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Aluminum ions stimulate the oxidizability of low density lipoprotein by Fe^{2+}: Implication in hemodialysis mediated atherogenic LDL modification

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Abstract

Objective: Al^{3+} stimulates Fe^{2+} induced lipid oxidation in liposomal and cellular systems. Low-density lipoprotein (LDL) oxidation may render the particle atherogenic. As elevated levels of Al^{3+} and increased lipid oxidation of LDL are found in sera of hemodialysis patients, we investigated the influence of Al^{3+} on LDL oxidation.

Materials and methods: Using different LDL modifying systems (Fe^{2+}, Cu^{2+}, free radical generating compounds, human endothelial cells, hemin/H_2O_2 and HOCl), the influence of Al^{3+} on LDL lipid and apoprotein alteration was investigated by altered electrophoretic mobility, lipid hydroperoxide-, conjugated diene- and TBARS formation.

Results: Al^{3+} could stimulate the oxidizability of LDL by Fe^{2+}, but not in the other systems tested. Al^{3+} and Fe^{2+} were found to bind to LDL and Al^{3+}could compete with Fe^{2+} binding to the lipoprotein. Fluorescence polarization data indicated that Al^{3+} does not affect the phospholipid compartment of LDL.

*Conclusions:*The results indicate that increased LDL oxidation by Fe^{2+} in presence of Al^{3+} might be due to blockage of Fe^{2+} binding sites on LDL making more free Fe^{2+} available for lipid oxidation.

Keywords: *LDL oxidation, aluminum ions, iron ions, hemodialysis, atherosclerosis*

Introduction

Aluminum ion (Al^{3+}) is a redox-inactive trivalent cation which has been shown to catalyze the peroxidation of lipids in liposomes and membrane vesicles *in vitro* [1–4]. Due to this ability high concentrations of aluminum have been implicated in the pathogenesis of several clinical disorders, such as Alzheimer's disease or dialysis dementia, a fulminant neurological disorder that develops in patients undergoing renal dialysis [5–8]. Recently, Pratico et al. [6] have shown that aluminum, by increasing oxidative stress as measured by isoprostane (8,12-*iso*-iPF$_{2\alpha}$-VI) formation, modulates brain amyloidosis in amyloid precursor protein (APP) transgenic mice [6]. Aluminum caused acceleration of amyloid β peptide formation and plaque deposition. This was inhibited by vitamin E pointing to the role of lipid oxidation in this pathological process [9]. Increased plasma lipid peroxidation and platelet peroxidation were found in patients receiving regular hemodialysis (HD) treatment and were correlated to their higher serum

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aluminum levels [10–12]. Accelerated atherosclerotic plaque formation is found in HD patients [13]. In the pathophysiological process of atherogenesis the oxidative modification of low density lipoprotein (LDL) may play a significant role [14–16]. Taking these observations into account one could speculate, that aluminum ions might stimulate the oxidative modification of LDL. Therefore, in the present paper, we have evaluated the effect of aluminum ions on LDL oxidation using different established model systems of *in vitro* atherogenic (oxidative) modification of LDL.

Materials and methods

Chemicals and reagents

2,2′-azobis(2-amidinopropane)hydrochloride (AAPH) was from Polysciences, PA USA. Hemin (bovine) and SIN-1 (morpholinosydnonimine) were from Sigma. Laurdan (N,N-Dimethyl-6-dodecanoyl-2-naphthylamine) was supplied by Fluka. Sodium hypochlorite (NaOCl) solution was from Aldrich Chemical Company. The iron-complexes Venofer® (ferric saccharate) and INFeD® (ferric dextran) were from Vifor and Schein Pharmaceuticals, respectively. Aluminum nitrate was from Merck. All other chemicals used were of analytical grade.

Metal ion stock solutions

Stock solutions of aluminum nitrate, copper sulfate and iron ammonium sulfate were prepared in distilled water (20 mmol/l) and further diluted in water or in 0.15 mol/l NaCl, 10 mmol/l Tris-HCl pH 7.4 (TBS).

Lipoprotein isolation

LDL was isolated by ultra centrifugation as reported previously [17]. The final preparations were dialyzed against 150 mmol/l NaCl containing 0.1 mmol/l EDTA and filter-sterilized. Protein was estimated according to [18] using bovine serum albumin as a standard. All LDL concentrations are given as mg protein/ml. Three different LDL preparations were used in this study.

LDL oxidation

Prior to LDL oxidation, the lipoprotein was equilibrated in 0.15 mol/l NaCl, 10 mmol/l Tris-HCl pH 7.4 (TBS) using Sephadex G-25 chromatography (PD-10 columns, Pharmacia).

Metal ion dependent oxidation

LDL (0.2 mg/ml TBS) was incubated in the absence or presence of Cu^{++} sulfate (5 μmol/l) or Fe^{++} as ferrous-ammonium sulfate (25 μmol/l) at 37°C for the indicated time. LDL oxidation mediated by hemin (2.5 μmol/l) or the respective iron-complex (2.5 μmol/l) was done in the presence of H_2O_2 (100 μmol/l). Reactions were stopped by EDTA (100 μmol/l). Hemin stock solutions were prepared in 20 mmol/l NaOH and further diluted in TBS. Metal ion solutions were freshly prepared daily.

Metal ion independent oxidation

LDL (0.2 mg/ml TBS) was incubated in the absence or presence of AAPH (1 mmol/l) or SIN-1 (1 mmol/l) for 4 h at 37°C.

Apoprotein modification

Apoprotein modification was done by incubating LDL (0.5 mg/ml) with hypochlorite (5 mmol/l) for 60 min at 37°C. Protein modification was estimated by the change in relative electrophoretic mobility (REM) as given below. Stock NaOCl concentration was estimated spectrophotometrically in 0.01 mol/l NaOH using ε_{292} as 350 $M^{-1}\,cm^{-1}$[19].

LDL interaction with hemin and therapeutic iron-complexes

LDL in TBS (0.5 mg/ml containing 20 μmol/l BHT) was incubated with hemin, INFeD® or Venofer® with the indicated concentrations in the absence or presence of aluminum ions (100 μmol/l) for 30 min at room temperature. Interactions of hemin and iron complex with LDL were analyzed by agarose gel electrophoresis. LDL protein was stained with Coomassie blue.

Endothelial cell culture

Endothelial cells (HUVEC) were isolated from human umbilical veins and maintained in culture as reported previously [20]. For experiments cells were passaged into 35 mm culture dishes. Only passage numbers 1 and 2 were used. All incubations were done in RPMI-1640 medium.

Estimation of LDL oxidation

Thiobarbituric acid reactive substances (TBARS) were estimated as reported [21]. Samples were measured at 532 and 620 nm. Absorbance $A_{532}-A_{620}$ was used for calculation of TBARS. A molar extinction coefficient of $15.6 \times 10^4\,M^{-1}\,cm^{-1}$ was used to calculate malondialdehyde formed.

Conjugated dienes. LDL lipid oxidation was analyzed by monitoring diene conjugation as the increase in absorbance at 234 nm ($\varepsilon = 2.95 \times 10^4\,M^{-1}\,cm^{-1}$) [22]. Values are given as μmol/l in the assay.

Lipid hydroperoxides. Total lipid hydroperoxides were measured with the CHOD-iodide color reagent ($\varepsilon_{365} = 1.73 \times 10^4\,M^{-1}\,cm^{-1}$; E. Merck) [23].

Lipoprotein electrophoresis

Aliquots (10 μl) of treated or untreated LDL were applied to agarose gels (1% in TRIS-buffer) and run for 90 min, and lipoproteins were detected according to the supplier of the analytical system (Lipidophor All In, Technoclone). Measurement of REM was taken as an indicator of LDL oxidation [16], setting the electrophoretic mobility of native (untreated) LDL arbitrarily as 1.

Laurdan fluorescence

The lipophilic probe Laurdan was incorporated into LDL as reported [24,25]. In brief, after Sephadex gel chromatography LDL was diluted to 50 μg/ml TBS and Laurdan (stock solution 1 mmol/l ethanol) was added to a final concentration of 0.5 μmol/l and the samples were incubated for 45 min at 37°C resulting in constant fluorescence intensity. Emission spectra (excitation 360 nm) were recorded between 400 and 550 nm using a Hitachi spectrofluorimeter. The generalized polarization values (GP) were calculated as described [24]: $GP = (I_{440} - I_{490})/(I_{440} + I_{490})$ where I_{440} and I_{490} are the emission intensities at 440 and 490 nm, respectively ($n = 5$).

Atomic absorption spectrophotometry

LDL (1 mg/ml TBS) was incubated in the presence of Fe^{2+} or Al^{3+} for 30 min at 37°C and subsequently subjected to gel chromatography. The eluate containing the protein fraction was analyzed for bound iron or aluminum by atomic absorption spectrophotometry using a Hitachi Z-8200 Polarized Zeeman Atomic Absorption Spectrophotometer.

LDL aggregation by aluminum or physical forces

LDL (0.2 mg/ml TBS) was pre-incubated for 5 min in the absence or presence of Al^{3+}(6.25–100 μmol/l). Particle aggregation was measured as the increase in absorbance at 300 nm [4]. Aggregation by physical forces was induced by vortexing 1 ml LDL (0.2 mg/ml TBS) in round bottom 12 mm diameter borosilicate tubes using a Heidolf vortexer at maximal speed up to 60 s [17].

Release of iron from hemin and therapeutic iron-complexes

Released iron was estimated by the ferrozine—method [26]. Hemin (10 μmol/l) or the respective iron-complex (10 μmol/l) was incubated in TBS-buffer in the presence of 100 μmol/l H_2O_2 at 37°C. At the indicated time an aliquot of the mixture was withdrawn and the reaction was stopped by catalase (300 nmol/l). After 10 min of incubation at room temperature the ferrozine-reagent was added and after 30 min the absorbance was read at 540 nm. Fe^{3+} chloride was used as a standard.

Results

Figure 1 shows the influence of Al^{3+}(200 μmol/l) on free transition metal ion-initiated LDL oxidation as measured by lipid hydroperoxide formation in isolated human LDL.

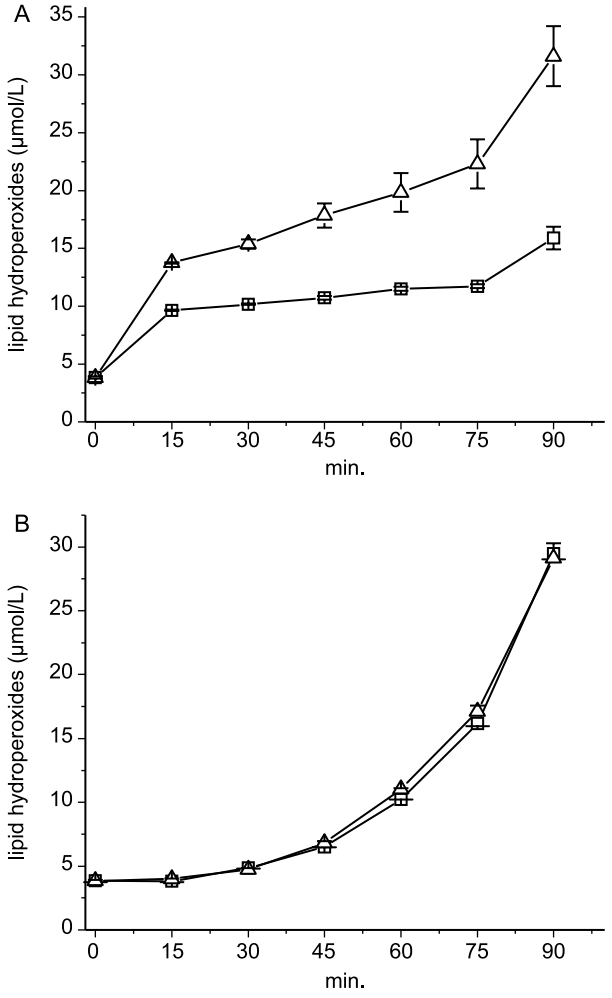

Figure 1. Influence of aluminum ions on LDL oxidation initiated by Fe^{2+} (A) or Cu^{2+} (B). LDL (0.2 mg/mL TBS) was pre-incubated in the absence (□) or presence (△) of Al^{3+}(200 μmol/l) for 10 min. and subsequently LDL oxidation was initiated by Fe^{2+}(25 μmol/l) or Cu^{2+}(5 μmol/l) ($n = 3$). Incubations were done at 37°C and lipid hydroperoxides were estimated as given in Methods.

In contrast to the LDL oxidation initiated by Cu^{2+}(5 μmol/l) the lipid oxidation of LDL by Fe^{2+}(25 μmol/l) was considerably enhanced in presence of Al^{3+}. Similar results were obtained when LDL oxidation was measured in parallel as TBARS formed in the lipoprotein (Figure 2).

Preformed lipid hydroperoxides in LDL can stimulate further oxidation by copper ions [27]. Thus, we have tested the influence of Al^{3+} on the lipid oxidation of copper ions in LDL which has been enriched in LPOs by pre-incubation with Cu^{2+}. As seen with native LDL, Al^{3+} did not influence the oxidation of pre-oxidized LDL by copper ions (not shown). Free radical generating compounds (like AAPH and SIN-1) have been used to study LDL oxidation *in vitro* [28,29]. Al^{3+}(0–200 μmol/l) did not stimulate lipid oxidation initiated by these compounds (both 1 mmol/l, data not shown). *In vivo*, the atherogenic modification of LDL may be mediated by vascular cells, HOCl, the reaction product of the activated myeloperoxidase system of granulocytes and macrophages and free hemin as proposed by [30–32]. Therefore, we tested the influence of Al^{3+} on the endothelial cell mediated lipid oxidation in LDL. Using HUVEC cell cultures the results show that Al^{3+}(0–100 μmol/l) did not stimulate the oxidation of LDL by endothelial cells (Figure 3). Al^{3+}(100 μmol/l) was not toxic to endothelial cells as measured by LDH release (control: 21.7 ± 2.1 U/l; Al^{3+}: 26.0 ± 4.4 U/l, difference not significant; $n = 3$).

HOCl can attack the apoprotein in LDL resulting in the formation of an atherogenic particle [30] which shows altered electrophoretic mobility. When LDL (0.5 mg/ml) was incubated with reagent NaOCl (5 mmol/l) the REM increased to 1.6 indicating apolipoprotein modification (not shown).

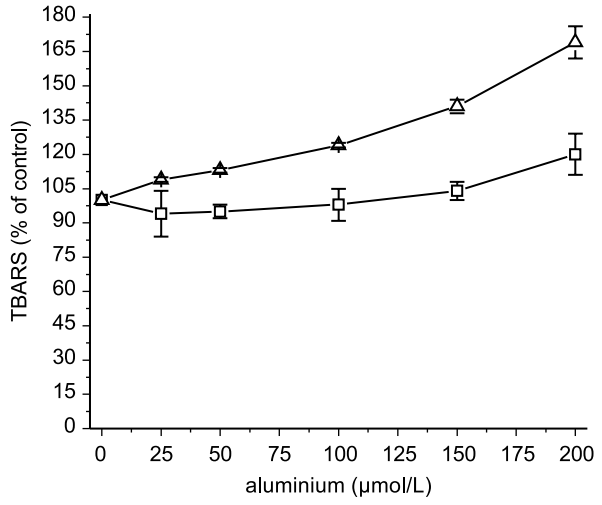

Figure 2. TBARS formation in Fe^{2+} oxidized LDL. LDL (0.2 mg/mL TBS) was pre-incubated in the absence (□) or presence (△) of increasing concentrations of Al^{3+}(25–200 μmol/l) for 10 min. and subsequently LDL oxidation was initiated by Fe^{2+}(25 μmol/l) for 90 min at 37°C ($n = 3$). TBARS formation was estimated as given in Methods.

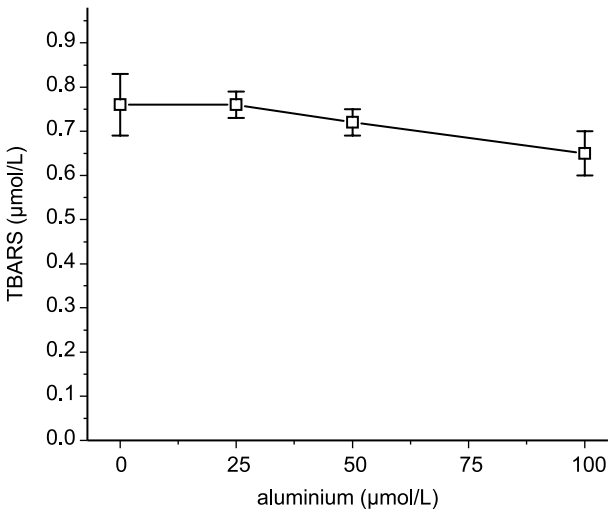

Figure 3. Influence of aluminum on endothelial cell mediated LDL oxidation. HUVEC cultured as described in Methods were incubated in RPMI-1640 containing 0.2 mg/ml LDL in the absence or presence of 0–100 μmol/l aluminum ions for 18 h at 37°C ($n = 4$). TBARS were estimated in the supernatants as given in Methods.

Aluminum ions (up to 100 μmol/l) had no influence on the hypochlorite—mediated modification of LDL. It has been reported that hemin—a Fe^{3+} porphyrin-complex—can induce LDL oxidation in presence of H_2O_2 [37,32]. When LDL was incubated with hemin/H_2O_2 (2.5 and 40 μmol/l, respectively) there was a rapid increase in conjugated diene formation monitored (not shown) indicating LDL oxidation.

Aluminum ions had no effect on lipid oxidation in this particular system of atherogenic LDL modification. Hemin has been shown to bind to LDL and the LDL/hemin complex can be scavenged by macrophages [31]. As analyzed by agarose gel electrophoresis, increasing concentrations of hemin (25–100 μmol/l) caused an increase in REM of LDL [37] indicating binding of hemin to LDL. Aluminum ions did not show any effect on hemin/LDL interactions (not shown). Venofer® and INFeD® are therapeutically used Fe^{3+}-complexes [33,34]. Thus, we tested both compounds (2.5 μmol/l) in their ability to induce lipid oxidation in presence of H_2O_2 (40 μmol/l). Under these conditions Venofer® and INFeD® were unable to oxidize LDL and Al^{3+} showed no effect in this system. In addition both Fe^{3+}-complexes showed no interactions with LDL in the absence or presence of aluminum ions (not shown).

Aluminum and other trivalent metal ions have been found to cause aggregation and fusion of liposomes [44]. Others and we have shown that aggregate and complex formation of LDL can result in altered oxidizability of the lipoprotein particle [17,35]. Aluminum (0–100 mol/l), when added to LDL,

caused aggregation of the lipoprotein particle (Figure 4A).

If aggregation of the lipoprotein by aluminum ions is the reason for altered (increased) oxidizability by Fe^{2+}, aggregation by physical forces should also result in increased oxidation of LDL by Fe^{2+}. When LDL was vortexed to cause aggregation [17] and the aggregates were incubated for 90 min at 37°C with 25 mol/l Fe^{2+}, the results show that aggregation did not result in increased lipid oxidation (Figure 4B).

Alterations of the phase components of phospholipid membranes by aluminum ions have been discussed by various authors investigating the effect of Al^{3+} on iron-induced peroxidation in liposomes and erythrocytes [2,3]. Such changes can be indicated by alterations in the fluorescence polarization spectrum of lipophilic probes. Using the lipophilic compound Laurdan as a probe [24,25] we found no alteration of the GP values in presence of 0–100 μmol/l Al^{3+} (not shown).

Assuming that Fe^{2+} like Cu^{2+} may bind to LDL as an initial step in lipid oxidation and Al^{3+} may support this binding resulting in increased LDL oxidation, we have estimated the binding of Fe^{2+} (and Al^{3+}) to LDL as well the influence of Al^{3+} on the binding of Fe^{2+} to LDL using atomic absorption analysis. As seen in Figure 5 both metal ions bound to LDL. However, when LDL was pre-incubated for 10 min with Al^{3+}(100 μmol/l) the binding of Fe^{2+} was strongly reduced.

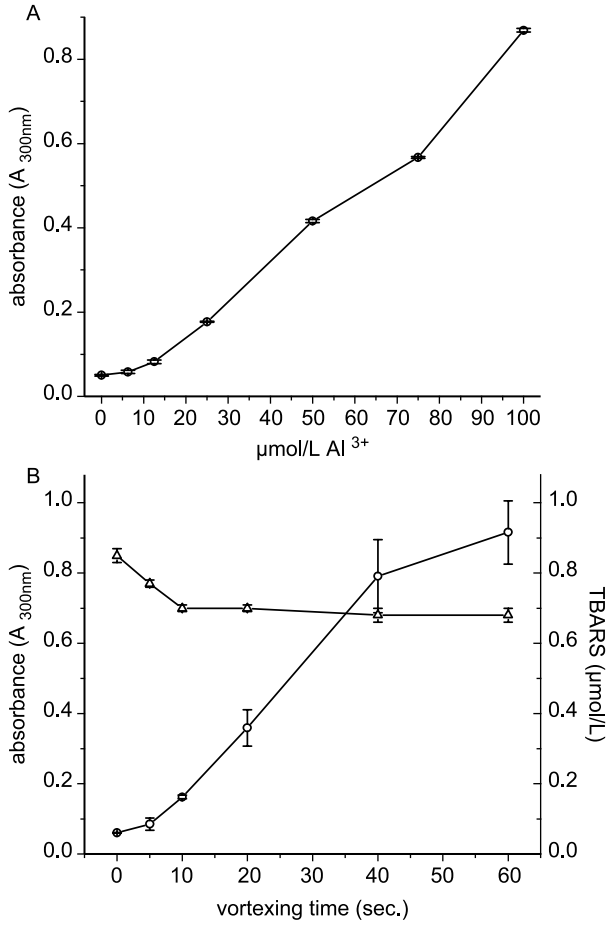

Figure 4. LDL aggregation and sensitivity of the aggregates to oxidation by Fe^{2+}. (A) LDL (0.2 mg/ml) TBS was incubated for 5 min at room temperature with the indicated concentrations of aluminum ions (0–100 μmol/l). Aggregation (o) was monitored as the increase in absorbance $A_{300\,nm}$. (B) LDL (0.2 mg/ml TBS) was aggregated (o) by vortexing for the indicated times as given in Methods. Subsequently, the aggregates were incubated with 25 μmol/l Fe^{2+} for 90 min at 37°C and TBARS formation (△) was estimated as given in Methods ($n = 3$). TBARS without Fe^{2+} addition were 0.15 ± 0.04 μmol/l.

Discussion

The toxic effect of aluminum ions has gained some interest in respect to neurological diseases like Alzheimer's disease. Aluminum accumulation has also been reported in renal impairment, anemia and other clinical complications in HD patients. In addition, age-dependent aluminum deposits were found in human aorta and cerebral artery [36]. Experimental aluminum overload in mice is associated with lipid peroxidation [6]. Using liposomal and brain microsomal systems, some authors have reported on the positive, i.e. stimulating effect of aluminum ions on lipid oxidation induced by Fe^{2+} ions in these model systems [1–4]. Higher lipid oxidation had been found in plasma of HD patients [10–12]. As LDL oxidation may play a significant role in atherogenesis—a complication of HD patients—we have tested the influence of Al^{3+} on LDL oxidation by Fe-ions and other pathophysiologically significant LDL oxidizing systems. The results showed that aluminum ions only increased the metal ion-induced oxidation of LDL in presence of Fe^{2+}, but not Cu^{2+}. Al^{3+} did not stimulate

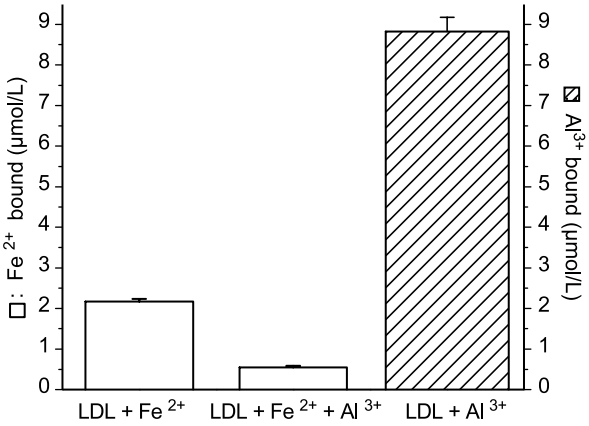

Figure 5. Influence of aluminum ions on Fe^{2+} binding to LDL. LDL (1 mg/ml TBS) was pre-incubated with Al^{3+}(100 μmol/l) for 10 min, subsequently Fe^{2+}(25 μmol/l) was added and further incubated for 30 min. Incubations containing Al^{3+}(100 μmol/l) or Fe^{2+}(25 μmol/l) alone were run in parallel ($n = 3$). LDL bound metal ions were estimated by atomic absorption as given in Methods.

the free radical-mediated, metal ion-independent lipid oxidation induced by peroxyl/alkyl radicals (AAPH) and peroxynitrite (SIN-1). The endothelial cell (HUVECs)-mediated LDL oxidation was not stimulated in the presence of aluminum ions. HOCl, a strong bio-reagent, has been identified as an agent inducing the formation of atherogenic LDL mostly via its reaction with the apoprotein in LDL [37,38]. Aluminum ions had no influence on the apoprotein modification induced by HOCl. The Fe^{3+}-porphyrin complex hemin has been identified as a strong pathophysiological agent inducing lipid oxidation and protein modification in LDL [37,32]. In contrast to uncomplexed iron ions, Al^{3+} did not stimulate LDL oxidation and protein modification mediated by hemin. Venofer® and INFeD®, both Fe^{3+}—complexes used therapeutically, were unable to modify LDL in absence or presence of Al^{3+}. Thus the stimulating effect of Al^{3+} on LDL oxidation might be specific for the oxidation induced by free or loosely bound Fe-ions. For the copper ion-induced LDL oxidation it has been shown that Cu^{2+} binds to specific sites in LDL inducing lipid oxidation [39]. Thus, one may assume that Al^{3+} may facilitate the binding of iron ions to LDL resulting in increased lipid oxidation. However, the results show that in presence of Al^{3+} much less Fe^{2+} was bound to the lipoprotein. Thus one may speculate that due to the binding of Al^{3+} to LDL putative iron binding sites are saturated by aluminum and therefore more free iron ions may be available to cause increased oxidation in the lipid phase.

Aggregation of LDL has been shown to modulate the sensitivity of LDL to oxidative stimuli [17]. The results show that aluminum can aggregate LDL as has been reported for liposomes. However, the present data indicate, that lipoprotein aggregation *per se* did not increase lipid oxidation induced by Fe^{2+}. On the other hand, the binding of aluminum ions may alter the lipid arrangement in LDL causing localized freezing of phospholipid movement, so facilitating the propagation of lipid peroxidation. A mechanism that has been proposed for the effect of Al^{3+} and Pb^{2+} on Fe^{2+}-induced lipid oxidation in liposomes and erythrocytes [2]. However, using Laurdan® as a lipophilic probe, we did not find any indication for an alteration of the lipid environment in LDL in presence of Al^{3+}. Similar observations have been reported for the effect of aluminum ions on liposomal membranes. Recently, it has been hypothesized by Exley that the pro-oxidant activity of aluminum in iron-driven biological oxidation might be explained by the formation of an aluminum superoxide semi-reduced radical ion ($AlO_2^{\cdot 2+}$). $O_2^{-\cdot}$ is formed in the presence of Fe^{2+} and O_2. In presence of aluminum the superoxide radical anion is captured ($O_2^{-\cdot} + Al^{3+} \leftrightarrow AlO_2^{\cdot 2+}$). $AlO_2^{\cdot 2+}$ can reduce Fe^{3+} to Fe^{2+} and thereby aluminum may facilitate iron-driven biological oxidations. Taken together our results show that aluminum ions can stimulate the atherogenic modification of LDL by Fe^{2+}. This may be of clinical relevance under pathophysiological circumstances where elevated aluminum levels are found (such as in HD patients) and which are associated with the release of free iron due to heme degradation such as intravascular hemolysis [11,32]. From a clinical point of view it is of interest to note that the application of iron complexes can result in release of redox-active iron into the circulation which is followed by a rise in plasma total peroxide and malondialdehyde concentrations [42–45]. Moreover, detailed studies on the cellular level revealed that iron can lead to endothelial cell injury and therefore may pose a risk for atherosclerosis [46].

Acknowledgements

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