Redox Biology 2 (2014) 732–738

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

# Potential role of proteasome on c-jun related signaling in hypercholesterolemia induced atherosclerosis

Erdi Sozen, Betul Karademir, Burak Yazgan, Perinur Bozaykut, Nesrin Kartal Ozer*

*Department of Biochemistry, Faculty of Medicine, Genetic and Metabolic Diseases Research Center (GEMHAM), Marmara University, 34668 Haydarpasa, Istanbul, Turkey*

ARTICLE INFO

*Article history:*
Received 26 December 2013
Received in revised form 21 February 2014
Accepted 21 February 2014

*Keywords:*
Atherosclerosis
Hypercholesterolemia
Proteasome
JNK1
AP-1
Vitamin E

ABSTRACT

Atherosclerosis and its complications are major causes of death all over the world. One of the major risks of atherosclerosis is hypercholesterolemia. During atherosclerosis, oxidized low density lipoprotein (oxLDL) regulates CD36-mediated activation of c-jun amino terminal kinase-1 (JNK1) and modulates matrix metalloproteinase (MMP) induction which stimulates inflammation with an invasion of monocytes. Additionally, inhibition of proteasome leads to an accumulation of c-jun and phosphorylated c-jun and activation of activator protein-1 (AP-1) related increase of MMP expression. We have previously reported a significant increase in cluster of differentiation 36 (CD36) mRNA levels in hypercholesterolemic rabbits and shown that vitamin E treatment prevented the cholesterol induced increase in CD36 mRNA expression. In the present study, our aim is to identify the signaling molecules/transcription factors involved in the progression of atherosclerosis following CD36 activation in an *in vivo* model of hypercholesterolemic (induced by 2% cholesterol containing diet) rabbits. In this direction, proteasomal activities by fluorometry and c-jun, phospo c-jun, JNK1, MMP-9 expressions by quantitative RT-PCR and immunoblotting were tested in aortic tissues. The effects of vitamin E on these changes were also investigated in this model. As a result, c-jun was phosphorylated following decreased proteasomal degradation in hypercholesterolemic group. MMP-9 expression was also increased in cholesterol group rabbits contributing to the development of atherosclerosis. In addition, vitamin E showed its effect by decreasing MMP-9 levels and phosphorylation of c-jun.



## Introduction

Atherosclerosis is a chronic inflammatory disease which is associated with the presence of fatty plaques in the arterial wall [1]. During atherosclerosis, smooth muscle cells become activated by oxLDL, start to proliferate, and migrate into the intima of the arterial wall where they form foam cells [2]. Many studies have shown that increase of oxLDL have significant roles in the induction of oxidative stress related changes [3]. Hypercholesterolemia and increased oxidative stress have been involved in the development of atherosclerosis [4,5]. In addition, plasma levels of oxidized LDL and malondialdehyde (MDA)-modified LDL are related to each other which are also known as parameters of oxidative stress in acute and stable coronary artery disease [6]. Meanwhile alpha tocopherol, the most active form of vitamin E, shows its protective effects by inhibiting smooth muscle cell proliferation [7,8] and reduction of scavenger receptor CD36 expression in hypercholesteromic rabbits [9].

During atherosclerosis, the low density lipoprotein (LDL)-induced signaling pathway is stimulated following the uptake of cholesterol into the cell *via* oxLDL including mitogen-activated protein kinase (MAPK), JNK, c-jun and AP-1, known as a transcription factor which plays a key role on MMP expression [10]. In macrophages, supplementation with oxLDL give rise to recruitment of Lyn and activation of JNKs, known as stress-activated protein kinases, in a CD36-dependent manner. Studies using JNK inhibitors demonstrated a significant decrease in the uptake of oxLDL and foam cell formation [11]. The transcription factor AP-1 is a dimer protein, composed of basic region-leucine zipper (bZIP) proteins that belong to the Jun and Fos families [12]. Oxidative activation of JNK1 promotes the phosphorylation of c-jun and leading to the transactivation of AP-1-regulated genes [13].

Proteasomes are very complex machines that are involved in the proteolytic degradation of proteins for the regulation of cell homeostasis which includes "quality control" of newly synthesized proteins (ERAD), transcription factor regulation, neurodegenerative diseases, atherosclerosis and inflammatory processes [14]. Many studies show

*Abbreviations:* AP-1, activator protein-1; CD36, cluster of differentiation 36; ERAD, endoplasmic-reticulum-associated protein degradation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK, c-Jun amino terminal kinase; LDL, low density lipoprotein; MAPK, mitogen-activated protein kinase; MDA, malondialdehyde; MMP, matrix metallo proteinase; oxLDL, oxidized low density lipoprotein; TNF $\alpha$, tumor necrosis factor $\alpha$; UPS, ubiquitin-proteasome system; HPLC, high-performance liquid chromatography; TBA, thiobarbituric acid.

* Corresponding author.
*E-mail address:* nkozer@marmara.edu.tr (N. Ozer).

2213-2317/$ - see front matter 
http://dx.doi.org/10.1016/j.redox.2014.02.007

that, oxidative stress mediated protein aggregates have a key role in the inhibition of proteasome [15]. Both components of AP-1, c-jun and c-fos are known to be degraded by the proteasome. In a recent study, UVA mediated induction of protein aggregate formation results in the inhibition of proteasome which is accompanied by phosphorylation of c-jun and increased AP-1 activity leading to an enhanced MMP mRNA expression [16]. OxLDL may also stimulate the invasion of monocytes which results in inflammation by increasing the MMP production [1]. MMPs increase matrix degrading activity and accelerate leukocyte leakage into the regions of atherosclerotic plaques. Clinical studies improve the important role of MMPs in morphogenesis, and tissue remodeling, in the progress of arthritis, atherosclerosis, asthma and tumor formation [17]. MMP-9 degrades type IV collagen, the major constituent of basement membranes, and is released by macrophages, smooth muscle cells, and endothelial cells [18]. Enhanced MMP-9 levels in plasma have been shown in cardiovascular diseases [19].

In the present study, we investigated proteasomal activity, mRNA expressions of c-jun, JNK1, MMP-9 and the protein expressions of c-jun, phospho c-jun, JNK1, MMP-9 protein expressions in rabbit aorta. Since MMP-9 is a crucial enzyme for the progression of atherosclerosis, the underlying pathway for its induction should be highlighted in hypercholesterol induced atherosclerosis. In addition, the role of vitamin E in this pathway was investigated. The results show that increased MMP-9 activity resulted in an induction of atherosclerotic development in cholesterol group rabbits. This increase was found to be correlated with a decrease in proteasomal activity which is thought to be the reason for c-jun phosphorylation following the decrease of its degradation. In this direction, vitamin E showed its protective role by decreasing MMP-9 expressions correlated with a decrease of c-jun phosphorylation. But in vitamin E treated group proteasome was not found to be directly related to this pathway.

## Material and methods

### *Animal model*

All experimental procedures were approved by the Marmara University Ethics Committee, Istanbul (protocol number 062008). Twenty-one male albino rabbits (2–3 months old) were assigned randomly to three groups which were fed with 100 g/day of vitamin E poor diet. The first control group was only fed with vitamin E poor diet. The second group was fed with vitamin E poor diet containing 2% cholesterol and the third group was fed with vitamin E poor diet containing 2% cholesterol with daily intramuscular injections of vitamin E (50 mg/kg). After 4 weeks, following overnight fasting, rabbits were anesthetized using 50 mg/kg ketamine hydrochloride and 5 mg/kg xylazine hydrochloride. The blood was taken for cholesterol, vitamin E and MDA measurements. The aortic tissues of each animal were removed and fixed in formalin for microscopic examination, in RNA stabilization reagent for quantitative RT-PCR and were rapid-frozen in liquid nitrogen, stored at −80 °C for immunoblotting experiments.

### *Measurement of cholesterol, vitamin E and MDA in Serum*

Serum cholesterol levels were determined using an automated enzymatic technique by Hitachi Modular system P800 (Roche). The levels of alpha-tocopherol were determined in serum samples by using reversed-phase high-performance liquid chromatography (HPLC) according to Nierenberg and Nann [20]. Briefly, samples were dissolved in ethanol and applied to a Waters Symmetry C18 column (5 μm, 4.6 × 250 mm). MeOH: dH2O (95:5, v/v) was used as mobile phase and detections were performed by UV detector (Waters) at 294 nm.

MDA was determined according to Wong et al. [21] with modifications of Sommerburg et al. [22] as the thiobarbituric acid (TBA) reactive substance. Phosphoric acid (440 mM), sample or MDA standard, and TBA solution (42 mM) were incubated at 100 °C for 60 min. After the incubation, samples and standards were cooled on ice and diluted 1:1 (v: v) with NaOH (0.1 M) in methanol. After then, all samples were centrifuged at 10,000*g* for 2 min. Aliquots of the derivatized samples were applied to the reversed phase HPLC and separated by isocratic elution with phosphate buffer (50 mM, pH 6.8) containing 40% (v/v) methanol. TBA–MDA complex was detected by means of fluorescence using an excitation wavelength of 525 nm and emission of 550 nm.

### *Microscopic examination*

The samples were fixed in 10% buffered formaldehyde for 4 h then dehydrated and incubated in xylol for 1 h. This process was repeated and the tissue slices were embedded in paraffin and sectioned in 5 μm thickness. Sections were stained with hemotoxylene eosin for the microscopic examination.

### *Proteasome activity analysis by fluorometry*

Aortic tissues were lysed in 1 mM dithiothreitol by vigorous shaking for 1 h at 4 °C. The lysates were centrifuged at 14,000*g* for 30 min and supernatants were incubated in 225 mM Tris buffer (pH 7.8) containing 7.5 mM MgOAc, 7.5 mM $MgCl_2$, 45 mM KCl, and 1 mM dithiothreitol. The fluorogenic peptide succinyl-LLVY-methyl coumarin was used as a substrate at a concentration of 200 M to measure chymotrypsin-like activity of the proteasome. After 30 min of incubation at 37 °C, methyl coumarin liberation was measured with a fluorescence reader (360 nm excitation/485 nm emission) and calculated using free methyl coumarin as standards. To exclude other protease activities, the selective proteasome inhibitor lactacystin with the final concentration of 20 μM was used in the reaction, and proteasome activity was calculated as the difference between the total activity and the remaining activity in the presence of lactacystin.

### *Measurement of mRNA expressions in aortic tissue with quantitative RT-PCR*

Total RNAs were isolated with RNA Midi Kit (QIAGEN) from 200 mg of rabbit aorta. Smartspec spectrophotometry (BIO-RAD) was used for the determination of purity and amount of isolated RNA. cDNA was synthesized with Transcriptor High Fidelity cDNA Synthesis kit (ROCHE) using 100 ng total RNA. Quantitative reverse transcriptase PCR was applied to cDNA by using QuantiTect PCR Sybr Green kit (QIAGEN) and Rotor Gene Q-RT PCR system (QIAGEN). The results normalized to GAPDH mRNA expression results. The sequences of primers used were

rabbit JNK1 forward, 5′-GTGCTTTTCCCAGCTGACTC-3′;
rabbit JNK1 reverse, 5′-ATCGTGTGTTCCCTTTCGTC-3′;
rabbit c-jun forward, 5′-ACAGAGCATGACCCTGAACC-3′;
rabbit c-jun reverse, 5′-TTGCTGGACTGGATGATGAG-3′;
rabbit MMP-9 forward, 5′-AACACACACGACGTCTTCCA-3′;
rabbit MMP-9 reverse, 5′-TGCAGGATGTCAAAGCTCAC-3′;
rabbit GAPDH forward, 5′-GCGCCTGGTCACCAGGGCTGCTT-3′;
rabbit GAPDH reverse, 5′-TGCCGAAGTGGTCGTGGATGACCT-3′.

### *Immunoblotting to measure protein expressions in aortic tissue*

200 mg of thoracic aorta was homogenized at 20,000 rpm for 20 s with Ultraturrax homogenizator and centrifuged at 15,000*g* for 20 min. The protein concentrations of the supernatants were determined with the Bradford protein assay. 40 μg of samples were separated with 10–12% SDS-PAGE gels, and transferred to a nitrocellulose membrane. The antibodies against JNK (AnaSpec), MMP-9