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Original Contribution

Effects of vitamin E on peroxisome proliferator-activated receptor γ and nuclear factor-erythroid 2-related factor 2 in hypercholesterolemia-induced atherosclerosis

Perinur Bozaykut [a], Betul Karademir [a], Burak Yazgan [a], Erdi Sozen [a], Richard C.M. Siow [b], Giovanni E. Mann [b], Nesrin Kartal Ozer [a,*]

[a] Department of Biochemistry, Faculty of Medicine, Genetic and Metabolic Diseases Research Center, Marmara University, 34668 Haydarpasa, Istanbul, Turkey
[b] Cardiovascular Division, British Heart Foundation Centre of Research Excellence, School of Medicine, King's College London, London SE1 9NH, UK

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ABSTRACT

Atherosclerosis and associated cardiovascular complications such as stroke and myocardial infarction are major causes of morbidity and mortality. We have previously reported a significant increase in mRNA levels of the scavenger receptor CD36 in aortae of cholesterol-fed rabbits and shown that vitamin E treatment attenuated increased CD36 mRNA expression. In the present study, we further investigated the redox signaling pathways associated with protection against atherogenesis induced by high dietary cholesterol and correlated these with CD36 expression and the effects of vitamin E supplementation in a rabbit model. Male albino rabbits were assigned to either a control group fed with a low vitamin E diet alone or a test group fed with a low vitamin E diet containing 2% cholesterol in the absence or presence of daily intramuscular injections of vitamin E (50 mg/kg). To elucidate the mechanisms by which vitamin E supplementation alters the effects of hypercholesterolemia in rabbit aortae, we measured peroxisome proliferator-activated receptor γ (PPARγ), ATP-binding cassette transporter A1 (ABCA1), and matrix metalloproteinase-1 (MMP-1) mRNA levels by quantitative RT-PCR and the expression of MMP-1, nuclear factor-erythroid 2-related factor 2 (Nrf2), and glutathione *S*-transferase α (GSTα) protein by immunoblotting. The increased MMP-1 and decreased GSTα expression observed suggests that a cholesterol-rich diet contributes to the development of atherosclerosis, whereas vitamin E supplementation affords protection by decreasing MMP-1 and increasing PPARγ, GSTα, and ABCA1 levels in aortae of rabbits fed a cholesterol-rich diet. Notably, protein expression of Nrf2, the antioxidant transcription factor, was increased in both the cholesterol-fed and the vitamin E-supplemented groups. Although Nrf2 activation can promote CD36-mediated cholesterol uptake by macrophages, the increased induction of Nrf2-mediated antioxidant genes is likely to contribute to decreased lesion progression. Thus, our study demonstrates that Nrf2 can mediate both pro- and antiatherosclerotic effects.

Atherosclerosis is characterized by the presence of fatty plaques in the arterial wall and is a leading cause of cardiovascular disease [1]. It is a progressive disease involving the proliferation of smooth muscle cells (SMCs) and accumulation of lipids in the vessel wall, leading to macrophage foam cell formation [2]. Many studies have shown that oxidative stress and inflammation play significant roles in the pathogenesis of atherosclerosis [3,4]. Vascular oxidative stress contributes to oxidation of LDL through enhanced generation of ROS by endothelial cells, SMCs, and macrophages, leading to their uptake by macrophages. Normally, native LDL is not taken up by macrophages but once oxidized it is recognized by scavenger receptors such as CD36 [5]. Thus, the balance between generation of pro-oxidants and levels of endogenous antioxidants in the vessel wall plays a significant role in the pathogenesis of atherosclerosis [6].

Abbreviations: ABCA1, ATP-binding cassette transporter A1; AP-1, activator protein-1; ARE, antioxidant-response element; CD36, cluster of differentiation 36; GSTα, glutathione *S*-transferase α; HDL, high-density lipoprotein; HNE, 4-hydroxynonenal; IL-1, interleukin-1; Keap1, Kelch-like ECH-associated protein 1; iNOS, inducible nitric oxide synthase; LDL, low-density lipoprotein; LXRα, liver X receptor α; MMP-1, matrix metalloproteinase-1; NF-κB, nuclear factor κB; NOX, NADPH oxidase; Nrf2, nuclear factor-erythroid 2-related factor 2; oxLDL, oxidized low-density lipoprotein; PPARγ, peroxisome proliferator-activated receptor γ; ROS, reactive oxygen species; SOD1, superoxide dismutase 1; TNFα, tumor necrosis factor α

* Corresponding author. Fax: + 90 216 4181047.
E-mail address: nkozer@marmara.edu.tr (N.K. Ozer).

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The redox-sensitive transcription factor Nrf2 mediates cellular defenses against oxidative stress [7–10]. Oxidative and electrophilic stresses lead to Nrf2 activation and nuclear translocation, which in turn upregulates expression of antioxidant defense genes (heme oxygenase-1) and phase II detoxifying enzymes (glutathione *S*-transferase, GST, and NAD(P)H dehydrogenase quinone 1) [5,11,12]. GST upregulation is important for the redox state of the cell because GST catalyzes the conjugation of the reduced glutathione, which is a major intracellular antioxidant [11]. On the other hand, Nrf2 has been shown to regulate CD36 expression [8] and this has been reported to have proatherosclerotic effects [5,8,13,14].

PPARγ is a ligand-binding transcription factor that belongs to the nuclear receptor superfamily, which regulates expression of genes involved in lipid metabolism, inflammatory responses, and other biological processes [15,16]. PPARγ also regulates cholesterol efflux from macrophages by inducing the expression of liver X receptor α (LXRα), which in turn activates the expression of the transporter ABCA1 [1,17]. Furthermore, PPARγ activators inhibit the expression of matrix metalloproteinases MMP-1 [18,19] and MMP-9 [20] in vitro, which also contributes to the antiatherosclerotic effects of this transcription factor.

Studies have reported that vitamin E affords protection against the progression of atherosclerosis by reducing inflammatory gene expression in addition to its direct antioxidant effects [21]. Vitamin E is transported by lipoproteins in blood and thus 90% of vitamin E in serum is found in the LDL and HDL fractions, which acts to protect them from oxidation [21,22]. α-Tocopherol, which is the most active form of vitamin E, exerts protective effects by inhibiting smooth muscle cell proliferation [23,24] and downregulating monocyte recruitment [25] and scavenger receptor CD36 expression [26].

Although the mechanisms by which oxLDL regulates cellular gene expression are not fully elucidated, recent evidence suggests that transcriptional and signaling pathways mediate many biological effects of oxidized lipids [27]. Our aim was therefore to investigate cellular defenses activated by transcriptional and signaling factors associated with atherosclerosis induced by a high-cholesterol diet and to determine the effects of vitamin E on hypercholesterolemia-mediated changes in gene expression in vivo. We investigated the expression of the redox-sensitive transcription factor Nrf2, in addition to GSTα, PPARγ, ABCA1, and MMP-1, to elucidate whether cholesterol efflux is linked to cholesterol uptake by macrophages via CD36.

Material and methods

Animals and diets

All experimental procedures were approved by the Marmara University Animal Care and Use Committee, Istanbul (Protocol 062008). Twenty-one male albino rabbits (2–3 months of age) were assigned randomly to three groups, which were fed: (i) vitamin E-poor diet, (ii) vitamin E poor-diet containing 2% cholesterol, or (iii) vitamin E-poor diet containing 2% cholesterol with daily intramuscular injections of vitamin E (50 mg/kg). After 4 weeks, after overnight fasting, rabbits were anesthetized using 50 mg/kg ketamine hydrochloride and 5 mg/kg xylazine hydrochloride. Aortic tissues were kept in formalin for microscopic examination or in RNAlater stabilization buffer for PCR analysis and the remainder were stored at −80 °C for immunoblotting analysis.

Blood analysis

Serum cholesterol and α-tocopherol levels of the rabbits were determined with blood collected from the ear vein before the experiment started and from the heart after overnight fasting just before euthanasia. Serum cholesterol measurements were carried out by an automated enzymatic technique (Hitachi Modular system P800; Roche). α-Tocopherol levels were determined according to the high-performance liquid chromatography method of Nierenberg and Nann [28]. Briefly, samples were dissolved in ethanol and applied to a Waters Symmetry C18 column (5 μm, 4.6 × 250 mm); MeOH/dH_2O (95/5, v/v) was used as mobile phase and measurements were carried out at 294 nm.

Light-microscopic examination

Tissue sections were examined by light microscopy as described in our previous study [29]. Briefly, the samples were fixed in 10% buffered formaldehyde for 4 h and then dehydrated and incubated in xylol for 1 h twice, embedded in paraffin, and sectioned at 5-μm thickness. Sections were stained with hematoxylin and eosin before microscopic examination.

Chemiluminescence detection of ROS generation

Aortic tissues were dissected into 2-mm^2 pieces and added to white 96-well plates containing Krebs Henseleit buffer (in mM: NaCl 131.0, KCl 5.6, $NaHCO_3$ 25.0, NaH_2PO_4 1.0, Hepes 5.0, D-glucose 5.0, $CaCl_2$ 1.0, and $mgCl_2$ 1.0; pH 7.4) and L-arginine (100 μmol/L). Tissues were then incubated in Krebs Henseleit buffer containing lucigenin [30] (5 μmol/L) and NADPH (100 μmol/L) under dark conditions. Chemiluminescence was immediately recorded over 40 min in a microplate luminometer (Chameleon V; Hidex) at 37 °C after the addition of lucigenin. Maximal luminescence values obtained over a 10-min period were averaged for each treatment condition, and values from three different tissues with three replicates per tissue per condition were expressed as mean light units/mg protein.

Detection of mRNA in aortic tissue by quantitative reverse transcriptase PCR (RT-PCR)

Total RNA isolation was carried out using an RNA Midi Kit (Qiagen) and 250 mg of the rabbit aortae. The amount and purity of the RNA extracts were determined via Smartspec spectrophotometry (Bio-Rad). cDNA was synthesized with a Transcriptor High Fidelity cDNA synthesis kit (Roche) using 100 ng total RNA. Quantitative RT-PCR was performed using a Rotor Gene Q-RT PCR system (Qiagen) and QuantiTect PCR Sybr Green kit (Qiagen). PCR products were separated on a 2.4% agarose gel to control the product base pairs and the bands were extracted using a QIAquick gel extraction kit (Qiagen). The dsDNA concentration of the gel extractions was determined by spectrophotometry and 10^1–10^{10} dilutions were prepared for a standard curve for quantitative analysis. The samples and the standard curve were obtained simultaneously in one run, and the results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. The sequences of the primers used were rabbit PPARγ forward, 5'-CACGAAGAGCCTTC-CAACTC-3'; rabbit PPARγ reverse, 5'-TATGAGACATCCCCACAGCA-3'; rabbit ABCA1 forward, 5'-CTGGCCAGGATATTCAGCAT-3'; rabbit ABCA1 reverse, 5'-CGTCCTGCAGAAAAGATGTG-3'; rabbit MMP-1 forward, 5'-GCCCAATGGAAAGACCTACT-3'; rabbit MMP-1 reverse, 5'-CACCTTCAGCTTCTGGTTGT-3'; rabbit GAPDH forward, 5'-GCGCCT-GGTCACCAGGGCTGCTT-3'; rabbit GAPDH reverse, 5'-TGCCGAAGT-GGTCGTGGATGACCT-3'.

Immunoblotting

Thoracic aortic tissue (100 mg) was homogenized using 1/4 w/v lysis buffer at 24,000 rpm for 20 s with an Ultraturrax homogenizer and then centrifuged for 20 min. Protein amounts were determined