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].
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 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 GSTx, 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/dH2O (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-mm2 pieces and added to white 96-well plates containing Krebs Henseleit buffer (in mM: NaCl 131.0, KCl 5.6, NaHCO3 25.0, NaH2PO4 1.0, Hepes 5.0, d-glucose 5.0, CaCl2 1.0, and mgCl2 1.0; pH 7.4) and i-arginine (100 μm/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 aorta. 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 101–105 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′-CACGAGAAGCCTTCC-CAACTC-3′; rabbit PPARγ reverse, 5′-TATGGATCATCCCCACAGCA-3′; rabbit ABCA1 forward, 5′-CTGGCACCATATTCAGCAGC-3′; rabbit ABCA1 reverse, 5′-CTGCTCCACGAAAGATGTCG-3′; rabbit MMP-1 forward, 5′-GCCCAATGGAAGACACTT-3′; rabbit MMP-1 reverse, 5′-CACCTTTCGCTCTGGTGTTG-3′; rabbit GAPDH forward, 5′-GGCGCTT- GTTCACAGGCGTGGT-3′; rabbit GAPDH reverse, 5′-TGCAGAAGGT- GGTCGGCTGAGAC-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
in supernatants (Bio-Rad Bradford protein assay) and 30 μg of samples was run on SDS–PAGE and transferred onto nitrocellulose or polyvinylidene difluoride membranes. Primary antibodies against Nrf2 (Santa Cruz, sc-722), GSTα (Abcam, ab53940), MMP-1 (Millipore, MAB3307), GAPDH (Pierce, MA1-22670), or α-actin (Sigma, A2547) and horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence substrate (Cell Signaling Technology) were used. Blots were visualized using X-ray film and quantified by densitometry using ImageJ software.

Statistical analyses

Data denote measurements in aortic tissue that were obtained from three to seven rabbits. Statistical analysis was performed using Prism 4 (GraphPad) software. For determination of statistical significance of differences, one-way ANOVA was performed followed by multiple comparisons using the Bonferroni multiple comparison test. A \( p < 0.05 \) was considered statistically significant.

Results

Effects of cholesterol and vitamin E supplementation on blood serum levels

Supplementation with 2% cholesterol for 4 weeks resulted in ~30-fold increase in serum cholesterol in the cholesterol and cholesterol + vitamin E groups compared to the control group (Table 1). Intramuscular vitamin E injections increased serum vitamin E levels ~11-fold in the cholesterol + vitamin E group (Table 1). In the cholesterol group, serum vitamin E levels appeared to increase, mostly as vitamin E is a fat-soluble vitamin carried by LDL cholesterol in blood, but the values corrected for serum cholesterol levels were of a similar order of magnitude [24].

Effects of cholesterol and vitamin E supplementation on the atherosclerotic plaques of rabbit aortic tissue

Thoracic aortae stained with hematoxylin and eosin were examined by light microscopy (Fig. 1). In the control group, there was no impairment of the aorta integrity and all layers remained intact, whereas the cholesterol-fed rabbits exhibited atherosclerotic lesion formation, characterized by endothelial damage and a significant thickening of the intimal layer compared to the control group. We also observed clusters of foam cell formation due to lipid accumulation and lipid droplets between smooth muscle cells in the cytoplasm of the intimal cells. Notably, lipid accumulation and foam cell formation were detectable in the intima and media layers, and the structure of elastic fibers of the medial layer was normal in animals fed cholesterol and treated with vitamin E compared to cholesterol-fed group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum cholesterol level (mg/dl)</th>
<th>Serum vitamin E level (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 0</td>
<td>Week 4</td>
</tr>
<tr>
<td>Control</td>
<td>92.6 ± 9.8</td>
<td>79.0 ± 43.7</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>89.0 ± 24.0</td>
<td>2869.2 ± 586.6(^{\ast\ast})</td>
</tr>
<tr>
<td>Cholesterol + vitamin E</td>
<td>102.5 ± 29.4</td>
<td>2337.8 ± 926.8(^{\ast\ast})</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD.

\(^{\ast\ast}\) 0.001, 4 weeks of cholesterol and cholesterol + vitamin E vs 4 weeks of control group.

\(^{\ast\ast}\) 0.001, week 0 vs week 4 of the same group (n = 7).

Cholesterol supplementation induces ROS generation and vitamin E inhibits cholesterol-mediated increases in ROS

To detect the effects of a cholesterol-enriched diet and vitamin E treatment on ROS generation in thoracic aortic tissues, we monitored ROS generation using lucigenin-enhanced chemiluminescence (Fig. 2). The high-cholesterol diet resulted in a significant increase in lucigenin chemiluminescence, whereas ROS generation was significantly inhibited in cholesterol-fed animals administered vitamin E.

Vitamin E, but not cholesterol, modulates mRNA expression of PPARγ and ABCA1 in aortic tissue

To further investigate the effects of a cholesterol-enriched diet and vitamin E treatment in vivo, mRNA expression of PPARγ and ABCA1 was determined by quantitative RT-PCR. A 2% cholesterol diet for 4 weeks resulted in a small, but not significant, decrease in PPARγ (Fig. 3A) and ABCA1 (Fig. 3B) expression. However, a significant increase in both PPARγ and ABCA1 expression was observed in vitamin E-treated rabbits compared to the cholesterol-fed group, suggesting that changes in PPARγ and ABCA1 mRNA expression may correlate with one another.

Dietary lipid-induced hypercholesterolemia has significant effects on protein and mRNA expression of MMP-1

To gain a better understanding as to whether MMP-1 is affected in atherosclerosis, mRNA and protein expression of MMP-1 was determined. Both mRNA and protein expression increased significantly in the cholesterol-fed group (Fig. 4A and B). Vitamin E treatment inhibited the cholesterol-induced MMP-1 expression, which seems to be correlated with our findings for PPARγ expression.

Cholesterol and vitamin E induce Nrf2 protein expression in aorta

In this study, we aimed to elucidate whether Nrf2 expression is modulated in a rabbit model of atherosclerosis in vivo. As shown in Fig. 5A, both cholesterol and vitamin E significantly increased protein expression of Nrf2. In cholesterol-fed rabbits, we observed an approximately twofold increase, which was found to be three-fold in vitamin E-treated rabbits compared to the control group. The increase in the cholesterol-fed group supports our previous findings regarding CD36 levels and further suggests that vitamin E may afford protection via the antiatherogenic effects of Nrf2.

Vitamin E affords protection by GST induction in aorta from rabbits fed a high-cholesterol diet

Consistent with our finding that Nrf2 levels are increased in the vitamin E-supplemented group, GSTα protein expression was also elevated significantly in this group compared to animals fed
cholesterol only (Fig. 5B). GSTα expression was decreased in the cholesterol group, whereas vitamin E-induced upregulation of GST expression was correlated with elevated Nrf2 expression. These results suggest that vitamin E may afford protection against oxidative stress in atherogenesis via an Nrf2 redox-regulated pathway.

Discussion

A high-cholesterol diet leads to metabolic changes involving endothelial damage, an increase in extracellular matrix synthesis, smooth muscle cell proliferation, and altered antioxidant and pro-oxidant enzyme activity related to the progression of atherosclerotic lesions. To investigate the relationship between hypercholesterolemia and atherogenesis in vivo, feeding rabbits a 2% cholesterol-containing diet for 4 weeks was found to be sufficient to elicit formation of atherosclerotic lesions in a manner similar to dietary-mediated vascular changes in humans [24]. Many epidemiological studies have shown the protective effects of vitamin E against the development of atherosclerosis [31,32], due to its important roles in modulating signaling mechanisms and gene expression in addition to its radical-scavenging properties [21,32]. Vitamin E inhibits signal transduction pathways such as protein kinase C, the growth of vascular smooth muscle cells, and gene expression of CD36 and collagenase [21]. In our previous studies, vitamin E supplementation in rabbits was shown to protect against the development of atherosclerotic lesions in the aorta [23,24]. In the present study, we have further investigated the effects of a high-cholesterol diet in rabbits and vitamin E supplementation on the signaling pathways and redox-mediated transcriptional factors known to play key roles in the progression of the atherogenesis.

In our study, serum analysis showed that a high-cholesterol diet resulted in ~30-fold increase in serum cholesterol, whereas vitamin E supplementation increased its serum levels ~11-fold (Table 1). The development of atherosclerosis after 4 weeks of cholesterol feeding was confirmed by light-microscopic analysis (Fig. 1), revealing significant morphological changes associated with atherogenesis, including endothelial layer disruption, thickening of the intimal layer, lipid accumulation, and marked foam cell formation in the neointima. The analysis of serum cholesterol levels and presence of atherosclerotic lesions is consistent with this in vivo model of rabbit atherosclerosis [29]. In the cholesterol diet group receiving vitamin E supplementation, serum levels of vitamin E increased and the incidence and severity of atherosclerotic lesions

![Fig. 1. Morphology of rabbit aortae from each group. (A) Control group, (B) cholesterol group, (C) cholesterol + vitamin E group. Aortic tissue was 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 light-microscopic examination (× 20 original magnification). Images depict one representative aorta from each group (n = 3).](Image)

![Fig. 2. High-cholesterol diet induces reactive oxygen species generation in rabbit aortae. Aortic tissues were added to 96-well microtiter plates and preequilibrated with Krebs buffer and L-arginine (100 μmol/L) and then incubated in Krebs buffer containing lucigenin (5 μmol/L) and NADPH (100 μmol/L). ROS generation was measured by enhanced chemiluminescence over 40 min and expressed as mean light units (MLU) per milligram of protein averaged over 10 min. Data denote mean ± SD from each dietary group (n = 3). ***p < 0.001 vs control, ###p < 0.001 vs cholesterol.](Image)
High-cholesterol diet and vitamin E treatment affect mRNA and protein levels of MMP-1 in rabbit aortae. (A) Aortic tissue MMP-1 mRNA expression was determined by quantitative RT-PCR and normalized to GAPDH mRNA expression. Data denote mean ± SD for (A) PPARγ and (B) ABCA1. *p < 0.05 vs cholesterol (n = 5).

Fig. 3.

The oxidative modification of LDL in the arterial wall by ROS contributes to atherosclerosis [29]. These highly reactive species include superoxide, hydroxyl ion, hypochlorite, peroxynitrite, and hydrogen peroxide [33]. Accumulating evidence suggests that risk factors for atherosclerosis such as LDL can increase the production of ROS, not only in endothelial cells, but also in smooth muscle and adventitial cells [34,35]. Hypercholesterolemia stimulates the production of superoxide in vascular smooth muscle cells, leading to increased oxidation of LDL [36]. Our findings suggesting that hypercholesterolemia is associated with increased superoxide generation in aortic tissue from cholesterol-fed rabbits are consistent with the literature on vitamin E supplementation significantly inhibiting cholesterol-mediated increases in aortic ROS generation (Fig. 2). Vitamin E is likely to have reduced ROS generation by altering intracellular redox signaling pathways investigated in this study.

Reactive oxygen species and electrophilic agents interact with cysteine residues on the cytosolic binding protein Keap1 [37,38], resulting in activation of Nrf2 and upregulation of ARE-mediated gene expression both in vitro and in vivo [39–41]. We previously reported a significant increase in CD36 mRNA levels in cholesterol-fed rabbits [29], and studies by Ishii et al. of oxidized LDL-treated murine macrophages [8] provided the first evidence that activation of Nrf2 upregulates CD36 expression. In the present study, we observed an increase in Nrf2 protein expression in aortae of both cholesterol-fed and vitamin E-supplemented rabbits (Fig. 5A). The increase in Nrf2 expression in the cholesterol group suggests that Nrf2 may lead to increased lipid accumulation via upregulation of CD36 (Fig. 6), resulting in foam cell formation [10]. A recent study demonstrated that Nrf2 may control CD36 expression independent of the PPARγ pathway in both murine and human macrophages [42] and these findings are consistent with our data. We observed that Nrf2 regulates CD36 expression separately from PPARγ and may thus contribute to the development of the atherosclerosis. The correlation between elevated levels of serum cholesterol and increased expression of Nrf2 also supports a recent report that Nrf2 regulates lipid metabolism in Nrf2-deficient mice [14]. However, significantly elevated levels of Nrf2 expression in vitamin E-supplemented aortae compared to the cholesterol diet group may in part account for the protection against the development of atherosclerosis via upregulation of antioxidant defense enzymes. Nrf2 regulates the transcriptional induction of a battery of antioxidant genes including GST (Fig. 6). Vitamin E supplementation may increase the binding of Nrf2 to the ARE in the promoter region of GST, resulting in increased GST expression, and thus may partly play a protective role against cholesterol-diet-induced atherosclerosis. GST has also been shown to contribute to reactive aldehyde detoxification, and GST-mediated conjugation of glutathione is critical in neutralizing HNE in vascular cells [43]; thus GST upregulation after vitamin E supplementation may play an additional protective role against...
cholesterol-diet-induced atherosclerosis. In vitro studies have also demonstrated that GSTα protects cells against toxicity mediated by acrolein, HNE, and other aldehydes [44–46]. GSTs have also been suggested to participate in upregulation of iNOS and to regulate NO generation through activation of the NF-κB pathway [46]. In addition to regulating detoxification and the cellular redox state, the glutathione/GST system has additional roles to modulate cell-cycle control and cell proliferation, which are critical in the atherosclerotic process [47].

Our study demonstrates that a high-cholesterol diet slightly decreased PPARγ and ABCA1 mRNA expression, whereas vitamin E supplementation significantly increased these mRNA levels by approximately twofold compared to the high-cholesterol group (Figs. 3A and 3B). The correlation between PPARγ and ABCA1

Fig. 5. Effects of high-cholesterol diet and vitamin E treatment on protein levels of Nrf2 and GSTα in rabbit aortae. (A) Representative immunoblots for Nrf2 and densitometric analysis of Nrf2 protein expression relative to actin. (B) Representative immunoblots for GSTα and densitometric analysis of GSTα protein expression relative to actin. Data denote mean ± SD, *p < 0.05, **p < 0.01 vs control; #p < 0.05 vs cholesterol (n = 3).

Fig. 6. Schematic model highlighting upregulation of redox signaling in hypercholesterolemia-induced atherosclerosis and the protective role of vitamin E. Abbreviations used: ABCA1, ATP-binding cassette transporter A1; AP-1, activator protein-1; ARE, antioxidant-response element; CD36, cluster of differentiation 36; CE, cholesterol ester; GST, glutathione S-transferase; HO-1, heme oxygenase-1; HDL, high-density lipoprotein; LXRα, liver X receptor-α; Keap1, Kelch-like ECH-associated protein 1; MMP-1, matrix metalloproteinase-1; Nrf2, nuclear factor-erythroid 2-related factor 2; oxLDL, oxidized low-density lipoprotein; PPARγ, peroxisome proliferator-activated receptor γ; ROS, reactive oxygen species; RXR, retinoid X receptor; sMAF, small musculoaponeurotic fibrosarcoma.
expression supports the hypothesis that PPARγ inhibits lipid accumulation and foam cell formation through the activation of cholesterol efflux from macrophages through ABCA1 in vivo [1,27]. Reverse transport of cholesterol is mediated by the activation of LXRx via PPARγ and thus indirect activation of the ABCA1 gene by PPARγ [1]. Vitamin E may stimulate the PPARγ–LXRx–ABCA1 transduction pathway (Fig. 6), thereby preventing foam cell formation by the induction of cholesterol efflux from macrophages [17]. Several studies have shown that PPAR ligands can modulate antioxidant genes such as thioredoxin-1, glutathione peroxidase-3, γ [19,55]. Our results are consistent with these genic effect mediated, in part, by PPARγ and, furthermore, that vitamin E treatment elicits an antiatherogenic gene expression through the upregulation of PPARγ and Nrf2 activity. This inhibition might also result via activation of the transcription factor AP-1 [18]; however, further studies are required to examine the contribution of other transcription factors in vivo.

Conclusions

In summary, our findings demonstrate that a high-cholesterol diet significantly enhances lipid accumulation via Nrf2-mediated induction of CD36 expression during atherogenesis. We also provided evidence for the role of vitamin E supplementation on gene expression through the upregulation of PPARγ and Nrf2 and induction of their downstream targets ABCA1 and GSTξ, respectively, and through inhibition of MMP-1. ABCA1 upregulation results in cholesterol efflux from macrophages and inhibition of MMP-1 results in reduced inflammation. Additionally, vitamin E significantly upregulates aortic Nrf2 levels and may thereby mediate enhanced antioxidant defense through the induction of GST expression. Thus, our study establishes that Nrf2 may mediate both pro- and antiatherosclerotic effects in the development of atherosclerosis and other vascular diseases.

Acknowledgments

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