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***In vitro* Glycooxidized Low-Density Lipoproteins and Low-Density Lipoproteins Isolated from Type 2 Diabetic Patients Activate Platelets *via* p38 MAPK**

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**Structured abstract**

Context: Platelet hyperactivation contributes to the increased risk for atherothrombosis in type 2 diabetes and is associated with oxidative stress. Plasma low-density lipoproteins (LDL) are exposed to both hyperglycemia and oxidative stress and their role in platelet activation remains to be ascertained.

Objective: The aim of this study was to investigate the effects of LDL modified by both glycation and oxidation *in vitro* or *in vivo* on platelet arachidonic acid (AA) signaling cascade. The activation of platelet p38 MAPK, the stress kinase responsible for the activation of cytosolic phospholipase A<sub>2</sub>, and the concentration of thromboxane B<sub>2</sub>, the stable catabolite of the pro-aggregatory AA metabolite thromboxane A<sub>2</sub>, were assessed.

Results: Firstly, *in vitro* glycoxidized LDL increased the phosphorylation of platelet p38 MAPK as well as the concentration of thromboxane B<sub>2</sub>. Secondly, LDL isolated from plasma of poorly controlled type 2 diabetic patients stimulated both platelet p38 MAPK phosphorylation and thromboxane B<sub>2</sub> production and possessed high levels of malondialdehyde but normal alpha-tocopherol concentrations. By contrast, LDL from sex- and age-matched healthy volunteers had no activating effects on platelets.

Conclusions: Our results indicate that LDL modified by glycooxidation may play an important contributing role in platelet hyperactivation observed in type 2 diabetes via activation of p38 MAPK.

**Abbreviations:** AA, arachidonic acid; BHT, butylated hydroxytoluene; EDTA, ethylene diamine tetraacetic acid; MDA, malondialdehyde; p38 MAPK, p38 mitogen-activated protein kinase; TBARS, thiobarbituric acid reactive substances; TxA<sub>2</sub>, thromboxane A<sub>2</sub>; TxB<sub>2</sub>, thromboxane B<sub>2</sub>.

## **Introduction**

Oxidative stress has been identified as one of the factors closely associated with platelet hyperactivation in type 2 diabetic patients (1) even in the absence of any vascular complications (2). It remains to be determined whether oxidative stress is inherent to platelets and/or is a consequence of circulating factors that could influence platelet function. Among them, low-density lipoproteins (LDL) are submitted to both glycation and oxidation in diabetes (3). In this context, the aims of our study were: 1) to determine the effect of *in vitro* glycoxidized LDL on platelets and compared it with either control, glycated or oxidized LDL. 2) to investigate the effect of LDL isolated from plasma of type 2 diabetic patients compared to healthy volunteers on platelets. Anti-/pro-oxidant status of LDL was assessed and the activation of platelet p38 mitogen-activated protein kinase (p38 MAPK) as well as the formation of thromboxane B<sub>2</sub> (TxB<sub>2</sub>) were determined.

## **Subjects and Methods**

### ***In vitro experiments***

#### *Low-density lipoprotein isolation from healthy subjects*

LDL were isolated from plasma by density gradient ultracentrifugation (density 1.019-1.063 g.ml<sup>-1</sup>) (4). The concentration of protein was estimated using the Bradford assay (5). LDL were stored at 4°C in the dark under nitrogen and used within 2 days after preparation.

#### *In vitro low-density lipoprotein modification*

LDL (3.5 mg protein/ml) in phosphate buffered saline (PBS, pH 7.2) were all incubated at 37°C in the dark, under nitrogen in the presence of sodium azide (1.5 mmol/liter) :

- Control LDL were prepared by incubating LDL with ethylenediamine tetraacetic acid (EDTA) (1 mmol/liter) and butylated hydroxytoluene (BHT) (5 µmol/liter) for 5 days.

- Glycated LDL were LDL incubated with 50 mmol/liter D-glucose for 5 days in the presence of EDTA (1 mmol/liter) and BHT (5  $\mu$ mol/liter).

- Oxidized LDL were prepared by incubating LDL for 5 days and then treating them with 1  $\mu$ mol/liter  $\text{CuSO}_4$  for 1 hour at 37°C.

- Glycoxidized LDL consisted of LDL incubated with 50 mmol/liter D-glucose for 5 days and treating them with 1  $\mu$ mol/liter  $\text{CuSO}_4$  for 1 hour at 37°C.

LDL preparations were then dialyzed against PBS before their interaction with platelets.

#### *LDL characterization*

LDL concentrations of  $\alpha$ -tocopherol were determined by reversed-phase HPLC (6). Briefly, LDL samples, containing tocol (6-hydroxy-2-methyl-2-phytylchroman) and  $\delta$ -tocopherol as internal standards, were extracted with 4 volumes of hexane following the addition of 1 volume of ethanol. Tocopherol isomers were separated onto a Nucleosil  $\text{C}_{18}$  column, 5 $\mu$ m (4 x 150 mm) and detected fluorimetrically (excitation 295nm, emission 340nm).

Overall lipid peroxidation was evaluated by quantitation of malondialdehyde (MDA) by reversed-phase HPLC (7). LDL samples were mixed with thiobarbituric acid (TBA) (10 mmol/liter) and acetic acid in the presence of BHT (5 mmol/liter) and incubated at 95°C for 1 hour. TBA-MDA adduct was extracted with ethyle acetate, separated on a Nucleosil  $\text{C}_{18}$  column 5 $\mu$ m (4.6 x 250 mm) and detected fluorimetrically (excitation 515nm, emission 553nm).

The degree of glycation was determined by the trinitrobenzene sulfonic acid assay (8). LDL glycation was expressed as the percentage of relative reduction of the detected amino groups of lysine of modified LDL compared with control LDL.

#### *Diabetic and control subjects*

Ten type 2 diabetic patients (5 men and 5 women, aged  $58 \pm 2$  yr) from the Department of Endocrinology and Metabolic Diseases were matched for sex and age to ten healthy subjects (5 men and 5 women, aged  $54 \pm 2$  yr). Exclusion criteria for diabetic patients were smoking, antioxidant/vitamin supplementation, anti-aggregating drugs, insulin treatment. 6/10 were on metformin or sulfamides, 3/10 on glitazones and 7/10 took lipid-lowering drugs (statins). The patients had poorly controlled diabetes (fasting glycemia :  $11.9 \pm 2.0$  mmol/liter ; glycated hemoglobin HbA<sub>1C</sub> :  $8.8 \pm 0.6\%$ ). They had mild dyslipidemia with mild hypertriglyceridemia (triglycerides :  $2.0 \pm 0.2$  mmol/liter), normal LDL-cholesterol ( $2.7 \pm 0.3$  mmol/liter) and normal HDL-cholesterol ( $1.3 \pm 0.1$  mmol/liter). Control subjects were in good health as assessed by medical history and exclusion criteria were any pathology including diabetes and anti-aggregatory drugs. Written informed consent was obtained from all participants.

### ***Interaction between platelets and LDL***

#### *Platelet isolation and incubation with LDL*

Blood was collected at the regional blood center from healthy volunteers who had not ingested any aspirin or other non-steroidal anti-inflammatory drugs in the previous 10 days. Platelets were prepared (9) and incubated in the presence or absence of LDL (500 $\mu$ g/ml) for 2 hours at 37°C.

#### *Platelet p38 MAPK activation*

Platelets were lysed (10), proteins (25  $\mu$ g) were denatured for 10 min at 100°C, electrophoresed in 12% Tris-HCl polyacrylamide gels at 25 mA for 135 min and transferred to nitrocellulose membranes (100V, 30 min). The membranes were incubated with either 1/1000 anti-p38 MAPK or anti-phospho-p38 MAPK polyclonal antibodies (Cell Signaling Technologies, Beverly, MA, USA), washed and incubated with 1/2000 goat anti-rabbit horseradish peroxidase conjugate for 1 h. p38 MAPK and phospho-p38 MAPK were

visualized by enhanced chemiluminescence and bands were quantified by densitometry with an ImageMaster VDS-CL camera (Amersham Biosciences, Buckinghamshire, UK).

#### *Platelet thromboxane B<sub>2</sub> measurement*

Platelet TxB<sub>2</sub> was quantified by enzyme immunoassay (Amersham Biosciences, Buckinghamshire, UK). Coefficient of variation was lower than 10%.

#### *Statistical analysis*

Results are expressed as the mean  $\pm$  SD. Comparisons between groups were performed using a Wilcoxon test. Statistical significance was established at  $p < 0.05$ .

### **Results**

#### *Effects of in vitro modified LDL isolated from healthy subjects on platelets*

Glycoxidized LDL were compared with LDL incubated solely with glucose or CuSO<sub>4</sub> to differentiate the specific effects of glycation or oxidation from combined effects. The incubation of LDL with 50 mmol/liter glucose had no significant effects on LDL  $\alpha$ -tocopherol ( $7.70 \pm 1.84$  nmol/mg protein in glycated LDL vs  $8.53 \pm 2.27$  nmol/mg protein in control LDL, n=8) and MDA level compared to control LDL ( $0.19 \pm 0.16$  nmol/mg protein vs  $0.16 \pm 0.17$  nmol/mg protein, respectively, n=5). As expected, the addition of CuSO<sub>4</sub> to LDL resulted in a decreased  $\alpha$ -tocopherol ( $6.73 \pm 3.27$  nmol/mg protein) and an increased MDA levels ( $0.27 \pm 0.26$  nmol/mg protein) compared to control LDL. Glycooxidation of LDL neither led to significant additional decreases of  $\alpha$ -tocopherol ( $7.03 \pm 2.34$  nmol/mg protein) nor to further increases of peroxide levels ( $0.27 \pm 0.33$  nmol/mg protein) compared to oxidized LDL. Their percentage of glycation was  $34 \pm 6\%$ , similar to that measured in glycated LDL ( $35 \pm 9\%$ ).

The incubation of platelets with control LDL or glycated LDL had no significant effect on the activation of p38 MAPK (Fig. 1A) and TxB<sub>2</sub> concentration (Fig. 1B) as compared to platelets alone. The addition of oxidized LDL to platelets increased p38 MAPK phosphorylation by 2-fold and TxB<sub>2</sub> concentration LDL by 69%. Finally, glycoxidized LDL had the most pronounced effect on platelet p38 MAPK phosphorylation (3-fold increase) and TxB<sub>2</sub> level (2.4-fold increase) compared to platelets alone.

#### *Effect of LDL isolated from type 2 diabetic patients on platelets*

MDA levels increased by 6-fold in LDL from type 2 diabetic patients compared to LDL from control subjects ( $1.74 \pm 2.58$  nmol/mg protein vs  $0.29 \pm 0.29$  nmol/mg protein, respectively, n=10) whereas LDL  $\alpha$ -tocopherol concentrations did not differ between patients and control subjects ( $10.25 \pm 1.94$  nmol/mg protein vs  $10.26 \pm 3.25$  nmol/mg protein in LDL, respectively, n=10). The incubation of platelets with LDL from diabetic patients resulted in a 2.2-fold increase of phosphorylated p38 MAPK amount (Fig. 2A) and a 2-fold increased basal concentration of TxB<sub>2</sub> as compared to platelets ( $578 \pm 164$  pmol/10<sup>9</sup> platelets vs  $267 \pm 22$  pmol/10<sup>9</sup> platelets) (Fig. 2B). It is worth noting that neither significant phosphorylation of p38 MAPK nor increase of TxB<sub>2</sub> concentration were observed in platelets incubated with LDL from healthy volunteers.

## **Discussion**

Our results show that LDL modified by glycooxidation *in vitro* or *in vivo* activate platelets whereas control or glycated LDL have no effect. Previous studies on the interaction between platelets and LDL have shown that LDL do not induce platelet activation *per se* (11) but may increase the sensitivity of platelets to different agonists (12) *via* an increased phosphorylation of p38 MAPK (13). Such a discrepancy between results could be ascribed to differences in the

degree of LDL oxidation. We show that the incubation of LDL with glucose did not change their anti-/pro-oxidant status corroborating that glycation alone is insufficient to promote LDL oxidation (14). Moreover, glycated LDL had no stimulating effects on platelets. Although one study reported that glycated LDL caused an increased platelet aggregating response to ADP, the presence of high levels of TBARS in those LDL suggests that the effects were likely due to the combination of LDL glycation and oxidation (15). We also show that copper-oxidized LDL increased p38 MAPK phosphorylation by 2-fold and TxB<sub>2</sub> concentration by 69% confirming that LDL defined as minimally or mildly oxidized may activate platelets (12,16). Finally, glycoxidized LDL, reflecting at best the state that may occur in LDL from diabetic patients, were the most effective triggers of platelet AA signaling cascade. Although the presence of glucose has been shown to accelerate copper-induced LDL oxidation (17), we did not observe any significant differences between oxidized LDL and glycoxidized LDL in terms of vitamin E and MDA levels. This could be related to the copper-LDL ratios used which are much lower in our study than in the above published studies, as suggested by Knott *et al.* (14). Supporting our *in vitro* results, we present new data indicating that LDL isolated from poorly controlled type 2 diabetic patients increase platelet p38 MAPK phosphorylation and TxB<sub>2</sub> formation whereas those isolated from healthy control subjects do not. It has been shown that LDL isolated from type 1 diabetic patients increased platelet aggregation and TxB<sub>2</sub> release (18). Concerning the agents in LDL from diabetic patients responsible for platelet activation in our experiments, their identification seem rather difficult since LDL represent a heterogenous mixture of particles modified to different degrees of glycation and oxidation. However, an important feature is that LDL from selected patients possessed high concentrations of MDA which supports data reporting increased levels of plasma lipid peroxides in type 2 diabetic patients (19). No modification of vitamin E was observed ruling out an involvement of this antioxidant. Moreover, LDL isolated from selected diabetic

patients can be defined as glycoxidized LDL since the percent of HbA<sub>1C</sub> ( 8.8%) is known to be correlated with the percent of glycated LDL (20). In conclusion, LDL from type 2 diabetic patients – as well as glycoxidized LDL *in vitro* – activate platelet AA signaling cascade. The effect of LDL appears to be related to the combination of hyperglycemia and lipid peroxidation independently of vitamin E status. Thus, it suggests that glycoxidized LDL may act as one of the triggers of platelet activation occurring in type 2 diabetes.

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**FIG. 1 Effect of *in vitro* modified LDL on platelet p38 MAPK phosphorylation (A) and TxB<sub>2</sub> concentration (B).**

Platelets were incubated for 2 h at 37°C in the absence (0) or presence of 500µg/ml control LDL, glycated LDL (LDL + 50 mmol/liter glucose), oxidized LDL (LDL + 1µmol/liter CuSO<sub>4</sub>) or glycoxidized LDL (LDL + 50 mmol/liter glucose + 1µmol/liter CuSO<sub>4</sub>). Data are means ± SD of 5 to 8 experiments. <sup>a</sup>*P* < 0.05 vs platelets.

**FIG. 2 Effect of LDL from control subjects and type 2 diabetic patients on platelet p38 MAPK phosphorylation (A) and TxB<sub>2</sub> concentration (B).**

Platelets were incubated for 2 h at 37°C in the absence (0) or presence of LDL (500µg/ml). Results are means ± SD (n=10). <sup>a</sup>*P* < 0.05 vs platelets, <sup>b</sup>*P* < 0.05 vs platelets + LDL from control subjects.

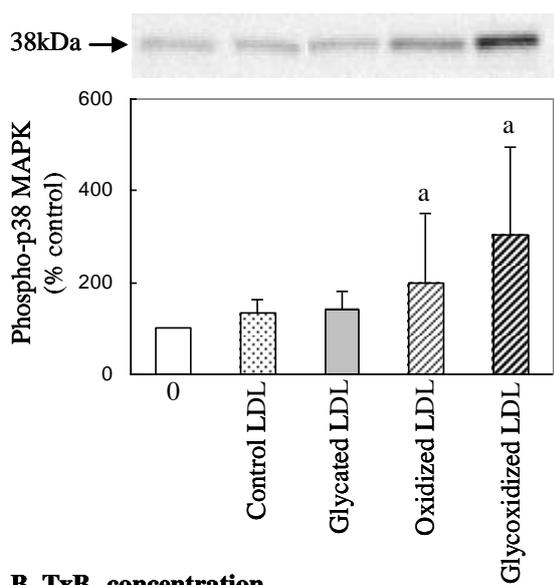
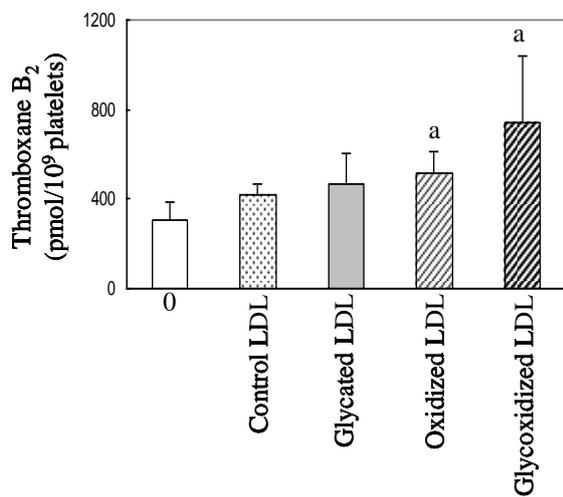
**A. p38 MAPK phosphorylation****B.  $\text{TxB}_2$  concentration**

FIG.1

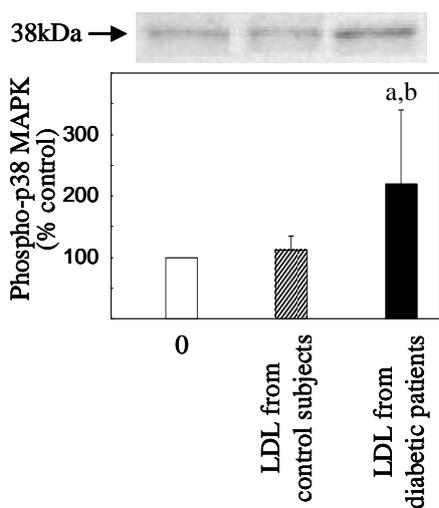
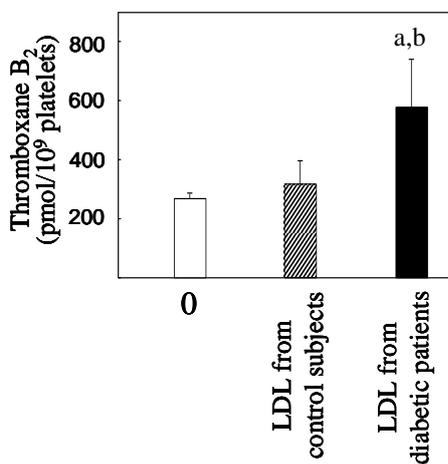
**A. p38 MAPK phosphorylation****B. TxB<sub>2</sub> concentration**

FIG. 2