# THE STANDARDIZED *GINKGO BILOBA* EXTRACT EGB-761 PROTECTS VASCULAR ENDOTHELIUM EXPOSED TO OXIDIZED LOW DENSITY LIPOPROTEINS

S. V. PIERRE∗, P. LESNIK^, M. MOREAU^, L. BONELLO#, M-T. DROY-LEFAIX¤,
S. SENNOUNE Ω, M-J. DURAN Ω, T. A. PRESSLEY Ω, J. SAMPOL#, J. CHAPMAN^,
J-M. MAIXENT§✉


§ INSERM U927, School of Medicine, University of Poitiers & CHU la Miléterie, Poitiers, France
∗ Department of Pharmacology and Physiology, University of Toledo, College of Medicine, Toledo, OH, USA.
^ INSERM U551 Université Pierre et Marie Curie, Hôpital de la Pitié-Salpêtrière. Paris, France.
¤ IPSEN. Paris France.
# INSERM U608. Faculté de Pharmacie. Université de la Méditerranée. Marseille, France.
Ω Dept. of Physiology, Texas Tech University Health Sciences Center, Lubbock, TX, USA
✉Corresponding author : Pr J.M. Maixent INSERM U927 CHU la Miléterie BP 5772, rue de la Miléterie, Poitiers Cedex 86022. France. E-mail: jmmaixent@gphy.campus.univ-poitiers.fr

**Abstract** – Dietary antioxidants are frequently proposed as protective agents for the vascular endothelium during the onset of atherosclerosis. This protection may occur at two distinct levels. First, they prevent oxidative modification of atherogenic lipoproteins (LDL). Second, they can provide a cellular protection against oxidized LDL-mediated endothelium dysfunction, although this mechanism remains poorly considered in many instances. To gain insight into the mechanism underlying such cellular protection against oxidized LDL, we examined the impact of a popular traditional medicine, an extract from *Ginkgo biloba* with well-known antioxidant properties, on two endothelial cells properties: cell adhesion and ionic homeostasis. Cellular lipoperoxides levels were also measured as a marker of cellular oxidative stress. Human umbilical-vein endothelial cells were exposed to native (nat-) or oxidized (ox-) LDL, the latter prepared to be compatible with clinically observed levels of oxidation. Although nat-LDL had little effect, ox-LDL increased endothelial adhesive properties (35%, p<0.01) and lipoperoxidation (45%, p<0.01). Na,K-ATPase activity, a key regulator of ionic homeostasis, was significantly decreased after exposure to nat-LDL (30%, p<0.01) and dramatically depressed after exposure to ox-LDL (65%, p<0.001). The standardized preparation of *Ginkgo biloba* EGb-761 totally protected adhesive properties and endothelial lipoperoxide levels. Moreover, it limited the decrease in Na,K-ATPase activity induced by ox-LDL to levels similar to nat-LDL. This suggests that EGb-761 protects endothelial adhesive properties and helps prevent the disruption of ionic homeostasis. The EGb-761-mediated inhibition of ox-LDL-induced lipoperoxide levels in endothelial cells appears to be an important mechanism by which *Ginkgo biloba* extract protects endothelial properties.

**Key words**: endothelium, oxidized low density lipoproteins, atherosclerosis, Na,K-ATPase, EGb 761, adhesion native LDL: nat-LDL; oxidized-LDL: ox-LDL.

## INTRODUCTION

High plasma concentrations of cholesterol, in particular those of low-density lipoprotein (LDL), have long been considered as one of the principal risk factors for atherosclerosis. The process of atherogenesis, however, is far more the simple accumulation of lipids within the artery wall. It is a series of highly specific cellular and molecular responses that can be best described as a complex inflammatory disease as reviewed by Ross (1999) and as we have recently reported in animals with a natural occurence of atherosclerotic lesions (El Aouafi et al. 2007). Although LDL and LDL cholesterol are key components in the initiation and progression of this inflammatory disease, an even more critical event appear to be their chemical modifications. Indeed, over the past decade, a growing body of evidence has implicated the oxidative

modification of LDL as a key contributor to the early stages of atherosclerosis. In its very early stages, the mechanism underlying the action of ox-LDL involves an alteration of vascular endothelium function. One of the most commonly reported changes in action is an activation of endothelial adhesive properties, an observation that accounts in large part for the consideration of atherosclerosis as an inflammatory disease (reviewed by Ross, 1999). Indeed, in humans, the precursor of the atherosclerotic lesion, the fatty streak, is a purely inflammatory lesion characterized by the accumulation of monocyte/macrophages within the intimal layer of the blood vessel (Stary et al, 1994). There is strong evidence that oxidized lipids derived from LDL initially facilitate monocyte deposition within the subendothelial space. For example, intravenous administration of ox-LDL results in an increase in leukocyte adherence to the vascular endothelium (Lehr et al, 1991) and incubation of endothelial cells with ox-LDL enhances monocyte binding to the endothelial cells (Jeng et al, 1993).

It follows that agents that minimize oxidation of LDL would be expected to limit the progress of atherosclerosis, and indeed, antioxidants such as vitamins E and C seem to have a clinically relevant protective effect on the vessel wall (Glass & Witztum, 2001, Salonen et al., 2003). Nevertheless, many studies in the past decades suggest that the mechanism underlying this protective effect goes beyond a simple prevention of LDL oxidation. Some antioxidants have been shown to provide direct protection of the vascular endothelium against oxidized LDL-mediated dysfunction (Kuzuya *et al*., 1991, Keaney *et al*., 1996, review by Diaz et al., 1997), including changes in adhesive properties (Weber *et al*., 1994; Erl et al., 1997; Erl *et al*., 1998, Mine et al., 2002, Yoshida et al., 2000, Cominacini et al, 1999, Li et al., 1998). Further indications that the protective effect involves more than one mechanism include the observations that not all antioxidants display the same properties and efficacy (Lehr et al., 1995; review by Frei, 1999). Despite several years of effort, the clinical efficacy of the best characterized antioxidants, vitamins E and C, remains controversial (Hodis et al., 2002; review by Salonen JT, 2002; reviews by Heinecke, 2001 and 2003). The current understanding of what would be an efficient anti-atherogenic antioxidant therapy is therefore quite open to discussion, and it has become apparent that the

antioxidant status of the patient (Steinberg and Witztum, 2002; commented by Violi et al., 2002) as well as the type and form under which the antioxidant is provided (Niki & Noguchi, 2002; Frei, 1999, Diaz et al., 1997), are primary issues that vary greatly from case to case. It is also likely that different patients would benefit from different antioxidant therapies. In short, we are still looking for an efficient antioxidant therapy in the prevention of atherosclerosis. Recently, new effects of EGb-761 on oxidized LDL-inflammatory response in endothelial cells and atherosclerosis have been described (Chen et al, 2003; Shafer et al, 2007; Rodriguez et al, 2007).

These issues have not escaped discussion by the lay press, and many traditional herbal preparations have become increasingly popular. Many of these preparations contain numerous antioxidants with diverse properties, but the heterogeneity of their composition also raises the possibility of a synergistic effect that could increase their efficacy relatively to simple anti-oxidants. Such a preparation, EGb-761, an extract from the leaves of the tree *Ginkgo biloba*, has become a widely prescribed drug in certain regions of the world, including Western Europe. Ginkgo extract proves to be a complex mixture of flavonoid glycosides, terpenoids (known as bilobalides and ginkgolides), and organic acids (DeFeudis, 1991; DeFeudis, 1998; Gohil, 2002). Not surprisingly, the complexity of its composition encourages a great deal of variability. One solution has been to employ standardized preparations, such as EGb-761, which minimize variations from batch to batch. Although the efficacy of Ginkgo extracts has been clinically demonstrated in the treatment of various cardiovascular and cerebral disorders (LeBars, 1997; Pietri, 1997; review by DeFeudis FV, 1998; Birks et al., 2002; Morgenstern, 2002), it is not known whether it may prevent early atherosclerotic events. The importance of EGb-761 in the treatment of the disease, however, is suggested by its ability to protect LDL against oxidative modification (Yan *et al*., 1995, Christen & Maixent 2002). In keeping with the more general protective effects of antioxidants, its effects also extend to regulation of inflammatory events (Gozin *et al*., 1998; Cheung et al., 2001, Daba et al, 2002, Christen et al. 2002). Whether these effects can be extended to the ox-LDL-induced inflammatory response of vascular endothelia has not yet been investigated.

The Na,K-ATPase is an ubiquitous transmembrane protein regulating the active

transport of sodium and potassium ions across the cell membranes. Therefore, Na,K-ATPase is essential for cellular homeostasis and could be considered as a very sensitive cellular sensor (Glynn, 1994). Surprisingly, there is no research linking membrane Na,K-ATPase activity in endothelial cells to ox-LDL. Recently Sukhanov et al. (2003) reported from microarray analysis an upregulation of the three subunits of Na, K-ATPase expression (ATP1B1, ATP1B, and ATP1AL1) in vascular cells. However it is well known that Na,K-ATPase is modified following oxidative stress (Maixent and Lelièvre 1987). Interestingly, we have found that EGb-761 prevents the brain tissue lipoperoxidation and impairment of Na,K-ATPase that accompanies unilateral disruption of cerebral blood flow (Pierre *et al*., 1999; Pierre *et al*., 2002). We reasoned that a similar effect might be occurring in vascular endothelia exposed to oxidized LDL, underlying some potential protective effect of the extract during early atherogenic events. We therefore evaluated the ability of EGb-761 to prevent disorders in two major endothelial functions: cell adhesion and ionic homeostasis, using a primary culture model of human umbilical vein endothelium. The results clearly show that EGb-761 prevents the changes in vascular endothelial adhesive properties and minimizes the changes in ionic homeostasis elicited by exposure to oxidized LDL. Its underlying mechanism seems to involve its ability to prevent endothelial lipoperoxidation. This study suggests that Ginkgo extract may have a protective effect on vascular endothelium during the onset of atherosclerosis.

## MATERIALS AND METHODS

### *Isolation and oxidation of LDL*

Samples of LDL were isolated from human subjects for subsequent exposure to endothelial cells in culture. Plasma from healthy, normolipidemic volunteers was subjected to sequential preparative ultracentrifugation (Mougenot et al. 1997), and LDL was obtained from the fraction corresponding to a density of 1.024-1.040 g/ml. Protein concentrations of the resulting isolates were determined by the method of Lowry *et al*. (1951). Oxidative modification of the isolated LDL was carried out by incubating native LDL (500 µg protein/ml) in phosphate-buffered saline in the presence of 2.5 µmol/l $CuCl_2$ at 37°C for 24 h. Both oxidized and native LDL were then diluted with RPMI 1640 (Gibco BRL, Cergy Pontoise, France), a cell culture medium that minimizes further oxidation.

### *Cell culture and incubation conditions*

Primary human umbilical vein endothelial cells (HUVEC) grown until confluence were used. The cells were isolated from cord blood as described previously (Jaffe

*et al*., 1973), and then maintained in RPMI 1640 medium containing 20% fetal calf serum (Gibco), 1% penicillin-streptomycin (Sigma Chemical Co., St Louis, Missouri, USA), 1.25% endothelial cell growth supplement (Sigma), 20 mmol/L L-Glutamine (Gibco) and 1% heparin (Sigma) at 37°C in 5% $CO_2$. HUVEC purity was assessed by morphological and immunological criteria, including expression of von Willebrand's factor. For a typical experiment, confluent monolayers of HUVEC were pre-incubated for 24 h in standard culture medium in the absence or presence of EGb-761 (25, 50 or 100 µg/ml), a standardized commercially-available preparation of *Ginkgo* extract (Beaufour IPSEN Institute, Paris, France). The culture medium was then removed, and the cells were incubated for an additional 24 h in normal or EGb761-supplemented medium, as appropriate, to which native or oxidized LDL had been added (100 µg/ml).

### *Cell Viability*

The viability of isolated HUVEC was assessed by monitoring the presence of functional mitochondrial enzymatic activity. HUVEC monolayers grown in 96-well microtiter plates were exposed to various experimental conditions for 24 h, and then 10 µl of a commercial reagent containing WST-1 (Boehringer Mannheim, Mannheim, Germany) was added to each well. After 2 hours, conversion of the tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenases produced an increase in absorbance at 450 nm in viable cells.

### *HUVEC Adhesive Properties*

The adhesive properties of HUVEC were assessed by monitoring the adhesion of monocytes to the monolayer. THP1 cells (an established line of human monocytes) were labelled with calcein-AM (Molecular Probes) using the procedures recommended by the manufacturer and then incubated for 30 min with confluent monolayers of HUVEC. The initial fluorescence was measured using a cytofluorimeter (Series 4000, Perseptive Biosystems, France). The monolayers were then washed gently three times with RPMI 1640 to remove non-adherent THP1 cells and the residual fluorescence was read. The percentage of adhesion was defined as (residual fluorescence)/(initial fluorescence) *100.

### *$Na^+$-$K^+$-ATPase activity*

The specific activity of $Na^+$-$K^+$-ATPase was measured as the activation of *p*-nitrophenyl phosphatase (pNPPase) activity produced by the addition of $K^+$ using a modification of a method described previously (Ward and Bowman, 1976, Wald *et al*., 1996). Briefly, assays were performed on HUVEC monolayers in 24-well plates. The culture medium was removed, and the monolayers were washed twice with buffered isotonic sucrose (250 mM sucrose, 20 mM imidazole, pH 7.4). After a 5-min preincubation at 37° C in a medium containing 6 mM $MgCl_2$, 20 mM imidazole, 250 mM sucrose, pH 7.4, and the absence or presence of 20 mM KCl, the enzymatic reaction was initiated by the addition of 8 mM *p*-nitrophenyl phosphate (pNPP, Sigma), and allowed to proceed for 0 or 5 min. at 37°C. An aliquot of the reaction mixture was then recovered and mixed with an equal volume of 1 M NaOH. After 5 min at 4°C to permit color development, the optical density was measured at 410 nm. $K^+$-stimulated pNPPase activity was estimated from the difference in *p*-nitrophenol production in the absence and presence of $K^+$, standardized

for protein and expressed as µmol of p-nitrophenol/mg protein/h.

*Lipid Peroxidation*

As an index of cellular oxidative stress, cell-associated thiobarbituric acid-reactive substances (TBARS) were measured in HUVEC monolayers as described by Wallin et al. (1993).

*Statistical Analysis*

All results are expressed as mean ± SEM. Multiple comparisons were performed by one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test. Values of p<0.05 were considered statistically significant.

# RESULTS

*Endothelial Adhesive Properties*

To determine the consequences of LDL exposure on the HUVEC model of vascular endothelia, we assessed a major indicator of endothelial function, cellular adhesion. Exposure for 24 h to native LDL (100µg/ml) had no effect on basal adhesive properties of HUVEC (Fig 1), as measured from the adhesion of fluorescence-labelled human monocytes. Similarly, treatment with *Ginkgo* extract produced no effect, either alone or in the presence of native LDL. In contrast, a 24-h exposure to oxidized LDL (100 µg/ml) increased cell adhesion by about 35% (p<0.01), a response that was blocked with concentrations of extract above 50 µg/ml. Indeed, the adhesion of monocytes to HUVEC exposed to oxidized LDL in the presence of a maximal concentration of extract (100 µg/ml) was indistinguishable from endothelial cells incubated under control conditions.
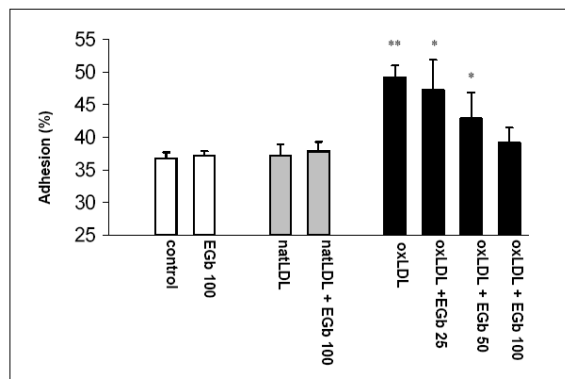


**Figure 1. Effect of LDL and EGb-761 on HUVEC adhesive properties**. HUVEC were pre-incubated for 24h in the presence or absence of EGb-761 (25 to 100 µg/ml). Cells were then exposed for 24 h to native (gray bars) or oxidized LDL (black bars) at the dose of 100 µg/ml, in the presence or absence of EGb-761 at the pre-incubation dose. Adhesion of human monocytic cell line THP1 on HUVEC monolayers was then assayed as described in the "Materials and Methods" section. Values are means ± SEM of 6 separated experiments. ** p<0.01 and * p<0.05 *vs.* control

group (HUVEC incubated 48 h with standard culture medium.

*Endothelial Na$^+$-K$^+$-ATPase activity*

Ionic homeostasis within the umbilical endothelia represents another potential function affected by atherogenic lipoproteins (Nayler, 1991). Earlier studies have suggested that the Na$^+$-K$^+$-ATPase may be implicated in the early pathological changes induced by LDL (Torkovskaia et al., 1983; Chen et al., 1995). We evaluated endothelial Na$^+$-K$^+$-ATPase by measuring the K$^+$-activated hydrolysis of *p*-nitrophenol phosphate, (i.e., pNPPase activity). Enzymatic activity following exposure to native LDL was decreased by about 30% (p<0.01). Exposure to oxidized LDL further decreased the K$^+$-activated pNPPase activity, by 65% (p<0.001) EGb-761 did not affect pNPPase activity in the absence of LDL nor the decrease in activity induced by native LDL. However, treatment with EGb-761 minimized the decrease in activity induced by oxidized LDL (p<0.05). The protection provided by the extract reduced the decrease from 65% to 30%, which was the level of inhibition observed for native LDL. Taken together, the observations of cellular adhesion and Na$^+$-K$^+$-ATPase suggest that EGb-761 can prevent the changes in endothelial function elicited by exposure to oxidized LDL.
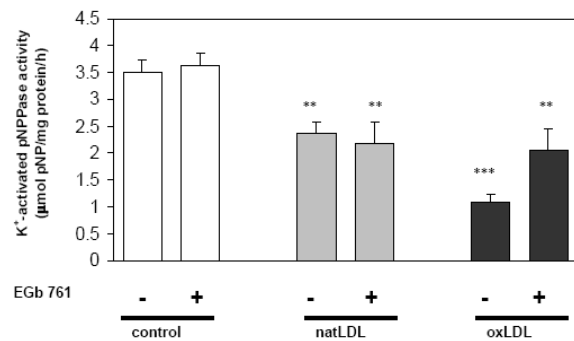


**Figure 2. Effect of LDL and EGb-761 on Na,K-ATPase function**. HUVEC were pre-incubated for 24h in the presence or absence of EGb-761 (100 µg/ml). Cells were then exposed for 24 h to native (gray bars) or oxidized LDL (black bars) at the dose of 100 mg/ml, in the presence or absence of EGb-761 at the pre-incubation dose (100 µg/ml). Na,K-ATPase activity was measured by the K$^+$-activation pattern of paranitrophenyl phosphatase (pNPPase) activity in HUVEC monolayers. Values are means ± SEM of 5 separated experiments. ** p<0.01 vs. control group, *** p<0.001 *vs*. control group (HUVEC incubated 48 h with standard culture medium).

*Cell Viability*

A trivial explanation for the effects of ox-LDL on endothelial function could be non-specific toxicity. We therefore ensured that the

native and oxidized forms of LDL employed under the conditions of this study had a negligible effect on HUVEC viability. Three sets of HUVEC isolated from three different donors were examined. Cell viability under control conditions was defined as 100%. Twenty-four hours of exposure to native or oxidized LDL (100 µg/ml) had no significant effect on cell viability as assessed by mitochondrial dehydrogenase activity (table 1). A similar lack of effect on viability was observed when HUVEC were pre- and co-incubated with a maximal concentration of EGb-761 (100 µg/ml).

**Table 1. Cell Viability**

|  | No Extract Exposure | *Ginkgo* Extract (100 µg/ml)[a] |
|---|---|---|
| Control | 100[b] | 100.42 ± 2.9 |
| Native LDL (100 µg/ml) | 88.8 ± 3.7 | 89.8 ± 4.2 |
| Oxidized LDL (100 µg/ml) | 83.5 ± 5.1 | 86.2 ± 1.4 |

[a] Pre-exposure to control medium or Ginkgo extract for 24 h, followed by 24 h in the absence or presence of LDL.
[b] All observations are relative to control conditions, mean ± SEM, $n=3$.

*Endothelial Lipoperoxidation*

We next examined the possible mechanisms that might mediate the protective effects of EGb-761. An obvious possibility is lipid peroxidation, which, if altered, could have significant effects on cellular function. As an index of lipoperoxidation, we measured the concentration of thiobarbituric acid reactive substances (TBARS). Exposure to native LDL did not affect basal TBARS levels in HUVEC (Fig. 3). However, a 24h exposure to the same concentration of oxidized LDL increased significantly TBARS levels by about 45% ($p<0.01$). EGb-761 (100 µg/ml) prevented this increase in endothelial lipoperoxidation induced by oxidized LDL, producing TBARS levels indistinguishable from controls.
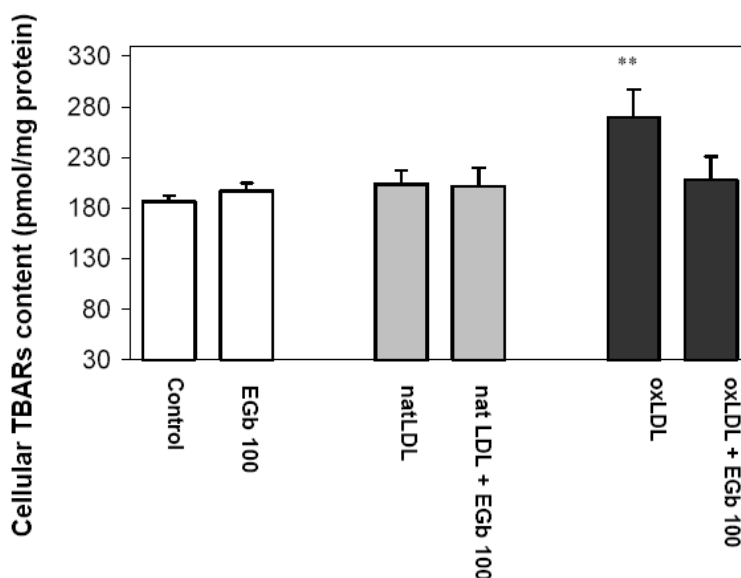


**Figure 3. Effect of LDL and EGb-761 on cellular Thiobarbituric Acid-Reactive Substances (TBARS) contents**. HUVEC were pre-incubated for 24h in the presence or absence of EGb-761 (100 µg/ml). Cells were then exposed for 24 h to native (gray bars) or oxidized LDL (black bars) at the dose of 100 µg/ml, in the presence or absence of EGb-761 at the pre-incubation dose (100 µg/ml). TBARS were then assayed as an index of lipoperoxidation. Values are means ± SEM of 7 separated experiments. **$p<0.01$ *vs.* control group (HUVEC incubated 48 h with standard culture medium).

# DISCUSSION

Epidemiologic studies have provided evidence of an inverse relationship between atherosclerosis and antioxidant intake. The oxidative-modification hypothesis implies that reduced atherosclerosis is a result of prevention of LDL oxidation (review by Diaz et al., 1997), but linking the reduced oxidation of LDL to a reduction in atherosclerosis has been problematic (Hodis et al., 2002). Several important mechanisms may underlie the role of antioxidants in preventing the clinical manisfestations of atherosclerosis. The present findings suggest that the standardized *Ginkgo biloba* extract EGb-761 protects endothelial cell against the deleterious effects of reactive oxygen species (ROS) formed at the cellular level (as suggested by TBARS measurements) during oxLDL exposure and preserves adhesive properties. Moreover, EGb-761 helps prevent ox-LDL-mediated disorders in ionic homeostasis.

These observations were made on a well-established model of human endothelial cells (HUVEC) in culture. Moreover, the atherogenic challenge that we imposed was designed to mimic that observed clinically in the intact animal. Indeed, the oxidized human LDL that was used has an oxysterol content compatible with that reported in human plasma (Mougenot *et al.*, 1997; Hodis *et al.*, 1994). There was no evidence of toxicity to the endothelial cells when exposed to either native or oxidized LDL, as monitored by cell viability, and the treated HUVEC displayed no evidence of abnormal morphology. The experimental design was therefore consistent with the clinical observation that the endothelium remains morphologically unchanged during the initial development of atherosclerosis, but the phenotype is changed to an activated state which favors inflammatory processes such as monocyte adhesion (Hajjar D. P. *et al.*, 1981) rather than the cytotoxicity and apoptosis seen at other stages (Bustamante et al, 2007). The range of the dose response for EGB-761 protection was similar to that seen by Chen et al. (2003) in endothelial cells from human aorta, another established experimental model. The mechanism by which ox-LDL increased endothelial adhesive properties is still not clear. During inflammatory processes, adhesion of monocytes to endothelium is mediated by a highly sequential and specific regulation of the expression of adhesion molecules by monocytes and endothelial cells. The signal transduction pathways for the ox-LDL-induced expression of binding molecules include the translocation of the redox-sensitive transcription factor NF-κB. Moreover, intracellular reactive oxygen species (ROS) are key intracellular messengers mediating this process (reviews by Lum & Roebuck ,2001; Schiffrin, 2002). EGb-761 has well known free radical scavenging properties. Consistent with this, our data show a total prevention of lipid peroxide formation that accompanies the lack of an increase in adhesion. It is therefore tempting to speculate that the protective effect of EGb-761 is mediated via its antioxidant properties as shown previously in mesangial cells (Akiba et al., 2004). However, since a role for the protein kinase C (PKC) pathway in ox-LDL-induced endothelial activation has also been reported (Mine *et al.*, 2002), a modulation of the PKC pathway by EGb-761cannot be excluded at this point (Bastianetto and Quirion, 2002). EGb-761 could reduce the ox-LDL/LDL quotient since this quotient seems of importance for the formation of unstable atherosclerotic plaques in human (Glass and Witztum, 2001).

One of the key events of the atherosclerotic process that leads to functional alterations seems to be perturbations in the physico-chemical properties of the endothelial membrane (Thorin *et al.*, 1995). Such modifications in membrane characteristics might influence protein functions and transmembrane ionic movements. The Na,K-ATPase is a membrane-bound enzyme that plays a crucial role in cellular ion homeostasis. We have characterized its expression in HUVEC in an earlier work (Pierre et al., 2001). During experimental atherosclerosis, its enzymatic activity is altered in arterial smooth muscle cells (Chen *et al*., 1995), an effect that could be linked to an alteration of the membrane composition by cholesterol oxidation products present in ox-LDL (Torkhovshaia et al. 1983; Peng and Morin, 1987). Because EGb-761 prevents lipoperoxidation and the impairment of active ion transport that accompanies cerebral ischemia (Pierre *et al*., 1999; Pierre *et al*., 2002), we reasoned that a similar effect might be occurring in vascular endothelia exposed to oxidized LDL. Our results on lipoperoxidation and Na,K-ATPase indeed support this hypothesis, but an additional, more unexpected piece of information came from the control experiments using native, non-oxidized LDL. According to the present results, exposure to native LDL induced a

decrease of about 30% in enzymatic activity, despite the lack of any evidence for lipid peroxidation.Although further investigation is warranted to understand the mechanism of native LDL-induced modulation of Na,K-ATPase activity, possible explanations include a modification of the lipidic environment of the Na, K-ATPase related to membrane phospholipid alterations and enzyme conformation or specific cellular localization of Na,K-ATPase isoform (Gerbi & Maixent, 1999, Rigoard et al., 2007). Clearly, oxidized LDL produced a more dramatic effect, reducing Na,K-ATPase activity by 60%. A component of LDL affecting ionic homeostasis is present in the lipoprotein before its oxidation. This hypothesis is reinforced by the fact that Na,K-ATPase activity was identical in endothelial cells exposed to native LDL, native LDL + EGb-761, or oxidized LDL + EGb-761, *i.e.*, 30% lower than in the control conditions. EGb-761 seems capable of protecting against changes linked to oxidation state, but other detrimental effects of LDL appear to be unaffected. It is also suggestive that two of the main components of LDL, phosphatidylcholine and cholesterol, have been previously shown to be potent inhibitors of endothelial Na,K-ATPase activity (Mayol et al., 1999).

There may also be a potential link between functional alteration of adhesion and alteration of Na,K-ATPase activity. Indeed, inhibition of endothelial Na,K-ATPase activity by the specific inhibitor ouabain has been shown to promote an increase in adhesive properties or tissue factor (Bereta et al., 1995, Stähli et al, 2007). In the present study, however, there was little evidence of a correlation between Na,K-ATPase inhibition and the increase in endothelial adhesive properties, as evidenced by the lack of an effect of native LDL on monocyte adhesion to endothelial cells, yet the same conditions produced a 30 % decrease in Na,K-ATPase activity. A tempting explanation, supported by recent reports, is that Na,K-ATPase inhibition *per se* does not explain the effect of ouabain on the expression of endothelial adhesion molecules, but that digitalis compounds such as ouabain are also able to trigger a specific signaling pathway through their binding to Na,K-ATPase, independently of its ion transporting properties (Aizman *et al.*, 2001, review by Xie and Askari, 2002). Recently it was reported that in human cultured endothelial cells, Na,K-ATPase regulates tumor necrosis factor induced tissue factor expression (Stähli et al, 2007). Given the

major role played by tissue factor in the initiation of thrombosis, and our previous observation that low ouabain concentrations prevent the TNF-induced alteration of human endothelial cells (Pierre et al, 2001, Pierre, S. 2000 : Doctoral Thesis), pleiotropic clinical applications linked to Na,K-ATPase protection could be found by targeting the endothelial cell.

In conclusion, the present study demonstrates that the standardized *Ginkgo biloba* extract EGb-761 protects endothelial adhesive properties and helps preventing the disruption of ionic homeostasis during exposure to oxidized LDL under conditions where ROS generation was prevented. The results clearly suggest the free radical scavenging properties of the extract as the main mechanism underlying this protection. This suggests that EGb-761 may be protective against the onset of atherosclerosis.

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