Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2011;27:363-372

Accepted: March 09, 2011

Pivotal Role for Platelet-Activating Factor Receptor in CD36 Expression and oxLDL Uptake by Human Monocytes/Macrophages

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Key Words
OxLDL • Macrophages • CD36 • Platelet-Activating Factor Receptor • Foam cells • Atherosclerosis

Abstract
The uptake of oxLDL by CD36 is not regulated by intracellular levels of cholesterol, leading to macrophage differentiation into foam cells which play a major role in atherosclerosis. Furthermore, oxLDL competes with PAF in macrophages for binding to PAF receptors (PAFR). Here we investigated the involvement of PAFR in CD36 expression and uptake of oxLDL by human monocytes/macrophages. Adherent peripheral blood mononuclear cells were treated with PAFR-antagonists (WEB2170, CV3988); inhibitors of ERK1/2 (PD98059), p38 (SB203580), JNK (SP600125) or diluents, before stimulation with oxLDL or PAF. After 24 h, uptake of FITC–oxLDL and expression of CD36 was determined by flow cytometry and phosphorylation of MAP-kinases by Western blot. It was shown that the uptake of oxLDL was reduced by PAFR antagonists. CD36 expression was up-regulated by oxLDL, an effect reversed by PAFR antagonists. The up-regulation of CD36 and oxLDL uptake both required MAP-kinases activation. The oxLDL–induced ERK1/2 and JNK but not p38 phosphorylation was reversed by PAFR-antagonists suggesting that oxLDL signalling involves PAFR dependent and independent pathways. In macrophages from PAFR$^{-/-}$ mice, oxLDL was unable to up-regulate CD36 expression and the oxLDL uptake was reduced compared to *wild type*. These results suggest that oxLDL interacts with PAFR in macrophages to increase CD36 expression and oxLDL uptake. Whereas pharmacological intervention at the level of PAFR would be beneficial in atherosclerosis remains to be determined.

Introduction

CD36 is a class B scavenger receptor expressed mainly on the membranes of monocytes/macrophages, platelets and adipocytes. It was first identified as a thrombospondin-1 receptor, but it is now known that this receptor is able to interact with different and

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unrelated ligands, including oxidised low density lipoprotein (oxLDL) and phospholipids present in the membrane of apoptotic cells [1].

The uptake of modified LDL by CD36 is not regulated by intracellular levels of cholesterol, leading to continuous uptake of oxLDL and the differentiation of macrophages into foam cells. It is well established that these cells play a major role in atherosclerosis [2, 3]. It was suggested that the expression of scavenger receptors is increased in atherosclerosis because of auto-regulation by components of the oxLDL particle [4]. The relevance of this receptor in atherosclerosis has been suggested by experiments using mice deficient in both CD36 and ApoE. These animals showed reduced atherosclerotic lesions and greatly reduced uptake of oxLDL by macrophages compared to their littermate controls [5, 6]. The mechanism of oxLDL-induced foam cell formation during the atherosclerotic process has been under intense investigation, and it seems to be dependent on several membrane and nuclear receptors which mediate, independently or synergistically, the cell response [4, 7]. It has been shown that although CD36 has a very short intracytoplasmic tail, oxLDL is able to induce the activation of JNK in macrophages. Moreover, oxLDL induces the activation of MAPK pathway through a pertussis toxin (PTX)-sensitive G-protein-coupled receptor [8] which suggests that other receptors besides CD36 are engaged by oxLDL.

The oxidation of LDL generates molecules which are structurally similar to the pro-inflammatory lipid mediator platelet activating factor (PAF) [9] and some effects of oxLDL binding to its receptors have been shown to be dependent on PAFR activation [10]. The receptor for PAF is expressed constitutively on macrophages and other cell types [11]. The PAFR expressed in human leukocytes was cloned in 1991 by Nakamura et al. [12] and presents homology to other G-protein-coupled receptors inducing phosphatidylinositol turnover, phospholipase A2 and D activation and stimulation of protein tyrosine kinases. It is known that PAFR engagement results in rapid uptake of the ligand and transcriptional activation of several genes [11].

We have previously suggested that PAF and oxLDL share common ligands based on the observation of cross-desensitisation between these receptors and by the inhibition of the effects of oxLDL by PAFR antagonists in murine macrophages [13]. In the present study, we investigated whether interactions between these receptors occur in human macrophages and its consequences on the uptake of oxLDL. We found that PAFR activation is a key event in the uptake of oxLDL and up-regulation of CD36. These effects of PAFR seem to occur via ERK1/2 and JNK phosphorylation.

Materials and Methods

Materials

The following drugs were purchased from Gibco (Long Island, NY, USA): Fetal Calf Serum (FCS), L-glutamine and HEPES-acid. RPMI-1640, penicillin, streptomycin, aprotinin, leupeptin, benzamidin, chloramphenicol, a protease inhibitor cocktail and PMSF were purchased from Sigma-Aldrich (St. Louis, MO, USA). The p38 MAPK inhibitor SB203580 and the JNK inhibitor SP600125 were purchased from Tocris Bioscience (Ellisville, Missouri, USA) and the ERK1/2 inhibitor PD98059 was purchased from Calbiochem (Merck Chemicals, Nottingham, UK). The PAFR antagonist WEB2170 was kindly supplied by Boehringer Ingelheim (Pharma KG, Biberach, Germany), and an additional PAFR antagonist CV3988 was purchased from Biomol (Enzo Life Sciences, New York, USA). PAF C16 was acquired from Cayman Chemical (Ann Arbor, Michigan, USA). Monoclonal phycoerythrin (PE)-anti-hCD36, the isotype control PE-mouse IgM, purified mouse IgA anti-mouse CD36 (clone JC63.1), biotin-conjugated rat anti-mouse IgA antibody (clone C10-1) and streptavidin-PE-conjugated were purchased from BD Biosciences (Franklin Lakes, NJ, USA). The monoclonal antibodies against phosphorylated and non-phosphorylated forms of ERK1/2, p38 and JNK and against rabbit IgG-HPR were purchased from Cell Signalling Technology (Beverly, MA, USA). The ECL reagent kit and the nitrocellulose membrane for western blot were purchased from GE Healthcare (Uppsala, Sweden), the Ficoll–Hypaque from Amersham–Pharmacia Biotech (Uppsala, Sweden) and the BCA kit from Thermo Scientific (Rockford, IL, USA).

Purification and oxidation of LDL

Blood was collected from normolipidemic blood donor volunteers and plasma was obtained after centrifugation at 1,000 *g*, 4 ºC, for 15 min in the presence of EDTA 1 mg/mL. Thereafter, were added benzamidine (2 mM), gentamicin (0.5%), chloramphenicol (0.25%), PMSF (phenyl-methyl-sulphonyl-fluoride) (0.5 mM), and aprotinin (0.1 units/mL). LDL (1.019-1.063 g/mL) was isolated by sequential ultracentrifugation (100,000 x *g*, 4 °C), using a P90AT-0132 rotor (CP70MX ultracentrifuge; Hitachi Koki Co., Ltd., Tokyo, Japan), dialysed (4 °C) against PBS, pH 7.4 containing 1 mM EDTA, filtered (0.22 μm) and stored at 4 ºC. The protein concentration was determined by the BCA kit. Part of the LDL was dialysed overnight against EDTA-free PBS, followed by incubation with $CuSO_4$ (20 μM per mg of LDL protein; 18 h; 37 °C). The oxidation was stopped by the addition of 1 mM EDTA. This procedure has been previously standardised and results in completely oxidised LDL [14].

Isolation and culture of human PBMC

Peripheral blood mononuclear cells (PBMC) were separated by a Ficoll–Hypaque gradient (density 1.077 g/mL) according to the manufacturer's instructions, resuspended and cultured in RPMI-1640 supplemented with 5% FCS, 15 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Cell cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. After 1 h, the non-adherent cells were washed out with warm PBS and the adherent monocytic cells were cultured in RPMI/10% FCS for 72 h. One day before the experiments, the medium was changed for RPMI/2% FCS. The cells were adjusted to 2×10^6 cells/ml.

Flow cytometry for CD36 expression

Macrophages were cultured in the presence of oxLDL for 24 h. The PAFR antagonists WEB2170 and CV3988 or the inhibitors of ERK1/2 (PD98059), p38 (SB203580) or JNK (SP600125) were added to the cell cultures 30 min before the addition of oxLDL. After the incubation time, the cell culture medium was replaced with cold PBS and plates were put on ice for 30 min, after that, the cells were scrapped with a rubber policeman cells, centrifuged at 300 x g for 10 min, washed and resuspended in blocking buffer (PBS with 10% human AB serum) for 15 minutes at 4 °C and then incubated in staining buffer (PBS, FCS 1%, sodium azide 0.1%) containing the labelled monoclonal antibodies diluted 1:100, PE-conjugated anti-human CD36 or the isotype control. The expression of CD36 was evaluated in flow cytometer (FACS Canto - Becton BD Biosciences) and the data were analysed by the software Summit® V4.3 (DakoCytomation).

Uptake of oxLDL

LDL was labelled with FITC, as described previously [15, 16]. The FITC concentration in LDL was determined by spectroscopy against FITC standard solution at 495 nm. The F/P (Fluorochrome/Protein) molar ration was admitted in the range of 2.4-3, calculated as described [17]. FITC-LDL was oxidised by $CuSO_4$ (20 µM per mg of LDL protein; 18 h; 37 °C). The uptake of oxLDL-FITC was determined by FACS and fluorescence microscopy. To investigate the role of the PAFR in the uptake of oxLDL, monocytes/macrophages (2×10^5 cells) were pre-treated with WEB2170 for 30 min, then incubated with FITC-oxLDL (30 µg/mL) for 1 h at 37 ºC in the dark. In another group of experiments, cells were pre-treated with specific inhibitors for ERK1/2, p38MAPK or JNK for 30 min and then incubated with oxLDL (30 µg/mL). After 24 h, the cells were washed twice with RPMI/5% FCS to remove the oxLDL from the supernatant and incubated over-night in RPMI/5% FCS. This procedure was performed for recovery the receptors for oxLDL to the plasma membrane. The uptake assay was performed by incubation with FITC-oxLDL for 1 h at 37 ºC. Cells were washed with cold PBS and fixed with 2% formaldehyde. The uptake of FITC-oxLDL was evaluated by flow cytometry using a FACS Canto (Becton BD Biosciences) and the analysis was performed using software Summit® V4.3 (DakoCytomation).

Western blot for phosphorylated forms of ERK1/2, p38-MAPK and JNK

Human monocytes/macrophages (2×10^6 cells/ml) were pre-treated with WEB2170 (50 µM) or CV3988 (30 µM) for 30 min, then treated with oxLDL for 10 min. Cells were washed with cold PBS and protein lysates were obtained by homogenising cells in lysis buffer (1% Nonidet® P40, 50 mM Tris, pH 8.0, 150 mM NaCl, SDS 0.1%) supplemented with a protease inhibitor cocktail and phosphatase inhibitors (sodium fluorate 10mM and sodium orthovanadate 1mM). Protein concentrations were determined using the BCA assay kit. Equal amounts of protein (15 µg) were separated by SDS-PAGE (10%) and transferred to a nitrocellulose membrane. The phosphorylated and total non-phosphorylated proteins were detected using monoclonal antibodies to ERK1/2, p38 and JNK. Proteins were visualised using horseradish peroxidase-conjugated IgG anti-rabbit (1:1,000 dilution, 1 h) and the ECL system. The resulting autoradiograms were analysed using the AlphaEaseFC™ software, V3.2 beta (Alpha Innotech).

Isolation of macrophages from PAFR deficient mice ($PAFR^{-/-}$)

Balb/c $PAFR^{-/-}$ mice were kindly provided by Professor Takao Shimizu (University of Tokyo) [18] and were bred and maintained under SPF conditions at Institute of Biomedical Sciences at the University of São Paulo. All procedures described here had prior approval from the local animal ethics committee. Resident peritoneal macrophages were obtained from eight- to ten-week-old Balb/c (*wild type*) and $PAFR^{-/-}$ mice by lavage with cold sterile PBS and centrifuged (300 × g, 10 min, 4 °C). Cells were cultured in RPMI/5% FCS. Cells were left to adhere on microplates for 2 h at 37 °C, 5% CO_2 in RPMI. Non-adherent cells were removed by aspiration of supernatants and the cultures supplied with fresh medium. The expression of CD36 was evaluated in non-activated or oxLDL- activated macrophages by flow cytometry after 24 h. The macrophages were detached as described before and incubated with mouse Fc block for 15 min at 4°C and then incubated with mouse IgA anti-mouse CD36 (1:400) for 20 min, 4 °C, washed and incubated with biotinylated anti-mouse IgA (1:250) and Streptavidin-PE (1:250) for 20 min at 4 ºC. Cells were washed with cold PBS and fixed with 2% formaldehyde. In order to determine the uptake of oxLDL, the adherent peritoneal macrophages were incubated with FITC-oxLDL (FITC-oxLDL (30 µg/mL) for 1 h at 37 ºC in the dark. Cells were washed with cold PBS and fixed with 2% formaldehyde. The uptake of FITC-oxLDL was evaluated as described previously.

Statistical analysis

Data are presented as mean ± standard error of at least five experiments with different donors. Each experiment was performed in triplicate. Analysis of variance (ANOVA), combined with the Student-Newman-Keuls test, was used to evaluate the statistical significance of the differences. $P < 0.05$ values were considered as significant.

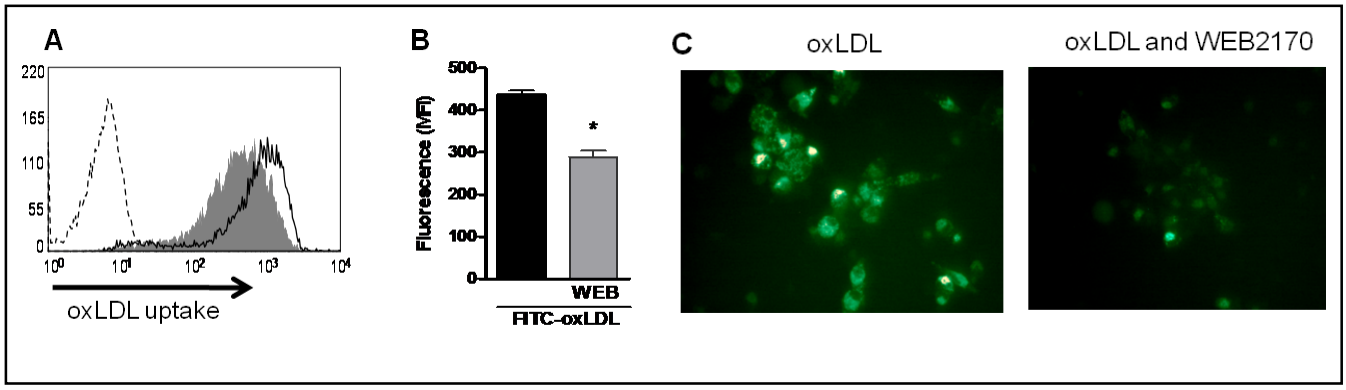

Fig. 1. The uptake of oxLDL is reduced by antagonism of the PAFR. Adherent human monocytes/macrophages were cultured for 72 h. The uptake assay was performed incubating the cells with FITC-oxLDL (30 µg/mL) for 1 h. The black line shows the uptake of FITC-oxLDL in unstimulated cells. In gray, the cells were pre-treated with 50 µM WEB2170 for 30 min and then incubated with FITC-oxLDL for 1 h. The dashed line shows the untreated control (A). In B is shown the mean of fluorescence intensity (MFI) of the FITC-oxLDL uptake in non-treated and WEB2170 pre-treated macrophages. The uptake of FITC-oxLDL was visualised by fluorescence microscopy (C). Results are representative (A and C) or presented as mean ± SEM of the MFI (B) of five donors. * $p < 0.01$ comparing untreated with WEB2170 pre-treated cells.

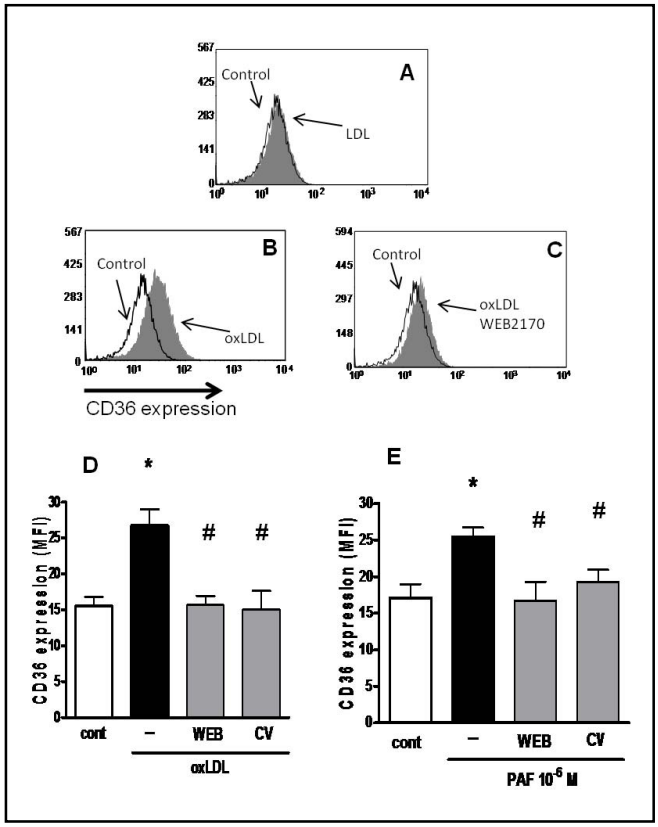

Fig. 2. Up-regulation of CD36 expression induced by oxLDL in human macrophages is dependent on PAFR. Adherent human monocytes/macrophage were stimulated with oxLDL (30 µg/mL) (B-D) for 24 h and pre-treated or not for 30 min with WEB2170 (50 µM) or with CV3988 (30 µM). Another group of cells (E) was stimulated with PAF (10^{-6} M) for 24 h and similarly pre-treated with the antagonists. The expression of CD36 was measured by FACS. In the upper panel, a representative histogram is showing the non-modified LDL (A). Data are presented as mean ± SEM of the Mean Fluorescence Intensity of five donors performed in triplicate. * $p < 0.01$ comparing cells stimulated with oxLDL or PAF with non-stimulated cells. # < 0.05 comparing WEB and CV treated with non-treated cells.

Results

PAFR is involved in the uptake of oxLDL and modulation of CD36 expression in human monocytes/macrophages

To investigate the involvement of PAFR we used two selective PAFR antagonists, WEB2170 [19] and CV3988 [20] and the concentration was taken from previous studies in murine macrophages [13]. Human monocytes/macrophages were incubated with FITC-oxLDL and the uptake was measured after 1 h by FACS and expressed as mean of fluorescence. It can be seen in Fig. 1 that oxLDL was strongly taken up by these cells. To investigate the involvement of PAFR in the uptake of oxLDL, the cells were treated with the PAFR antagonist WEB2170 (50 µM) before the addition of FITC-oxLDL. We showed here that the PAFR antagonist significantly decreased the uptake of

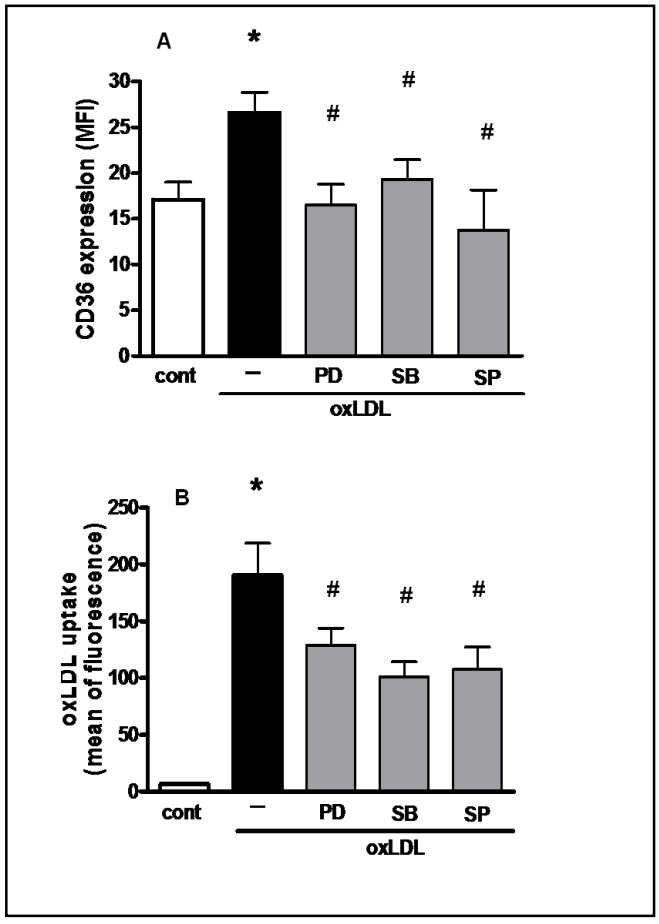

Fig. 3. The uptake of ox-LDL and CD36 expression requires ERK1/2, p38MAPK and JNK activation. Adherent human monocytes/macrophages were pre-treated with 10 μM of PD9805930 (ERK1/2 inhibitor), SB203580 (p38 inhibitor) or with SP600125 (JNK inhibitor) for 30 min before the addition of oxLDL (30 μg/mL). After 24 h, the expression of CD36 was measured by FACS (A). For the oxLDL uptake assay, the cells were similarly pre-treated with the drugs, incubated with FITC-oxLDL and the uptake was measured after 1 h by FACS (B). Data are presented as mean ± SEM of the Mean Fluorescence Intensity (MFI) of six (in A) or four (in B) different donors. * $p < 0.01$ comparing oxLDL stimulated cells with non-stimulated cells (cont). # < 0.05 comparing cells treated with the MAP kinase inhibitors with non-treated cells.

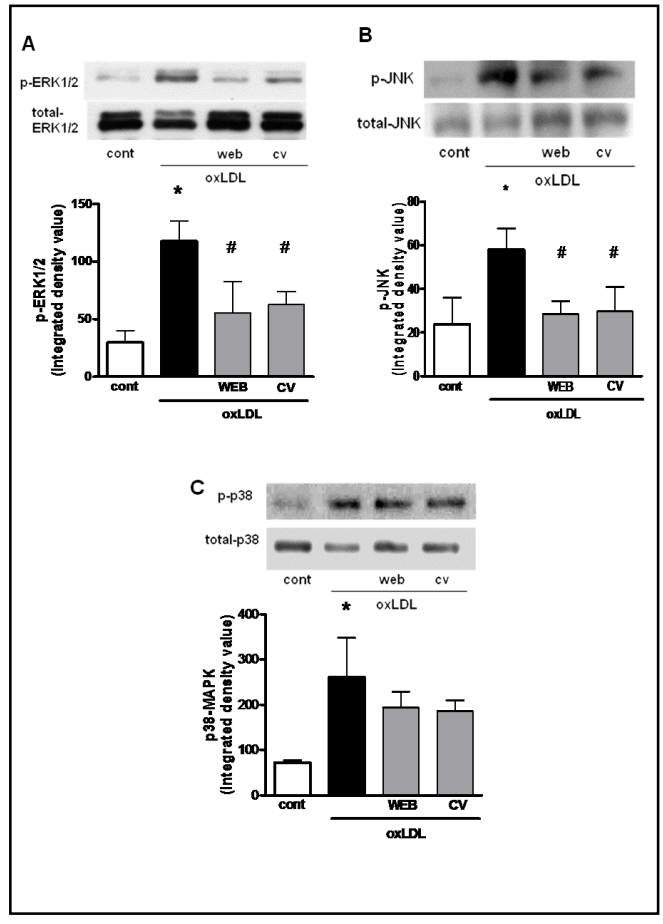

Fig. 4. ERK1/2 and JNK phosphorylation induced by oxLDL is inhibited by PAFR antagonists. Adherent monocytes/macrophages were cultured for 72 h and pre-treated or not with WEB2170 (50 μM) or with CV3988 (30 μM) for 30 min before stimulation with oxLDL (30 μg/mL) for 10 min. Western blot analysis of cell lysates was performed using antibodies against the phosphorylated and non-phosphorylated forms of ERK1/2, JNK and p38 (A, B and C, respectively). Data are presented as mean ± SEM of four donors. Protein expression was quantified by AlphaEaseFC™ software, V3.2 beta (Alpha Innotech). The autoradiographs show one representative experiment. * $p < 0.01$ comparing oxLDL-stimulated with the non-stimulated group (cont). # < 0.05 comparing WEB and CV treated with the non-treated group.

FITC-oxLDL. This is illustrated in Fig. 1C, where the uptake of FITC-oxLDL is visualized by fluorescence microscopy.

We have previously shown that incubation of the human monocytic THP-1 cells with oxLDL for 24 h increases the expression of CD36 on the cell membrane [14]. The same was observed in the present study with human monocytes/macrophages. Fig. 2B and 2D show that, in cells stimulated with oxLDL (30 μg/mL), the expression of CD36 was significantly increased (65% compared to control). This up-regulation of CD36 by oxLDL was reversed by treatment of the macrophages with PAFR antagonists WEB2170 or CV3988 before the addition of oxLDL (Fig. 2C and 2D). In the upper panel are the representative FACS histograms which show that the up-regulation of CD36 is restricted to oxLDL since it did not occur when non-modified LDL was used (Fig. 2A). The isotype control

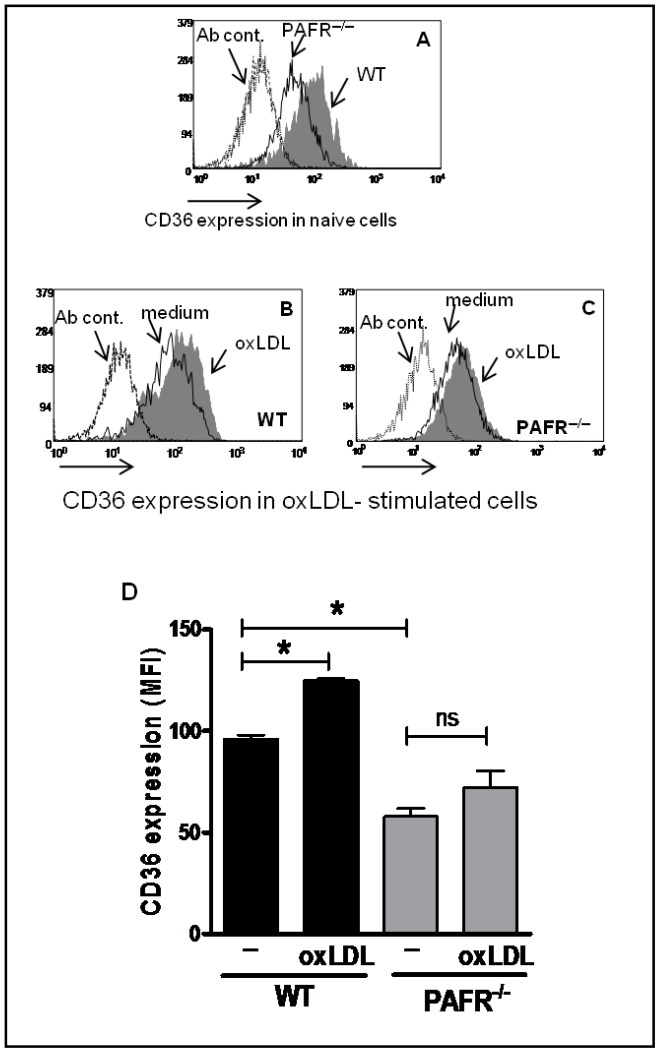

Fig. 5. PAFR is required for optimal CD36 expression in macrophages. Peritoneal macrophages were obtained from BALB/c *wild type* (WT) and PAFR$^{-/-}$ mice. In A is shown a representative histogram of CD36 expression in non-stimulated PAFR$^{-/-}$ and WT macrophages (basal expression). In B and C representative histograms of CD36 expression in macrophages from WT and PAFR$^{-/-}$ stimulated with oxLDL for 24 h. Ab control refers to the group treated with the secondary fluorescent antibody. In D, the data are presented as mean ± SEM of the Mean Fluorescence Intensity (MFI) of five animals in each group. * significantly different p<005; ns – non significantly different.

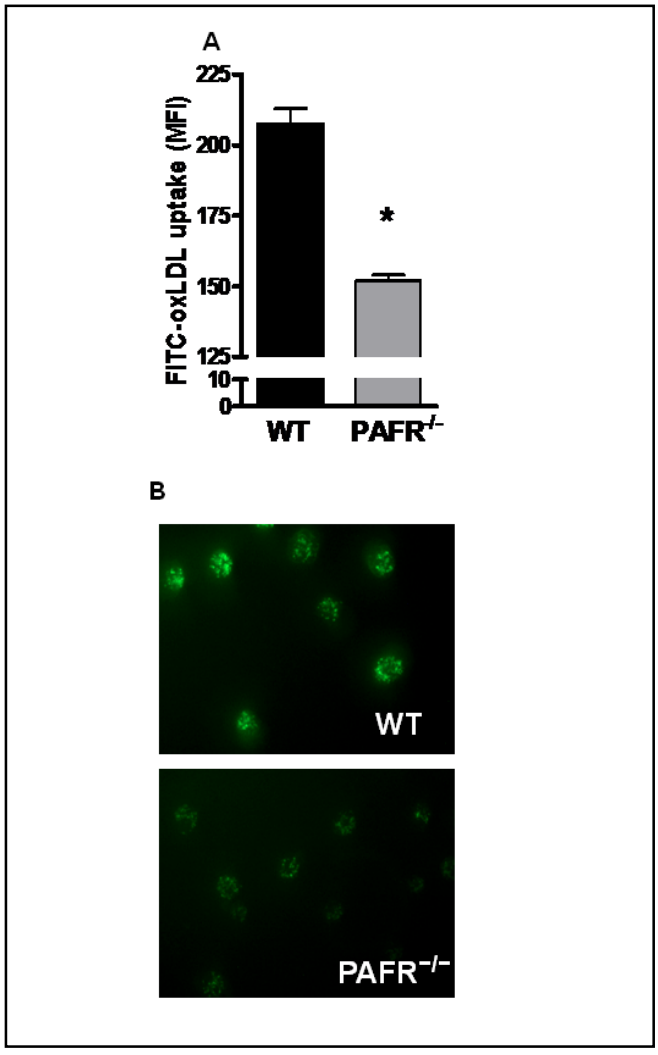

Fig. 6. PAFR regulates the uptake of FITC-oxLDL by mouse peritoneal macrophages. Peritoneal macrophages were obtained from BALB/c *wild type* (WT) and PAFR$^{-/-}$ mice and incubated with FITC-oxLDL (30 µg/mL) for 1 h. In A is shown the mean of fluorescence intensity (MFI) of the FITC-oxLDL uptake by PAFR$^{-/-}$ and WT macrophages. The uptake of FITC-oxLDL was visualised by fluorescence microscopy (B). Results are presented as mean ± SEM of the MFI (A) or one representative (B) of four animals in each group. * $p < 0.01$ comparing WT with PAFR$^{-/-}$ mice.

was also performed and showed minimal non-specific binding (not shown). In another set of experiments, the monocytes/macrophages were incubated for 24 h with PAF (10^{-6} M) instead of oxLDL. Fig. 2E shows that PAF is also able to up-regulate the expression of CD36 (49% increase compared to control) and this effect was reversed by pre-treatment of the cells with WEB2170 or CV3988 further confirming that the concentration of the antagonists used is effective. WEB2170 or CV3988 treatment modulated CD36 expression only in cells that were stimulated with oxLDL or PAF. The PAFR antagonists did not affect the basal expression of CD36 (not shown).

MAP-kinases activation by oxLDL is required for uptake and CD36 up-regulation and involves PAFR dependent and independent mechanisms

MAP kinases are involved in many cellular responses induced by oxLDL [21], and here we investigated which kinases are required for oxLDL-induced CD36 up-regulation and uptake. To this purpose, macrophages were treated with 10 mM of specific inhibitors of ERK1/2 (PD98059), p38 MAPK (SB203580) and JNK (SP600125) 30 min before addition of oxLDL and CD36 expression was measured after 24 h. Fig. 3A shows that the treatment of monocytes/macrophages with inhibitors of ERK1/2, p38 and JNK significantly reduced the expression of CD36 induced by oxLDL (96%, 76% and 100%, respectively). Fig. 3B shows that the uptake of FITC-oxLDL induced by oxLDL was reduced by the treatment of monocytes/macrophages with inhibitors of ERK1/2, p38 and JNK (33%, 48% and 44%, respectively). These results indicate that activation of the MAP kinases (ERK1/2, p38 and JNK) is required for both, oxLDL induced CD36 up-regulation and for the oxLDL uptake by human monocytes/macrophages.

We next investigated whether PAFR interferes with MAPKs activation induced by oxLDL. To this purpose we analysed the effect of blocking the PAFR in the oxLDL-induced activation of MAPKs. Human monocytes/macrophages were treated with PAFR antagonists, WEB2170 or CV3988, 30 min prior to oxLDL addition. After stimulation for 10 min, cell lysates were analysed by Western blot for phosphorylated and total MAP kinases. It was found that oxLDL induced the phosphorylation of ERK1/2, JNK and p38 (Fig. 4). Fig. 4A shows that treatment with WEB2170 or CV3988 inhibited the phosphorylation of ERK1/2 (71% and 62% inhibition, respectively) and in Fig. 4B these antagonists inhibited JNK phosphorylation (85% and 80% inhibition, respectively). However, the phosphorylation of p38 MAPK induced by oxLDL was not affected by the PAFR antagonists (Fig. 4C).

These data show that, although oxLDL induces the phosphorylation of ERK1/2, p38 MAPK and JNK, only the phosphorylation of ERK1/2 and JNK is dependent on PAFR activation. Thus it seems that oxLDL signalling involves both PAFR dependent and independent pathways.

Macrophages from PAFR$^{-/-}$ mice show defective uptake of oxLDL and CD36 expression

In order to confirm the involvement of PAFR in oxLDL uptake and CD36 expression we repeated the experiments in PAFR$^{-/-}$ mice. Resident peritoneal macrophages were obtained from PAFR$^{-/-}$ and *wild type* Balb/c mice, stimulated with oxLDL and the expression of CD36 was evaluated after 24 h. Fig. 5A shows that whereas oxLDL clearly up-regulated the expression of CD36 in macrophages from *wild type* mice, in PAFR$^{-/-}$ mice the expression of CD36 was only marginally increased. A representative histogram for oxLDL stimulated macrophages from *wild type* (Fig. 5B) and PAFR$^{-/-}$ mice (Fig. 5C) are shown. Fig. 5A shows that PAFR$^{-/-}$ mice express less CD36 than the *wild type* mice under basal conditions.

Next, we investigated the uptake of FITC-oxLDL by PAFR$^{-/-}$ macrophages. To this purpose, resident peritoneal macrophages from Balb/c PAFR$^{-/-}$ and *wild type* mice were incubated with FITC-oxLDL (30 μg/mL) and the uptake was measured after 1 h by FACS and expressed as mean of fluorescence intensity (MFI). Fig. 6A shows that macrophages obtained from PAFR$^{-/-}$ presented significantly lower capability of FITC-oxLDL uptake in comparison to macrophages obtained from WT mice. The difference between the groups is illustrated in Fig. 6B, where the uptake of FITC-oxLDL is visualized by fluorescence microscopy.

Discussion

In the present study, we investigated the role of PAFR in the uptake of oxLDL by human monocytes/macrophages. To this purpose we used two unrelated compounds that present PAFR antagonistic activity, WEB2170 and CV3988, and both were similarly effective. The concentration of the antagonists had been previously established to be effective in antagonizing the effects of PAF in murine macrophages [13] and this was confirmed in the present work where the concentration used reversed the PAF-induced up-regulation of CD36 expression. It was shown here that the uptake of oxLDL by human monocytes/macrophages was reduced by the treatment of cells with a PAFR antagonist suggesting the involvement of PAFR in the uptake of modified LDL.

It is known that the uptake of oxLDL by macrophages is directly associated with the expression of CD36 [1]. In our study, addition of oxLDL to macrophages increased CD36 expression, which is in accordance with previous findings that oxLDL exerts a positive feedback effect on the expression of its receptor in human and mouse macrophages [2, 14].

We found that PAFR antagonists reversed the up-regulated expression of CD36 induced by oxLDL. This indicates that PAFR is required for optimal expression of CD36.

These findings were further confirmed in experiments where we compared peritoneal macrophages from $PAFR^{-/-}$ mice with those from *wild type* mice. The results showed that the uptake of FITC-oxLDL is impaired in $PAFR^{-/-}$ mice. Also, whereas in *wild type* mice, oxLDL increased the expression of CD36, this did not happen in the $PAFR^{-/-}$ mice.

Another observation was that macrophages from $PAFR^{-/-}$ mice express lower levels of CD36 under basal conditions. This suggests that the intrinsic expression of CD36 is regulated by activation of PAFR by endogenous ligands. In human macrophages the PAFR antagonists did not affect the basal expression of CD36. This could be explained by the species difference or by the type of macrophages – peripheral blood monocytes differentiated *in vitro* to macrophages *versus* peritoneal macrophages. Moreover, whereas murine macrophages are constitutively deficient of PAFR, in the human macrophages, the PAFR was blocked only during the time of the experiment. Also, since human monocytes/macrophages express much lower levels of CD36 than mice peritoneal macrophages it is possible that an eventual effect of the PAFR antagonists could not be detected.

We have identified the signalling programs induced by oxLDL in human monocytes/macrophages critical for the up-regulated expression of CD36 and for the uptake of oxLDL since inhibitors of ERK1/2, p38 and JNK significantly reduced both outcomes. These results suggest that the phosphorylation of these kinases is a key event in the increased expression of CD36 induced by oxLDL as well as for the uptake of oxLDL by human monocytes/macrophages. It has been shown that activation of p38 by oxLDL is required for foam cell formation in J774 cells [22]. Ricci et al. showed that the inhibition of JNK reduced the formation of foam cells and the atherosclerotic lesion in animal models [23]. Whether inhibitors of these pathways would reduce foam cells formation in human disease require further studies.

We are proposing that oxLDL is able to interact with PAFR and others receptors through distinct moieties present in the oxLDL particle. In fact it has been described that during oxidation of the LDL particle are generated compounds which interact with PAFR [24]. In an attempt to understand at what level the crosstalk between receptors for PAF and oxLDL is occurring, we analysed the effect of PAFR antagonists in the MAP kinases pathways activated by oxLDL. It was found that when the PAFR is blocked, ERK1/2 and JNK phosphorylation is inhibited whereas phosphorylation of p38 is not affected. This suggests that oxLDL stimulates parallel and independent intracellular pathways via PAFR. Another possibility is that activation of PAFR or a scavenger receptor, such as CD36, would induce translocation of its partner receptor (eg. CD36 or PAFR) to lipid rafts, thus enhancing the activation of common signalling programs. We are presently investigating this hypothesis.

It has been described that oxLDL induces the activation of MAPK pathway in smooth muscle cells and suppress the NFκB activation in macrophages through a pertussis toxin (PTX)-sensitive G protein-coupled receptor [25, 26], which indicates that in addition to a scavenger receptor, a Gαi-coupled receptor is also required for oxLDL to activate cells signalling. Since the activation of PAFR also can be mediated by a Gαi-coupled receptor [27], it is plausible that for oxLDL to activate human monocytes/macrophages, an interaction between a scavenger receptor and PAFR would be required.

Regarding the involvement of PAFR in atherosclerosis, there is only one study that has shown that treatment of LDL receptor-deficient mice with PAFR antagonists reduced the formation of fatty streaks lesions, which are the first stage in the development of atherosclerosis [28]. However, the role of PAFR in the formation of the atherosclerotic plaque has never been addressed. It is known that foam cells produce high amounts of PAF [29], and that foam cells present in human atherosclerotic plaques express less PAFR than monocytes/macrophages [30]. Thus, in the atherosclerotic environment, which contains oxidised lipids, PAF and other inflammatory stimuli, macrophages would be recruited to the arterial intimae where they would take up oxLDL via CD36 and others scavenger receptors. This would lead to macrophage differentiation into foam cells since the uptake of oxLDL via CD36 is not regulated by intracellular levels of cholesterol [1]. We have shown here that this process is also dependent on PAFR. This would imply that PAFR antagonists could reduce the formation of foam cells, but this has not yet been investigated. Whereas pharmacological intervention at the level of PAFR activation would be beneficial in atherosclerosis remains to be determined.

Acknowledgements

The authors are grateful to Marlise Montes and Silvana A. Silva for technical assistance. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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