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Effect of Aluminium on Lipid Peroxidation of Human High Density Lipoproteins

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We investigated the effect of aluminium (Al^{3+}) on lipid peroxidation and physico-chemical properties of high density lipoproteins (HDL) isolated from human plasma.

Our results demonstrated that Al^{3+} enhances lipid peroxidation of human HDL as shown by the significant increase in lipid hydroperoxides in Al-treated HDL with respect to control HDL. The oxidative effect was higher at acid pH (pH 5.5) with respect to pH 7.4. Moreover, a stimulating effect of Al^{3+} on iron-induced lipid peroxidation of HDL was demonstrated.

The study of the effect of Al^{3+} on the physico-chemical properties of HDL, using the fluorescence polarization (Pf) of the probes TMA-DPH (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene iodide) and DPH (1,6-diphenyl-1,3,5-hexatriene), showed a significant decrease of Pf in Al-treated HDL with respect to control. These results suggest that Al^{3+} induces a decrease of molecular order at the lipoprotein surface. Moreover, the study of tryptophan (Trp) fluorescence demonstrated that aluminium induces structural modifications of HDL apoproteins and on HDL physico-chemical properties. The effect of Al^{3+} on lipid peroxidation of HDL was observed at aluminium concentrations similar to those observed in the brain of patients affected by neurological diseases. Aluminium-induced oxidative damage of HDL could be involved in the development of neurological diseases.

Keywords: Aluminium; High density lipoproteins; Iron; Lipid peroxidation; Physico-chemical properties; Neurological diseases

INTRODUCTION

Aluminium (Al^{3+}), an ubiquitous element found in many food products, is implicated in the development of some neurological diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD).[1,2] Increased concentrations of aluminium were observed in the brain of patients affected by neurological diseases[1,2] and an excessive accumulation of aluminium ions from dialysis fluids is known to cause encephalopathy in patients subjected to prolonged hemodialysis.[3]

Several studies suggested that lipid peroxidation and oxidative damage play an important role in the pathogenesis of neurological diseases.[4,5] In fact higher levels of biochemical markers of lipid and protein oxidation were observed in the brain of patients affected by neurological diseases.[6] Increased thiobarbituric acid reactive substances (TBARS) formation was observed in the brain of rat and chicken after dietary aluminium exposure.[7] Moreover, studies *in vitro* demonstrated that aluminium ions stimulate iron-induced lipid peroxidation in mouse brain and in model brain membranes[7–10] suggesting that the toxic effect exerted by aluminium on biological membranes and its involvement in the development of neurological diseases could be linked to the ability to trigger lipid peroxidation.

Other authors suggested that the toxicity of aluminium could be related to its effect on membrane physico-chemical properties (order, fluidity, polarity) as demonstrated in aluminium-treated (Al-treated) membranes, even in the absence of lipid peroxidation.[11–14]

The interactions between aluminium ions and human high density lipoproteins (HDL) and the effect

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exerted by aluminium on HDL lipid peroxidation have not been previously investigated. The interest in the study is supported by recent studies, which demonstrated the presence of HDL-like particles in human cerebrospinal fluid.[15,16] Moreover, a role of oxidized HDL in the development of AD and other neurological diseases was suggested.[17,18]

Aim of the present study was to investigate whether aluminium induces lipid peroxidation of HDL and exerts a stimulatory effect of iron-triggered lipid peroxidation of HDL isolated from human plasma. Moreover, the fluorescence polarization (Pf) of the probes TMA-DPH (1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene iodide) and DPH (1,6-diphenyl-1,3,5-hexatriene) incorporated in HDL and the study of tryptophan (Trp) intrinsic fluorescence were used to investigate the effect of aluminium ions on physico-chemical properties and on HDL apoprotein structures.

MATERIALS AND METHODS

Isolation of Human High Density Lipoproteins (HDL)

Blood was obtained from normolipemic healthy donors after overnight fasting. Plasma was prepared by centrifugation at 3000 rpm for 20 min, and thereafter, used for the isolation of HDL by ultracentrifugation using vertical rotor TV-865 B (Dupont Instruments).[19] After extensive dialysis against 10 mM phosphate-buffered saline (PBS) (pH 7.4), protein concentration of HDL was evaluated as described by Lowry *et al.*[20] Lipoproteins were used within 24 h after isolation.

Incubation of HDL with Aluminium

Incubation of HDL with aluminium was carried out using an aliquot (100 μg) of HDL resuspended in 10mM PBS (pH 7.4) or in 10 mM acetate buffer (pH 5.5) for 3 h at 37°C, in the absence or in the presence of increasing concentrations of $Al_2(SO_4)_3$ (10–500 μM). In some experiments HDL were treated with aluminium in the presence of the selective metal chelator such as 100 μM EDTA (ethylene-diaminetetraacetic acid) or 1 mM *o*-phenantroline.[9]

To investigate the stimulatory effect exerted by aluminium on iron induced lipid peroxidation, HDL were incubated with different concentrations of aluminium in the presence of 20 μM $(Fe_2(SO_4)_3)$.[8,9] The solutions of aluminium and iron were prepared daily before experiments.

Evaluation of Lipid Peroxidation

The extent of lipid peroxidation of untreated and Al-treated HDL (200 μg/ml) was evaluated by measuring the levels of lipid hydroperoxides by the ferrous oxidation xylenol orange assay at 560 nm as previously described by Jiang *et al.*[21,22] using a molar absorption coefficient of $4.3 \times 10^4\ M^{-1}\ cm^{-1}$[22]

Stimulation exerted by different concentrations of aluminium on iron induced lipid peroxidation in HDL was calculated as described by Verstraeten *et al.* (1998).[23]

$$\text{Stimulation} = \frac{(\text{Hydroperoxides with } Al^{3+} + Fe^{2+})}{(\text{Hydroperoxides with } Fe^{2+})}$$

Evaluation of Turbidity

The turbidity of untreated and Al-treated HDL (200 μg/ml) was evaluated by measuring the absorbance at 450 nm to verify whether aluminium induces aggregation of HDL.[24]

Extrinsic and Intrinsic Fluorescence Measurements

A stock solution (1 mM) of the fluorescent probes TMA-DPH (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene iodide) and DPH (1,6-diphenyl-1,3,5-hexatriene) were prepared in tetrahydrofuran (THF). The labelling of HDL was performed using a probe-lipid molar ratio of 1:1000 and by incubation of HDL with the fluorescent probes at 37°C until the fluorescence intensity reached a plateau.[25,26]

The fluorescence polarization (Pf) of the probes TMA-DPH and DPH was investigated at 37°C using 365 and 430 nm as excitation and emission wavelengths, respectively.

The intrinsic fluorescence of Trp in HDL was studied at room temperature using 100 μg HDL resuspended in 10 mM PBS (final volume, 2 ml).[26,27] Emission spectra (using 295 nm as excitation wavelength) were recorded using a Perkin Elmer LS 50B spectrofluorometer.

Statistics

All the results were presented as a mean ± SD. Student's *t*-test was used to analyse the significance of the results obtained in untreated and treated HDL. Values were considered to be significant at a p value less than 0.05.

RESULTS

The levels of lipid hydroperoxides in untreated HDL were 0.57 ± 0.03 nmol/100 μg. As shown in Fig 1A, higher levels of lipid hydroperoxides were observed in HDL incubated for 3 h at 37°C in the presence of

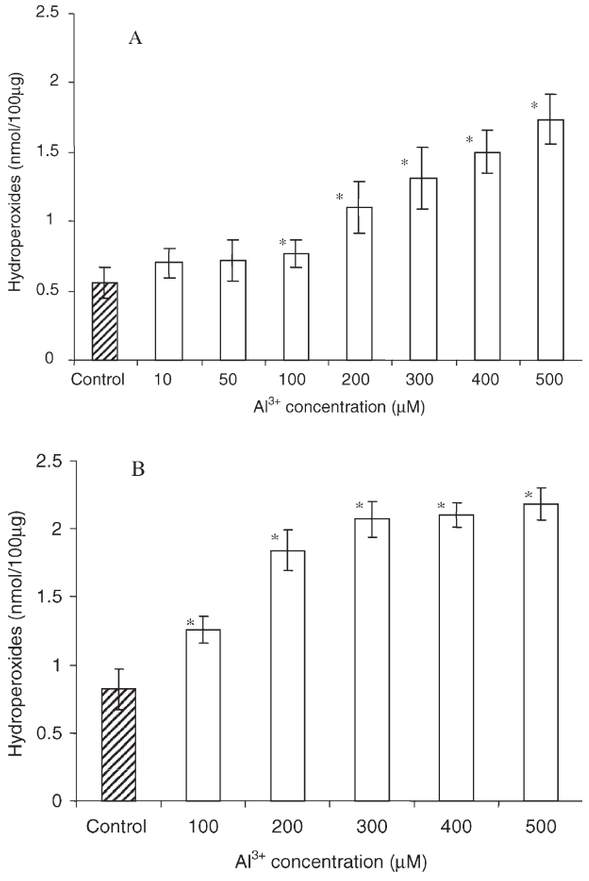

FIGURE 1 Levels of lipid hydroperoxides in HDL isolated from human plasma, incubated for 3 h in the absence (control) or in the presence of different concentrations of aluminium. (A) HDL incubated at pH 7.4; (B) HDL incubated at pH 5.5 (*$p < 0.001$ vs. control HDL).

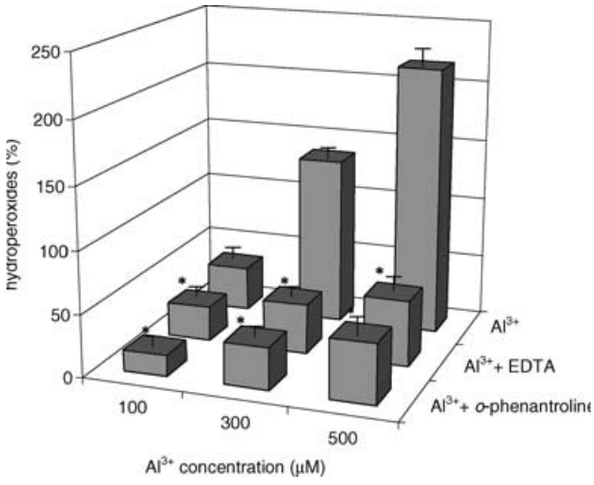

FIGURE 2 Effect of the selective metal chelators 100 μM EDTA and 1 mM *o*-phenatroline on aluminium induced lipid peroxidation of HDL isolated from human plasma. Results were shown as percentage increase of lipid hydroperoxides incubated for 3 h in the presence of different concentrations of aluminium with respect to HDL incubated without aluminium (*$p < 0.001$ vs. HDL treated with aluminium in the absence of chelators).

different concentrations of aluminium (pH 7.4) with respect to control HDL. The increase was statistically significant at the concentration of 100 μM aluminium and at higher concentrations of aluminium ($p < 0.001$). The results suggest that Al^{3+} exerts an oxidant effect on human HDL.

The levels of lipid hydroperoxides in control HDL incubated at pH 5.5 were slightly higher with respect to those observed at pH 7.4, but the differences were not significative. As shown in Fig 1B, the oxidant effect exerted by aluminium was observed also at pH 5.5. HDL treated with aluminium at pH 5.5 showed higher levels of lipid hydroperoxides with respect to the values at pH 7.4 ($p < 0.001$). These results suggest a higher oxidant effect of aluminium at acid pH, in agreement with previous studies in model membranes.[7,8]

To investigate the molecular mechanism involved in the oxidant effect exerted by aluminium on HDL, we compared the increase in lipid hydroperoxides in HDL treated with aluminium (100–500μM) in the presence of chelators (EDTA or *o*-phenantroline). The basal levels of lipid hydroperoxides were similar in HDL incubated alone or in the presence of selective chelators (0.57 ± 0.03nmol/100 μg in controls; 0.54 ± 0.05 nmol/100 μg in EDTA-HDL and 0.49 ± 0.07 in *o*-phenantroline-HDL). In our experimental conditions, an increase in lipid hydroperoxides realized in HDL incubated with aluminium either in the presence of 100 μM EDTA or 1 mM *o*-phenantroline. The increase was significant at all the concentrations of Al^{3+} used (100, 300 and 500 μM) in Al-EDTA-HDL with respect to EDTA-HDL ($p < 0.05$) and Al-*o*-phenantroline-HDL with respect to *o*-phenantroline-HDL ($p < 0.05$). As shown in Fig 2, the percentage increase in lipid hydroperoxides was significantly lower in HDL treated with aluminium in the presence of metal chelators.

No changes of turbidity were observed in HDL incubated with aluminium until the concentration of 1 mM, therefore, we can exclude that the effect of Al^{3+} on lipid peroxidation is due to aggregation of HDL (data not shown).

Effect of Aluminium on Iron Induced Lipid Peroxidation of HDL

To investigate whether aluminium exerts a stimulatory effect of iron-induced lipid peroxidation, HDL were incubated with 20 μM iron in the absence or in the presence of different concentrations of aluminium. The level of hydroperoxides in HDL incubated with 20 μM iron was significantly higher with respect to untreated HDL (6.2 ± 0.1 mmol/100 μg vs. 0.57 ± 0.03 nmol/100 μg, $p < 0.001$). The increase in lipid hydroperoxides in

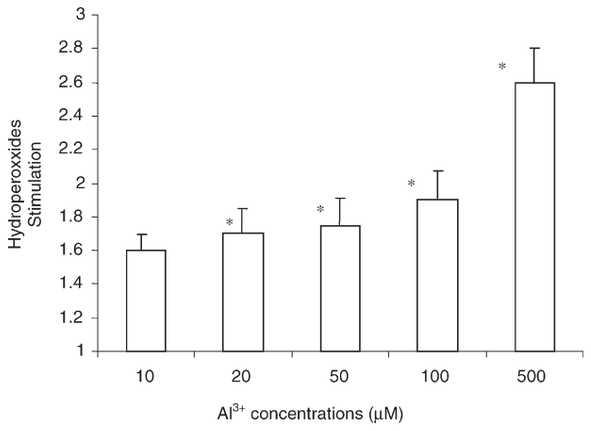

FIGURE 3 Stimulatory effect exerted by different concentrations (10–500 μM) of aluminium on iron induced lipid peroxidation of HDL isolated from human plasma. The stimulation induced by aluminium was calculated as: Stimulation (%) = (hydroperoxides with $Al^{3+} + Fe^{2+}$)/(hydroperoxides with Fe^{2+}) (See "Materials and Methods" section) (*$p < 0.001$ vs. HDL incubated for 3 h in the presence of 20 μM Fe^{2+} and 10 μM Al^{3+}).

HDL incubated in the presence of 20 μM iron realized at a lower concentration of aluminium (10 μM) with respect to HDL treated with aluminium in the absence of iron. As shown in Fig 3, the stimulation of aluminium on iron induced lipid peroxidation was dependent on aluminium concentration.

These results demonstrate that Al^{3+} stimulates iron induced lipid peroxidation in agreement with previous studies in model membranes.[8,9,24]

Effect of Aluminium on Pf value of the Probes DPH and TMA-DPH

As shown in Table I, Pf values of the fluorescent probe TMA-DPH in untreated HDL and in Al-treated samples were higher with respect to the DPH values (0.420 ± 0.001 and 0.300 ± 0.01 using TMA-DPH and DPH, respectively).

These results are in agreement with the different localization of the fluorescent probes in the lipoprotein particle.[25,26] In fact TMA-DPH, due to its cationic group, localizes at the lipoprotein surface, which is a more ordered region with respect to the core.[25]

Using the probe TMA-DPH, a significant decrease of Pf value was observed in Al-treated HDL. The decrease was Al^{3+} concentration-dependent (Table I). These results suggest that incubation in the presence of aluminium induces a significant decrease of molecular order at the surface of Al-treated HDL. Using DPH, which localizes in the lipoprotein core, no significant modifications in Pf value were observed in Al-treated HDL with respect to the control, suggesting that there are no alterations in the structural organisation of the environment surrounding DPH.

Effect of Aluminium on Trp Fluorescence

In our experimental conditions, the position of Trp maximum emission wavelength in control HDL was about 335 nm, in agreement with previous studies.[26] As shown in Table I, a blue-shifted position of the maximum emission wavelength of Trp fluorescence was observed in Al-treated HDL, with respect to control. The blue-shifted position of the maximum emission wavelength of Trp fluorescence suggests modifications in the environment of Trp residues of apoproteins in Al-treated HDL.

DISCUSSION

Our results demonstrate that aluminium ions trigger lipid peroxidation of human HDL. The oxidant effect exerted by aluminium on HDL was higher at acid pH (pH 5.5) with respect to pH 7.4, in agreement with previous studies on model membranes.[7,8] The effect is likely related with the higher aluminium solubility at acid pH. The action of aluminium at acid pH may well account for the increased accumulation of fluorescent "age pigments" in the nervous system of patients subjected to aluminium overload. Age pigments are formed within lysosomes, at an acid pH in the presence of a variety of metal ions.[9]

Moreover, our results demonstrate that aluminium exerts a stimulatory effect of oxidative action induced by iron salts in agreement with previous studies in model membranes.[9,10,24]

TABLE I Fluorescence polarization (Pf) values of TMA-DPH (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene iodide) and DPH (1,6-diphenyl-1,3,5-hexatriene) and tryptophan emission maximum in HDL incubated in the absence (control) and in the presence of different concentrations of aluminium

Aluminium concentration (μM)	TMA-DPH (Pf)	DPH (Pf)	Trp emission maximum (nm)
Control	0.420 ± 0.005	0.300 ± 0.010	340.4 ± 0.2
100	0.410 ± 0.006	0.300 ± 0.010	339.5 ± 0.6
250	0.400 ± 0.010*	0.300 ± 0.010	337.9 ± 0.3*
500	0.400 ± 0.010*	0.300 ± 0.010	337.2 ± 0.4*

*$p < 0.05$ vs. HDL control.

The mechanism by which aluminium facilitates lipid peroxidation has not been yet elucidated. Since aluminium has a fixed oxidation number of III, it cannot participate in the redox reactions that lead to radical formation, therefore previous studies suggested that the pro-oxidant effect aluminium, even if it is not a transition metal, could be likely a consequence of the interactions between Al^{3+} and the components of the lipoprotein surface and/or to modifications of lipid-apoprotein interactions and physico-chemical properties. In our experimental conditions, the significant decrease of Pf value of TMA-DPH incorporated in Al-treated HDL demonstrates that aluminum induces a decrease of the molecular order at the lipoprotein surface. Previous studies demonstrated that lipid–protein interactions of the lipoprotein surface modulate the susceptibility to lipoprotein peroxidation.[27,28] Therefore, we suggest that the binding and/or interactions of aluminium to the HDL surface could induce alterations of the phospholipid moiety of the lipoprotein monolayer which could promote the lipid peroxidation processes. This hypothesis is supported by previous studies on biological membrane.[11–13]

Furthermore, the blue-shifted position of Trp emission maximum fluorescence in HDL incubated with aluminium suggests modifications in the environment surrounding the Trp residues of HDL apoproteins. The position of the emission maximum of Trp fluorescence is sensitive to the hydrophobicity/hydrophilicity of the microenvironment in which it is localised, therefore, these results suggest modifications of apoprotein structure in Al-treated HDL with a decrease of the exposure of Trp residues of apoproteins to hydrophilic environment[26,29] that could be related to a direct effect exerted by Al^{3+} on apoprotein and/or to the modifications of lipid–protein interactions at the HDL surface. Previous studies demonstrated that a direct binding of aluminium to various proteins influences their secondary structures.[30–32]

Other hypotheses could be advanced to explain the role exerted by Al^{3+} on lipid peroxidation of HDL. It was suggested that the binding of Al^{3+} with membrane lipids could cause the displacement of metals initiators of lipid peroxidation from their specific lipid binding sites and promote lipid peroxidation.[9] In our experimental conditions, the increase in lipid hydroperoxides realized at a lower extent in HDL incubated with Al^{3+} in the presence of metal chelators, but it was non completely inhibited. Metal binding sites were observed in human HDL,[33] therefore, we cannot exclude that the pro-oxidant effect exerted by Al^{3+} on HDL, could be related to the non-specific binding of aluminium with the lipoprotein surface and the displacement of copper or iron, from their specific lipid binding sites at the HDL surface, facilitating the initiation and/or propagation of the oxidative damage.

Concerning the patho-physiological relevance of our results, it has to be stressed that in our experimental conditions, the effect of aluminium and iron on lipid peroxidation of human HDL was observed at concentrations similar to those observed in human cerebrospinal fluid. Normal aluminium and iron ions concentrations are about 18 μM and 0.88 mM, respectively.[34,35] Higher aluminium ions concentrations were observed in the brain of patients affected by neurological diseases (100 μM in AD, 1.1 mM in PD, 180 μM in dialysis encephalopathy syndrome),[36,37] therefore, our results suggest the possibility that *in vivo* Al-facilitated mediated lipid peroxidation of HDL might occur and Al-induced oxidative damage of HDL could be involved in neurological diseases.

Recent studies demonstrated the presence of lipoprotein particles in the density range of plasma HDL, in human cerebro-spinal fluid (CSF).[15,16] Moreover, the disruption of the blood–brain barrier observed in AD and in other neurological diseases could produce HDL levels higher than the normal values in brain districts, which could be susceptible to oxidation by free radicals. CSF lipoproteins contain polyunsaturated fatty acids, the major substrate for lipid peroxidation, and a higher susceptibility to oxidation of lipoproteins isolated from CSF of patients affected by AD was demonstrated.[38,39] Moreover, recent studies demonstrated that HDL oxidized in different experimental conditions exert a toxic effect on cultured cells with neuron degeneration and oxidative stress.[17,18]

In conclusion, our results demonstrate that Al^{3+} exerts an oxidant effect and stimulates iron-triggered lipid peroxidation of human HDL. Moreover, the present study demonstrates that aluminium ions induce modifications of physico-chemical properties and of apoprotein structure of HDL. Previous studies showed that lipoproteins isolated from CSF play a role in cholesterol homeostasis and in transport of amyloid protein and hydrophobic molecules.[40,41] Lipoprotein fluidity is considered an important factor that influences apoprotein conformation[25] and lipoprotein functional activities such as exchanges processes and affinity for enzymes of lipoprotein metabolism and membrane receptors.[33] Therefore, we can hypothesize that the modifications of molecular order and of apoprotein structure on Al-treated HDL could reflect in modifications of HDL functional activities in plasma and in CSF and that aluminium induced oxidative damage on HDL could be involved in the development of neurological diseases.

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