty acids produced by a phospholipase A\(_2\) activity (which is increased during lipoprotein oxidation [57]) might stimulate NADPH and NADH oxidases, as demonstrated in cultured vascular smooth muscle cells [61].

In conclusion, these data show that glycation of LDL increases its damaging influence on the vascular endothelium. The likely mechanism for attenuation of nitric oxide mediated dilator responses is increased formation of superoxide anion. This mechanism could play a crucial role in patients with diabetes mellitus and may contribute to disturbed organ perfusion.

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