

Oxidized LDL Promotes Apoptosis and Expression of Pro-Inflammatory Mediators in Alternatively Activated Macrophages

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ABSTRACT: Accumulation of lipid within non-adipose tissues can induce inflammation by promoting macrophage infiltration and activation. Oxidized lipoproteins (oxLDL) have been known to induce cellular dysfunction in resident macrophages through pro-inflammatory and pro-apoptotic properties. However research into the pro-inflammatory and pro-apoptotic effects of oxLDL in alternatively activated (M2) macrophages has been relatively sparse. In this study, the pro-inflammatory and pro-apoptotic effects of oxLDL (at concentrations of 1 and 40µg/ml) were investigated in M2 macrophages. Incubation of M2 macrophage for 24 hours with 1 or 40µg/ml oxLDL induced pro-inflammatory molecules (MCP-1 and IL-6) production. Induction of cell death via apoptosis was observed after 24 hours incubation with 1 or 40µg/ml, but statistical significant induction was only observed with 40µg/ml oxLDL. Taking into consideration that M2 macrophages have been proposed as an appropriate target for type 2 diabetes, these findings may be of use in enhancing our knowledge of the adverse effects of oxLDL, particularly in the context of obesity, type 2 diabetes and the metabolic syndrome.

INTRODUCTION

Insulin resistance associated with type 2 diabetes is linked with a group of metabolic abnormalities collectively known as “metabolic syndrome” (Lebovitz and Benerji, 2001). This group of metabolic abnormalities include visceral obesity, chronic inflammation and atherosclerosis (Smith, 2003). Metabolic syndrome and its related disorders are associated with higher levels of circulating oxidized low density lipoprotein (oxLDL) (Holvoet *et al.*, 2008). These high levels of oxLDL lead to lipid overload in non-adipose tissues – heart, pancreas, skeletal muscle, liver, kidney, and so play a vital role in the pathogenesis of type 2 diabetes and its complications (Unger, 2002). Lipid accumulation in non-adipose tissues can lead to cellular dysfunction and cell death: a phenomenon known as Lipotoxicity. Moreover, apoptosis is considered an essential pathophysiological phenomenon in atherosclerosis (Littlewood and Bennette, 2003) and a large body of evidence indicates that oxLDL are cytotoxic for several cell types including macrophages (Muller *et al.*, 2001; Heinloth *et al.*, 2002). Lipotoxicity may thus contribute to the increased risk of insulin resistance, type 2 diabetes and other cardiovascular complications associated with

obesity. However, the mechanisms by which lipids accumulation promote insulin resistance and type 2 diabetes are not fully understood.

Macrophages play an important role in the pathogenesis of several diseases due to their providing a balance between pro- and anti-inflammatory responses. Two distinct subsets of macrophage have been described: classically “pro-inflammatory” (M1) and alternatively “anti-inflammatory” (M2) activated macrophages (Gordon, 2003). M1 macrophages display a cytotoxic, proinflammatory phenotype while M2 macrophages suppress inflammatory responses. Macrophages isolated from adipose tissue in lean mice express M2 phenotypic markers, while macrophages isolated from adipose tissue of diet-induced obese mice express M1 phenotypic markers (Lumeng *et al.*, 2007).

The present study aimed to investigate the effect of oxLDL on the expression of classical activation markers, and on activation of apoptotic pathways. The study aimed to address these issues *in vitro* by using phorbol 12-myristate 13-acetate (PMA) differentiated M2 macrophages generated from the stimulation of human acute monocytic leukemia cell line (THP-1 cells) with interleukin-13 (IL-13) and the peroxisome

proliferators activated receptor gamma (PPAR γ) agonist rosiglitazone, in order to mimic the *in vivo* population of resident alternatively activated macrophages (Bouhleb *et al.*, 2007). Monocyte chemoattractant protein 1 (MCP-1) and interleukin-6 (IL-6) were selected as pro-inflammatory markers while haptoglobin-haemoglobin scavenger receptor (CD163) and mannose receptor (MR) as anti-inflammatory markers (Bouhleb *et al.*, 2007). Activity of caspase 3 and 7, which has been shown to play an important role in mammalian apoptosis (Garcio-Calvo *et al.*, 1999; Le *et al.*, 2002), was also evaluated. oxLDL induced the expression of M1 classical activation markers in THP-1 cells that are polarized towards an M2 alternative activation phenotype. Moreover, apoptosis was also found to be induced after oxLDL treatment of M2-polarized macrophages. Taking into consideration that M2 macrophages have been proposed as an appropriate therapeutic target for type 2 diabetes (Odegard & Chawla, 2008) and also the importance of resident macrophages in inflammation and atherosclerosis, understanding of oxLDL-triggered signaling events in these cells may prove to be extremely important in the context of obesity, type 2 diabetes and the metabolic syndrome.

MATERIALS AND METHODS.

Materials: All reagents were purchased from Sigma-Aldrich (Poole, UK) unless stated otherwise. oxLDL was obtained from Autogen Bioclear (UK). Rosiglitazone was obtained from GlaxoSmithKline (Uxbridge, UK). IL-13 was obtained from R&D Systems (UK). Human monocytic THP-1 cell lines were obtained from the European Collections of Cell Cultures (UK).

Maintenance of cells in culture: The human THP-1 monocyte cell line (THP-1Mon) was used for these studies. The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) foetal calf serum, 1% (v/v) penicillin (50IU/ml), 1% L-glutamine (2mM), 1% (v/v) non-essential amino acids and 1% (v/v) OPI supplement. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. The sub-cultured cells were maintained at 0.3 – 1.0 x 10⁶ cells/ml.

Generation of M2 macrophages: To generate M2 macrophages (Bouhleb *et al.*, 2007), cells

were seeded at 1x10⁶/ml in 6-well culture dishes and treated with 15ng/ml IL-13 and allowed to stand for 30 minutes before addition of 1 μ M rosiglitazone to activate M2 polarisation. Cells were incubated with IL-13/rosiglitazone for at least 72 hours. The resulting M2-polarised THP-1 monocytes were then differentiated into macrophages by treatment with 100ng/ml phorbol myristate acetate (PMA) for 72 hours.

Incubation of Cells with oxidized LDL:

After confirming that cells are fully differentiated, the media containing PMA was aspirated and replaced with fresh media. The resulting M2 macrophages were then treated for 24 hours with oxLDL (1 or 40 μ g/ml), or with apoptosis inducer, thapsigargin (100nM) as a positive control.

Apoptosis Assays: Apoptosis of M2 macrophages incubated with 1 or 40 μ g/ml oxLDL for 24 hours was assessed using Caspase-Glo 3/7 Assay (Promega, Southampton, UK) according to the manufacturers' instructions. The resulting luminescence data was read via a Dynex luminometer (Worthing, UK).

Cell Viability: The number of viable cells after 24 hours incubation with 1 or 40 μ g/ml oxLDL was determined colorimetrically using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) according to the manufacturers' instructions. The resulting optical density data was read via a Dynex plate-reading spectrophotometer (Worthing, UK).

RNA isolation and real-time quantitative PCR:

Total RNA were extracted with Trizol[®] reagent according to the manufacturer's instructions (Invitrogen, Paisley, UK). RNA samples were converted to cDNA using an Applied Biosystems[®] High-Capacity cDNA Archive Kit. MR, MCP-1, CD163, IL-6, CD36 and β -actin mRNA expressions were assessed using SYBR[®] Green Assays (Applied Biosystems, Warrington UK) and analysed using an Applied Biosystems 7500 Real-time PCR system. Semi-quantitative comparisons of mRNA expression levels were carried out using the 2^{- $\Delta\Delta$ CT} method where Δ CT equals the difference between CT values for target gene and the house-keeping gene, β -actin. The following primers were used:

MR. Fwd: 5'-CGAGGAAGAGGTTCCGGTTCACC-3'
 Rev: 5'-GCAATCCCGGTTCTCATGGC-3'
CD163. Fwd: 5-TTGCCAGCAGTTAAATGTG-3'
 Rev: 5'-AGGACAGTGTGGGGACTGG-3'
MCP-1. Fwd: 5'-ACTGAAGCTCGTACTCTC-3'
 Rev: 5'-CTTGGGTTGTGGAGTGAG-3'
IL-6. Fwd: 5'-GCCTTCGGTCCAGTTGCCTT-3'
 Rev: 5'-AGTGCCTCTTTGCTGCTTTCAC-3'
CD36. Fwd: 5'-GGAAGTGATGATGAACAGCAGC-3'
 Rev: 5'-GAGACTGTGTTGTCCTCAGCGT-3'
 β -actin. Fwd: 5'-TCCTGTGGCATCCACGAA-3'
 Rev: 5'-GAAGCATTTGCGGTGGAC-3'

Statistical Analysis: Data were expressed as mean \pm standard error of the mean. Statistical significance was determined with Student's *t*-test comparison between two groups of data sets. Significance levels were set at $P < 0.05$.

RESULTS

IL-13 Cytokine induced THP-1 Monocytes Activation and Polarization *in vitro*. 72 hours incubation of THP-1 cells with 15ng/ml IL-13 and 1 μ M rosiglitazone did not have a significant effect on cell viability (Fig 1a), indicating that the treatment was not cytotoxic to the cells. Analysis of M2 alternative activation showed that IL-13/Rosiglitazone brought about increases in both MR and CD163 mRNA (Fig 1b) (MR; 3.2fold \pm 0.023; CD163; 2.2 \pm 0.15, $P < 0.05$ in both cases). In contrast, PMA treatment of M2Mon did not have effect on polarisation as assessed by MR and CD163 mRNA expression (Fig 1c)

Induction of CD36 mRNA by oxLDL: The expression of CD36, a class B scavenger receptor responsible for macrophage uptake of oxLDL was studied during incubation of M2 macrophage with oxLDL. These receptors were not down-regulated; hence it appears that macrophages keep taking up oxLDL, and so accumulate massive amounts of lipids (Itabe 2003). oxLDL has been shown to increase the functional expression CD36 (Han *et al.*, 1997; Nakagawa *et al.*, 1998), hence the induction of CD36 mRNA after 24 hours treatment with both 1 and 40 μ g/ml oxLDL was observed (Fig. 2).

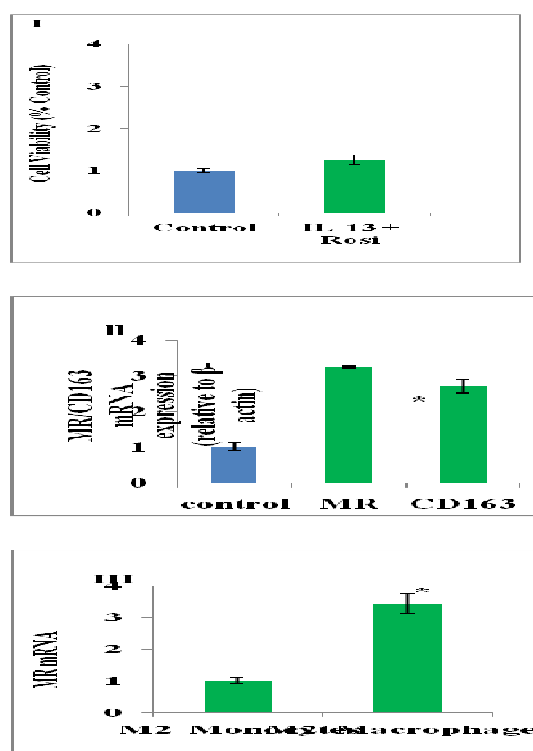


Figure 1. i): Effect of IL-13/rosiglitazone on THP-1 cell viability after incubation with the cytokine for 72 hours. The viability data show no decrease in cell viability indicating that the treatment was not cytotoxic to the cells. ii): Increased expression of alternative activation (M2) markers in THP-1 monocytes treated with 15ng/ml IL-13 and 1 μ M rosiglitazone for 72 hours. Markers were analyzed using an Applied Biosystems 7500 Real-time PCR system. iii): Treatment of M2 polarized THP-1 cells with 100ng/ml PMA do not affect the expression of M2 markers. Data are expressed as mean \pm SEM. * = $P < 0.05$ vs. control.

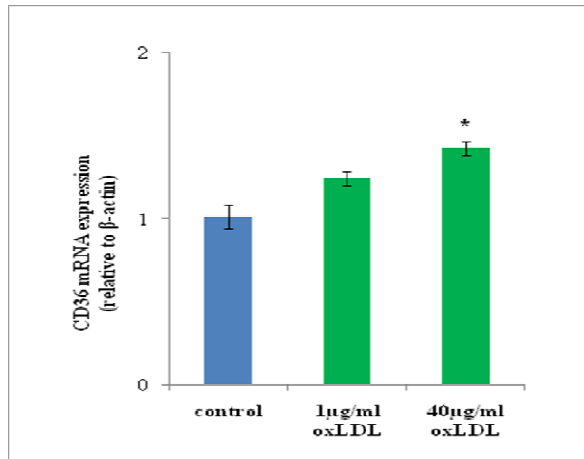


Figure 2. Effect of oxLDL on CD36 mRNA expression. M2 macrophages were incubated with the indicated concentrations of oxLDL for 24 hours. mRNA expressions were analysed using an Applied Biosystems 7500 Real-time PCR system. Data are expressed as mean ± SEM. * = $P < 0.05$ vs. control.

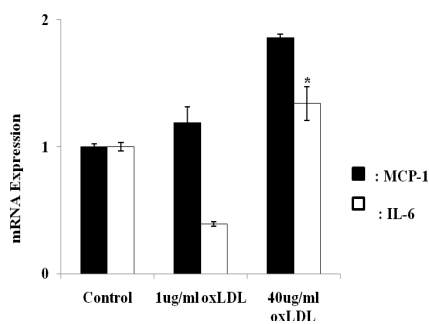


Figure 3. Effect of oxLDL on the expression of proinflammatory markers. M2 macrophages were incubated with the indicated concentrations of oxLDL for 24 hours. mRNA expressions were analysed using an Applied Biosystems 7500 Real-time PCR system. Data are expressed as mean ± SEM. * = $P < 0.05$ vs. control.

oxLDL induced the expression of M1 proinflammatory makers: MCP-1 and IL-6 have been used as markers of the proinflammatory M1 macrophages (Bouhlej *et al.*, 2007). These inflammatory markers play a vital role in the pathology of diseases associated with inflammation. As shown in Fig 3, oxLDL induced the upregulation of MCP-1 and IL-6 mRNAs in M2 macrophages after 24 hours incubation.

oxLDL Induced Apoptosis in M2 macrophage: Caspase -3 and -7 has been shown to play key effector roles in mammalian apoptosis (Garcio-Calvo *et al.*, 1999; Le *et al.*, 2002; Hitomi *et al.*, 2004) and therefore effect

of oxLDL on M2 macrophage apoptosis was investigated. As shown in Fig. 4, treatment with both 1 and 40 µg/ml oxLDL induced an increase in apoptosis. However, statistically significance activation was only seen in M2 macrophages incubated with 40 µg/ml oxLDL.

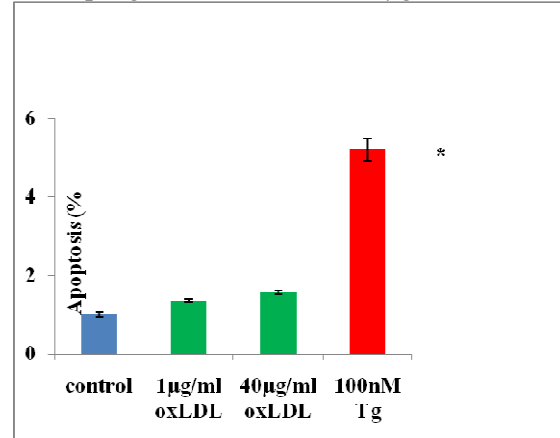


Figure 4. Induction of apoptosis by oxLDL. M2 macrophages were treated with the indicated concentrations of oxLDL or thapsigargin (positive control) for 24 hours. At the end of the incubation, the cells were analyzed for caspase -3/7 activity using Dynex luminometer. Data are expressed as mean ± SEM. * = $P < 0.05$ vs. control.

Cell viability: To further assess the physiological relevance of the findings obtained from apoptosis assay, the effect of oxLDL on cell viability after 24 hours of incubation was investigated. As shown in figure 5, both 1 and 40 µg/ml oxLDL induced a decrease in cell viability, confirming the cytotoxicity of oxLDL in M2 macrophages.

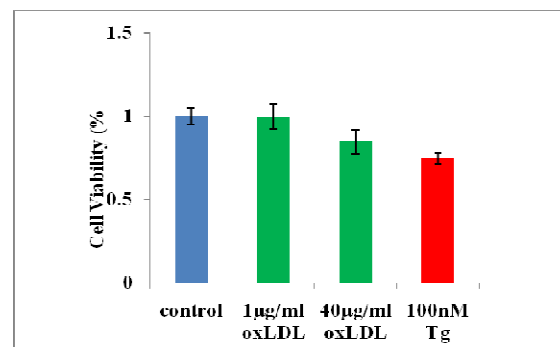


Figure 5. Effect of oxLDL on cell viability. M2 macrophages were incubated with the indicated concentrations of oxLDL or thapsigargin (positive control) for 24 hours. The number of live cells were determined by MTS reduction assay. Data are expressed as mean ± SEM. * = $P < 0.05$ vs. control

DISCUSSION

Evidence from human and animal studies suggests that lipid overload in non-adipose tissues, a condition known as lipotoxicity, contributes to the pathogenesis of several diseases. Lipotoxicity may thus contribute to the increased risk of insulin resistance, type 2 diabetes and other cardiovascular complications associated with obesity. However, the mechanisms by which lipid accumulation promotes insulin resistance and type 2 diabetes are not fully understood.

Elevated plasma low-density lipoprotein (LDL) leads to an increase in the adherence of circulating monocytes to arterial endothelial cells and rate of LDL entry into the intima (Steinberg 1997). In the intima, LDL can undergo oxidative modification catalyzed by any of the cells of the vasculature, i.e. macrophages, smooth muscle cells or endothelial cells (Steinberg 1997) forming a modified form of LDL called oxidized LDL (oxLDL).

oxLDL has been shown to induce variety of biological and physiological functions *in vitro* and these may seem to be contradictory in some respects. For example, oxLDL induces macrophage and smooth muscle cell proliferation (Yui *et al.*, 1993; Auge *et al.*, 1995; Biwa *et al.*, 1998) and also macrophage viability and survival (Sakai *et al.*, 1996; Hamilton *et al.*, 2001). While conversely a large body of literature also exists that demonstrates the cytotoxic and/or pro-apoptotic effects of oxLDL (Reid *et al.*, 1993; Dimmiller *et al.*, 1997; Sata *et al.*, 1998; Coles *et al.*, 2001; Martinet & Kockx, 2001; Nahn *et al.*, 2003; Tabas 2005; Seimon *et al.*, 2009; Sanson *et al.*, 2009). The reasons for these contradictory reports might be due to differences in the concentration and oxidation processes of the oxLDL used in these different studies.

As described earlier, macrophages in human and animal model show two distinct phenotypic characteristics, M1 and M2 phenotypes (Gordon 2003). Odegaard *et al.*, reported the importance of M2 macrophage phenotypes in insulin resistance and type 2 diabetes and hence suggested that M2 macrophages might be an appropriate therapeutic target for type 2 diabetes (Odegaard & Chawla, 2008). Moreover, as most insulin signaling molecules has been shown to be expressed on macrophages (Liang *et al.*,

2007), it can be concluded that this cell type has an important role in the pathogenesis of type 2 diabetes. Importantly, while the effects of oxLDL in resident macrophages have been widely studied, literature on the effects of oxLDL on M2 macrophages is relatively sparse.

In this study, the effect of oxLDL on the expression of proinflammatory molecules and the induction of apoptosis in M2 macrophages were investigated. The concept of alternative macrophage activation was first investigated, keeping in mind the lack of unity arising from previous studies in this area. Gordon attributed alternative activation to IL-4 and IL-13, while suggesting that other anti-inflammatory cytokines should be regarded as deactivating macrophages (Gordon 2003). In line with this view, treatment with IL-13 significantly increased the expression of MR and CD163 in the current study (Fig 1b). Further more, this study showed that PMA-induced differentiation of M2 polarized monocytes into M2 polarized macrophages did not have a significant effect on the expression of M2 polarization markers *in vitro* (Fig 1c).

The first property of oxLDL to be discovered that makes it more atherogenic than native LDL is the fact that it is recognized by scavenger receptors, leading to accumulation of cholesterol in foam cells (Henriksen *et al.*, 1982). For example, the scavenger receptor CD36 has been shown to bind and internalize oxLDL in macrophage (Nicholson *et al.*, 2000). As mentioned earlier, oxLDL increases the functional expression of CD36 in macrophages. In line with this study, the induction of CD36 mRNA in M2 macrophages was investigated in the present study. As shown in Fig 2, both 1 and 40µg/ml oxLDL increased the functional expression of CD36 mRNA after 24 hours incubation. However, a statistically significance increase was only observed in M2 macrophages incubated with 40µg/ml oxLDL (Fig 2).

To examine whether oxLDL can induce the expression of proinflammatory molecules (MCP-1 and IL-6), M2 macrophages were incubated with 1 or 40µg/ml oxLDL. As shown in Fig 3, both 1 and 40µg/ml induce the expression of MCP-1 mRNA. In contrast, IL-6 was only significantly induced in M2 macrophages incubated with 40µg/ml oxLDL. This observation supports the view that macrophage activation depends on the stimuli

present in the micro-environment. Next, the possible outcome of proinflammatory molecules expression was investigated. As mentioned earlier, CD36 is not downregulated but rather upregulated upon oxLDL treatment. This observation led Han & Pak to suggest that macrophage accumulation of oxLDL, due to unregulated uptake might lead to apoptosis-like cell death (Han & Pak, 1999). In line with this suggestion CD36 mRNA expression was significantly increased upon oxLDL treatment (Fig 2). This could be expected to result in cells taking up more oxLDL, and thus subsequent pronounced apoptosis. As shown in Fig 4, apoptosis was observed in M2 macrophages incubated with either 1 or 40µg/ml oxLDL, while thapsigargin (a positive control) induced greater apoptosis. Han *et al.*, observed that oxLDL at concentrations lower than 100µg/ml promotes growth and survival of macrophages (Han & Pak, 1999). Clearly, this is contradictory to the observations in the present study (i.e. oxLDL at 1-40µg/ml concentrations could induce apoptosis in M2 macrophages). The reasons for these contradictory reports might be due to concentration and oxidation process of oxLDL as well as the phenotypic characteristics of the macrophage. To further assess the physiological relevance of the findings obtained from apoptosis assay, MTS assay show that oxLDL decrease M2 macrophage viability after 24 hours of incubation (Fig 5). In conclusion, the findings of this article suggest that oxLDL in addition to its reported effects in resident macrophages, could also induce the expression of proinflammatory molecules and apoptosis in M2 macrophages. Taking into consideration that M2 macrophages have been proposed as an appropriate therapeutic target for type 2 diabetes (Odegard & Chawla, 2008) and also the importance of resident macrophages in inflammation and atherosclerosis (Martinet & Kockx, 2001; Seimon *et al.*, 2009), the findings presented here could enhance our understanding of the diverse effects of oxLDL, particularly in the context of obesity, type 2 diabetes and the metabolic syndrome.

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