Oxidized LDL Mediated Upregulation of ADAMTS-4 in Monocytes/Macrophages Involves The ROS-NFκB Pathway

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Research Article

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Abstract

ADAMTS-4 is a protease enzyme which involves in vascular remodeling and atherosclerosis. It was found to be upregulated in macrophages seen in atherosclerotic lesions. The aim of this study was to investigate the expression and regulation of ADAMTS-4 in oxLDL induced human monocytes/macrophages system. PBMCs isolated from human blood(hPBMCs), treated with oxLDL (50µg/ml) were used as the model system for the study. mRNA and protein expressions were studied by qRT-PCR, ELISA, and western blot analysis. ROS production and cell viability were determined by fluorescence imaging and MTT assay respectively. In the presence of oxLDL, monocytes get differentiated into macrophages, which were confirmed by the increased expression of CD-36, b- D glucuronidase activity and by the morphological changes. OxLDL increased the mRNA and protein expression of ADAMTS-4 and TIMP-3 in monocytes/ macrophages. A significant increase in the mRNA and protein expression of TNF- α was also observed in oxLDL treated cells compared to untreated control. In the presence of NAC, the ROS scavenger, the production of NF κ B and ADAMTS-4 was decreased significantly. Our study suggests that oxLDL significantly upregulated the expression of ADAMTS-4 in the monocyte/macrophage system. OxLDL mediated upregulation of ADAMTS-4 in hPBMCs involves TNF- α and ROS- NF κ B pathway.

Introduction

Cardiovascular diseases are the leading cause of death all over the world. Atherosclerosis is characterized by the complete or partial blockage of medium and large-sized arteries by plaque formation [27]. It is a chronic inflammatory disease with a multifactorial origin. The major risk factors include obesity, sedentary lifestyle, alcohol consumption, smoking, high fat diet, inflammation, etc. [21]. These risk factors lead to an increase in blood LDL level and immune cell activation [11]. LDL and activated immune cells like monocytes and T-Lymphocytes cause endothelial damage and lead to endothelial dysfunction [5, 31, 39]. Activated monocytes enter the arterial intima by a process called extravasation. LDL gets oxidized under the highly reduced environment in the arterial extracellular matrix (ECM) [38, 44]. The monocytes get differentiated into macrophages and produce several pro-inflammatory cytokines, chemokines, etc., and thus initiate an inflammation cascade. Macrophages express a number of lipid scavenging receptors including CD36 [5, 22]. Oxidized LDL binds to the LDL scavenging receptors and is engulfed by the macrophages. The macrophages gradually become foam cells and they form the core of the lipid-rich plaque [10]. The inflammatory molecules formed in various steps leading to the activation and migration of smooth muscle cells from the media layer to the intima of the arterial wall. In intima, smooth muscle cells (SMCs) get proliferated and produce many matrix components like aggrecan, versican, collagen, elastin, etc, which form the outer cap of the plaque. The activated cells also produce several proteases which degrade the matrix components [32]. Matrix metalloproteinases (MMPs) and A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) family of proteinases cleave ECM components like aggrecan, collagen, elastin, versican, etc [26]. The progress of atherosclerosis is

associated with an increase in the expression of matrix-degrading proteinases, which play a key role in the development and rupture of plaque [40].

ADAMTS family of matrix-degrading enzymes belongs to the M12 sub-family of matrix proteinases, consists of 19 members with various substrate specificities [2, 15]. They are known as metzincin as they need Zn^{2+} for their activity [17]. The protein consists of a signal peptide domain at the N-terminal, followed by a pro-domain, a catalytic domain, a disintegrin-like domain, thrombospondin motif, cysteine-rich domain, spacer region, and a number of thrombospondin repeats. These enzymes have different substrate specificities based on their C-terminal domain and they can cleave a number of substrates like aggrecan, syndecan, versican, brevican, biglycan, etc [2, 17, 23]. Hence they have a key role in physiological conditions like embryonic development, menstrual cycle, as well as pathological conditions including cancer, atherosclerosis, arthritis, etc [3, 17].

Among ADAMTS members, ADAMTS-1, 4, 5, 8, and 9 were reported to have role in atherosclerosis [4]. ADAMTS-4 is the major aggrecanase, also known as aggrecanase I. ADAMTS-4 has found to be expressed in macrophages-rich areas in atherosclerotic plaques. It was also found co-localized with VSMCs in atherosclerosis [35, 36]. In high-fat diet-fed ADAMTS $-/-$ mice, intra-plaque macrophage number decreased by reducing cell proliferation and increasing apoptosis [18]. ADAMTS-4 is reported to enhance atherosclerosis by promoting monocyte migration, differentiation, proliferation, and survival via promoting the production of pro-inflammatory cytokines such as GM-CSF, CCL2, and CXCL12 [18]. ADAMTS-4 cleaves versican, one of the major structural components of vascular ECM [37, 43]. Wagsater et al (2008) reported that cytokines like TNF- α and IFN- γ induce ADAMTS-4 production in PMA differentiated THP-1 macrophages. TIMP-3 is the potent endogenous tissue inhibitor of ADAMTS-4 that binds to the C-terminal thrombospondin domain other than the catalytic domain and blocks the substrate-binding [12]. Monocytes are differentiated into macrophages in the vessel wall in response to a number of signaling molecules like cytokines and chemokines and play a key role in the initial steps of atherosclerotic plaque formation [49]. Hyperlipoproteinemia is a major risk factor for the development of atherosclerosis. LDL undergoes modification in the vessel wall and oxLDL uptake by macrophage is thought to be one of the earliest events in the nascent plaque and results in foam cell formation [49, 51]. The extracellular matrix-degrading proteinases produced by foam cells play an important role in plaque vulnerability [33].

But there is no data available on the effect of oxLDL on the expression and regulation of ADAMTS-4 in monocyte/macrophage. ROS formed during mo-m ϕ differentiation act as a signaling molecule and it can induce activation of NF κ B [30]. NF κ B is reported to regulate the expression of ADAMTS-4 in nucleus pulposus cells[13]. Hence we investigated the role of oxidized LDL on the expression of ADAMTS-4 in monocytes/macrophages (mo-m ϕ) and the role of ROS using oxLDL- treated human PBMCs as the model system.

Materials And Methods

Preparation of oxLDL

Serum LDL was precipitated using Heparin-MnCl₂ solution. Isolated LDL treated with 10µM CuSO₄ for 24h at 37°C for oxidation [6]. After oxidation, it was dialyzed against 1X PBS containing 0.05M EDTA for 48hrs. The degree of oxidation was measured in terms of thiobarbituric acid reactive substances (TBARS) using malondialdehyde as standard. The level of TBARS was compared to native LDL [6].

PBMCs isolation and treatment

PBMCs isolated from voluntary donors were used for the study with approval from the human ethical committee, University of Kerala (ULECRIHS/UOK/2018/25). The cells were isolated by density gradient centrifugation using Histopaque-1077, washed with 1X PBS, and were resuspended in RPMI-1640, and seeded onto collagen I coated culture plates [34]. After 4hrs incubation, unattached cells were removed and attached cells were treated with oxLDL. The cells and culture supernatant were used for mRNA and protein expression studies.

MTT assay

The effect of oxLDL on cell viability was determined by using 3-(4, 5 Dimethylthiazol-2-yl) – 2,5 diphenyltetrazolium bromide (MTT) assay [7]. For MTT assay cells were seeded in 96 well plates and at 70% confluency, the cells were treated with different concentrations of oxLDL for 48 hrs. MTT solution (5mg/ml) was added and incubated for 4 hrs at 37°C. Then MTT solvent (Isopropanol) was added and read the absorbance at 570nm.

β-D- Glucuronidase assay

The cells were harvested and lysed in citrate buffer (0.1M, pH 4.6) containing 0.2% Triton X-100. The cell lysate was used to assay the β-D- glucuronidase enzyme using para nitrophenyl β-D glucuronide (N1627, Sigma Aldrich) as substrate at 37°C. The p-nitrophenol formed was determined by measuring absorbance at 405nm [14].

ELISA

ELISA was performed by the method of Engvall and Perlmann(1971). Samples were coated in a 96-well plate and kept overnight at 37°C. Then washed the wells with 1X PBS and incubated with primary antibodies of TNF-α (NBP1-19532, Novus Biologicals), ADAMTS-4 (AV41556, Sigma Aldrich), and TIMP-3(SAB4502973, Sigma Aldrich) at room temperature for 2hrs. Unbound antibodies were washed with 1X PBS- Tween20 and incubated with a secondary antibody. The color was developed using O-Phenylenediamine (P8787, Sigma Aldrich) in citrate phosphate buffer (pH 5) and H₂O₂ and measured at 490nm.

qRT- PCR (quantitative Real-Time - PCR)

Total RNA was isolated using Trizol reagent (1596018; Invitrogen) and converted into cDNA with Superscript first-strand cDNA synthesis Kit (1708891; Biorad) according to manufacturer's instruction. Real-time PCR was performed using Syber green (1725121; Biorad) with Eppendorff realplex2 master cycler. The sample was used for real-time PCR of genes ADAMTS-4, TIMP-3, CD-36, TNF- α , and GAPDH with specific primers. Samples were tested in triplicates. The results are expressed as fold changes to control. Primer sequences are given as follows: ADAMTS-4 Forward, CAAGGTCCCATGTGCAACGT., ADAMTS-4 Reverse, CATCTGCCACCACCAGTGTCT., TIMP-3 Forward, GGACACTAACTCTTCCCAGATG., TIMP-3 Reverse, CCAGGAGGATAGTTCCCAATAAA., TNF- α Forward, TGCACTTTGGAGTGATCGGC., TNF- α Reverse, ACTCGGGGTTTCGAGAAGATG., CD-36 Forward, TCACTGCGACATGATTAATGGTACA., CD-36 Reverse, ACGTCGGATTCAAATACAGCATAGAT., GAPDH Forward, ACAACTTTGGCATTGTGGAA., GAPDH Reverse, GATGCAGGGATGATGTTCTG.

Detection of cellular ROS

Cellular ROS production was detected using DCFDA (6883, Sigma Aldrich), a fluorescent dye, and images were taken by fluorescent microscopy. The isolated PBMCs were treated with oxLDL for 48hrs and untreated cells served as control. The cells were incubated with DCFDA in 1X PBS for 45min, washed with 1X PBS, and observed under a fluorescent microscope (Zeiss Axio Vert.A1). ROS generation was detected by the oxidation of DCFDA to green fluorescence [45].

Immunoblot analysis

Immunoblot was performed to determine the level of NF κ B p65 and ADAMTS-4. The cell lysate was mixed with reducing sample buffer and kept at 95°C for 10 min. Then samples were run on 10% PAGE gel [19]. After electrophoresis, the proteins were transferred to a nitrocellulose membrane using the wet transfer technique. The membrane was incubated with primary antibodies of NF κ B p65 (#8242, Cell Signaling Technology), ADAMTS-4 (AV41556, Sigma Aldrich), and β Actin (#4970, Cell Signaling Technology) at 4°C overnight and was incubated with Anti-rabbit secondary antibody (#7074, Cell Signaling Technology) for 2h at RT [41]. After washing, the membrane was incubated with enhanced chemiluminescence (ECL) reagent (1705060, Bio-Rad). The positive immunobands were quantified with ChemiDoc XRS+ Imager (Bio-Rad) and compared to control.

Statistical analysis

Each experiment was performed at least thrice. Data were expressed in Mean $\pm$ SEM (standard error of the mean) and were analyzed by Graph Pad Prism version 5.01. The significance of the difference between the two groups was determined by Student's t-test, and multiple groups were determined by one-way analysis of variance (ANOVA).

Results

1. Effect of oxLDL on cell viability

MTT assay was conducted to check the viability of cells. Isolated hPBMCs were maintained in culture in the presence of different concentrations of oxLDL (10, 20, 50, 100, 150µg/ml) for 48 hrs. The cells were incubated with MTT reagent and the formation of formazan was measured at 540nm. The cells showed $\geq 80\%$ viability in presence of oxLDL concentration up to 150µg/ml (Fig.1).

2. Confirmation of monocyte-macrophage differentiation in the presence of oxLDL

Isolated hPBMCs were incubated with oxidized LDL at different time intervals (24, 48, and 72hrs). The cells were harvested and analyzed for the β -D glucuronidase enzyme activity, which is used as a marker for monocyte-macrophage differentiation. The results showed that there was a significant increase in the β -D glucuronidase enzyme activity in oxLDL treated cells compared to untreated control cells (Fig2a). mRNA expression of CD36, the scavenger receptor expressed by the macrophages was studied by qRT-PCR. There was a significant increase in CD36 mRNA expression in oxLDL treated cells compared to control (Fig2b). The morphology of cells showed characteristic changes during mo-m ϕ differentiation when observed under phase-contrast microscope (Zeiss Axio Vert.A1) (Fig2c&d).

3. Effect of oxLDL on the production of ADAMTS-4 and TIMP-3

To analyze whether the exposure of oxLDL to monocytes has any effect on the production of ADAMTS-4 and TIMP-3, isolated hPBMCs were treated with different concentrations of oxLDL (25, 50, 100, 150µg/ml) for 48hrs. The culture supernatants were analyzed for ADAMTS-4 and TIMP-3 by ELISA using specific antibodies. Our results showed that the production of ADAMTS-4 was increased progressively with an increase in the concentration of oxLDL up to 100µg/ml (Fig3a). The production of its endogenous inhibitor TIMP-3 was increased on oxLDL treatment compared to control and decreased with the increase in oxLDL concentration (Fig3b).

4. Expression of ADAMTS-4 and TIMP-3 during monocyte-macrophage differentiation

Since ADAMTS-4 is localized in macrophage-rich regions of atherosclerotic plaque, we have studied the production of ADAMTS-4 during mo-m ϕ differentiation. To study the expression of ADAMTS-4 and TIMP-3, hPBMCs were incubated in presence of oxLDL (50µg/ml) for different time intervals (24, 48, and 72hrs). Culture supernatants and cells were analyzed for ADAMTS-4 and TIMP-3 by ELISA. The result showed that ADAMTS-4 secretion increased during the mo-m ϕ differentiation compared to untreated control cells (Fig 4a). The mRNA expression of ADAMTS-4 increased in oxLDL treated cells compared to untreated control cells (Fig 4b).

Production of TIMP-3, the endogenous tissue inhibitor of ADAMTS-4 was increased by oxLDL treated cells when compared to control at 24hrs and 48hrs (Fig 4c). qRT-PCR result also indicates increased mRNA expression of TIMP-3 by oxLDL compared to control (Fig 4d).

5. Effect of oxLDL on the production of TNF- α

Previous reports indicated that TNF- α induces the production of ADAMTS-4 in human osteoarthritic chondrocytes and THP-1 macrophages [46, 48]. In our study oxLDL significantly upregulated the production of ADAMTS-4 in the mo-m ϕ system. Further to check whether the upregulation of ADAMTS-4 by oxLDL is mediated through TNF- α , we examined the effect of oxLDL on the production of TNF- α in hPBMCs. The cells were treated with different concentrations of oxLDL (25, 50, 100, and 150 μ g/ml) for 48hrs and the results showed that the production of TNF- α increased with an increase in the concentration of oxLDL (Fig 5a). The mRNA expression of TNF- α was also examined using oxLDL at 50 μ g/ml concentration. A significant increase in the production of TNF- α mRNA was observed in oxLDL treated cells compared to untreated control (Fig 5b). These results indicate that oxLDL induced the upregulation of TNF- α in hPBMCs, which may, in turn, induce the production of ADAMTS-4.

6. ROS- NF κ B mediated up-regulation of ADAMTS-4 in mo-m ϕ .

OxLDL induces the production of ROS in macrophages. This was confirmed by DCFDA staining and our result showed that in the presence of oxLDL, the cells produce more ROS which was indicated by the enhanced green fluorescence compared to untreated control cells (Fig.6a&6b). To study the role of ROS on oxLDL mediated production of ADAMTS-4 during monocyte-macrophage differentiation, PBMCs were pre-treated with 2mM N- Acetylcysteine (NAC) and maintained in presence of oxLDL for 48hrs. The cell lysate and culture supernatant were analyzed for the production of ADAMTS-4 using western blot and ELISA respectively. Production of NF κ B was analyzed by western blot. Our results indicated that in the presence of NAC, the ROS scavenger, the production of NF κ B was decreased compared to oxLDL treated cells (Fig6c). Since NF κ B is the known transcription factor of ADAMTS-4, the production and secretion of ADAMTS-4 by macrophages was also decreased in the presence of NAC (Fig 6d &6e). Our results indicated the ROS- NF κ B mediated up-regulation of ADAMTS-4 in oxLDL treated monocyte/macrophages.

Discussion

Hyperlipidemia is one of the major risk factors of atherosclerosis. Oxidized forms of lipoproteins initiate plaque formation by the activation of monocytes leading to endothelial dysfunction and foam cell formation [8, 51]. Macrophages and foam cells express a number of proteinases involved in ECM remodeling and atherosclerotic plaque rupture [32, 33]. ADAMTS is a group of matrix-degrading enzymes secreted by the cells associated with plaque and reported to be involved in vascular remodeling and atherosclerosis [37]. ADAMTS-4 and ADAMTS-8 were seen in macrophage-rich areas of human atherosclerotic plaques and ADAMTS-4 was upregulated during the development of atherosclerotic plaque in mice [46]. But the role of oxLDL on the production and regulation of ADAMTS-4 is poorly understood. OxLDL mediates monocyte activation and differentiation by inducing the expression of scavenger receptors, cytokines, etc [5]. In our study also the presence of oxLDL activated monocytes and were differentiated into macrophages, which was confirmed by the increased expression of LDL scavenger receptor CD-36 and increased β -D glucuronidase activity. A significant change in the morphology of cells was also seen in the presence of oxLDL compared to untreated control cells.

Here, our study is focused on the expression and regulation of ADAMTS-4 during mo-mφ differentiation using oxLDL activated human peripheral blood mononuclear cells (hPBMCs) as a model system. Our results showed that on treatment with different concentrations of oxLDL, production of ADAMTS-4 by hPBMCs was significantly increased when compared to control. During the differentiation process, we obtained a significant increase in mRNA expression and secretion of ADAMTS-4 in oxLDL treated hPBMCs compared to control, indicating the upregulation of ADAMTS-4 during oxLDL induced mo-mφ differentiation. A previous report showed that THP-1 monocytes express ADAMTS-4 and its expression was significantly upregulated on PMA stimulation [47]. Another study also reported that ADAMTS-1, 4, and 5 expressions were induced during PMA mediated monocyte-macrophage differentiation of THP-1 cells [4]. The mRNA expression of ADAMTS-4 was increased in THP-1 macrophage on stimulation with TNF-α and IFN-γ [46]. TNF-α is reported to induce the expression and activity of ADAMTS-4 in human osteoarthritic chondrocytes also [48]. In this study oxLDL significantly upregulated the production of TNF-α, which may, in turn, result in the upregulation of ADAMTS-4. Here, oxLDL mediated monocyte activation and subsequent differentiation to macrophages would have initiated the signaling pathway leading to the upregulation of ADAMTS-4.

TIMP-3 is the endogenous inhibitor of ADAMTS-4. It inhibits ADAMTS-4 activity by binding to the C-terminal domain of ADAMTS-4 with its N-terminal [16, 42]. Our result showed that TIMP-3 production was also significantly upregulated on treatment with oxLDL at lower concentrations and no further upregulation was noticed at higher concentrations. TIMP-3 mRNA and protein expression were also increased initially during mo- mφ differentiation. The level of TIMP-3 in healthy tissues may maintain the homeostatic balance of matrix turnover by inhibiting catabolic enzymes [20]. TIMP-3 is also a TACE (TNF-α converting enzyme) inhibitor, which regulates the activation of the pro-inflammatory cytokine, TNF-α [24, 28]. This reveals the regulatory role of TIMP-3 in inflammatory diseases like atherosclerosis.

A Previous study has shown the effect of oxLDL on the induction of ROS in THP-1 macrophages [25] and here also, the production of ROS during oxLDL-induced mo-mφ differentiation was confirmed by DCFDA staining. OxLDL induces the nuclear translocation of NFκB and intracellularly generates reactive oxygen species, which plays an important role in macrophage formation [8]. NFκB is reported to be one of the key transcription factors of ADAMTS-4 in leptin-induced human articular chondrocytes [50]. Ahamed et al showed IL-1 induced ROS and adaptor proteins mediated ADAMTS-4 expression by the activation of NFκB in human chondrocytes. In the present study, we demonstrate that N- Acetyl Cysteine (NAC), the ROS scavenger could downregulate NFκB production in oxLDL treated mo-mφ and it results in the downregulation of the production and secretion of ADAMTS-4. These results indicate that oxLDL upregulated the production of ADAMTS-4 through ROS- NFκB pathway in the mo-mφ system. OxLDL plays an important role in atherogenesis. Macrophage scavenger receptors bind and internalize oxLDL leading to the formation of foam cells, which are part of atherosclerotic plaque. A number of ECM remodeling enzymes are produced by foam cells and they play important role in plaque rupture [33]. In our study oxLDL significantly upregulated ADAMTS-4 in mo-mφ, indicating that ADAMTS-4 might be playing a role in atherosclerotic plaque rupture.

Declarations

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Competing Interests

Authors have no competing interests to declare that are relevant to the content of this article.

Author Contributions

Aswani. S.S and Saja. K conceptualized the study and designed the experiments, analyzed the data, and interpreted the results of the experiments. Aswani. S .S prepared the figures and drafted the manuscript. Aswani.S. S, Mithra. S. Mohan and Aparna N.S performed the experiments. Saja. K and P. T. Boban reviewed and edited the manuscript. All authors read and approved the manuscript.

Data availability

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Ethics Approval

Experiments in this research were reviewed and approved by the Human ethical committee, University of Kerala (ULECRIHS/UOK/2018/25).

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Figures

Figure 1

MTT assay: hPBMCs were maintained in culture with oxLDL for 48hrs. The cells were incubated with MTT reagent (5mg/ml) for 3 hrs. Formazan crystals were dissolved in DMSO and the absorbance was measured at 540nm. Results given are the average of triplicate experiments and expressed as a percentage of control $\pm$ SEM.

Figure 2

Effect of oxLDL on mo-mφ differentiation: Isolated hPBMCs were maintained in culture with oxidized LDL (50μg/ml) for different time intervals in presence of 5% CO₂ at 37 °C. (a) β-D glucuronidase activity during mo-mφ differentiation: The cell lysate was analyzed for β- D glucuronidase activity. (b) CD36 mRNA expression during mo-mφ differentiation: mRNA isolated was analyzed for CD36 expression. The mean values of three experiments, presented as mean and SEM and analyzed in one-way ANOVA, ***p< 0.001 and **p< 0.01 compared to control. Morphological changes by microscopy: The cell morphology of oxLDL treated cells (d) was analyzed under phase-contrast microscope (20X) and compared with untreated control cells (c).

Figure 3

The effect of oxLDL on the expression of ADAMTS-4 and its endogenous inhibitor TIMP-3: Isolated PBMCs were maintained in culture with different concentrations of oxidized LDL (25, 50, 100, and 150 µg/ml) for 48hrs in the presence of 5% CO₂ at 37°C. Culture supernatant was analyzed for (a) ADAMTS-4 and (b) TIMP-3 by ELISA. The results are the mean values of three experiments, presented as mean and SEM and analyzed in one-way ANOVA. ***P < 0.001 compared with control.

Figure 4

ADAMTS-4 and TIMP-3 production during mo-mφ differentiation: Isolated hPBMCs were maintained in culture with oxidized LDL (50µg/ml) for different time intervals in presence of 5% CO₂ at 37 °C. Culture supernatants and cells were collected at different time intervals were analyzed for ADAMTS-4 (a) and TIMP-3(c) by ELISA. The mRNA expression of ADAMTS-4 (b) and TIMP-3 (d) were analyzed by qRT-PCR. The results are the mean values of three experiments, presented as mean and SEM and analyzed in one-way ANOVA, ***p < 0.001, **p < 0.01, and *p< 0.05 compared to control.

Figure 5

Effect of oxLDL on the production of TNF-α: Isolated PBMCs were maintained in culture with different concentrations of oxidized LDL (25, 50, 100, and 150 µg/ml) for 48hrs in the presence of 5% CO₂ at 37°C. Culture supernatant was analyzed for TNF-α by ELISA (a). Isolated hPBMCs were maintained in culture with oxidized LDL (50µg/ml) for 4hrs in the presence of 5% CO₂ at 37 °C. mRNA isolated from the cells was analyzed by qRT-PCR (b). The results are the mean values of three experiments, presented as mean and SEM and analyzed in one-way ANOVA. ***P < 0.001, **P < 0.01 and *P < 0.05 compared with control.

Figure 6

Effect of N- Acetylcysteine on the production of ADAMTS-4: Isolated PBMCS were maintained in culture in presence of oxLDL (50μg/ml) and NAC (2mM) for 48 hrs. Untreated cells served as control. The cells were treated with DCFDA and ROS-producing cells showed green fluorescence **(a)** Control **(b)** O_xLDL. The cells were analyzed for NFκB by western blot **(c)** the culture supernatant and cell lysate was analyzed for ADAMTS-4 by ELISA **(d)** and western blot **(e)** respectively. The results given are the average of triplicate

experiments $\pm$ SEM and analyzed in one-way ANOVA. *** $p < 0.001$ compared compared with control, ### $p < 0.001$ compared with oxLDL treated group.

Supplementary Files

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