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ORIGINAL ARTICLE

Tempol attenuates atherosclerosis associated with metabolic syndrome via decreased vascular inflammation and NADPH-2 oxidase expression

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Abstract

Oxidative stress is an important factor in the generation of vascular injury in atherosclerosis. Chronic administration of fructose in rodents is able to facilitate oxidative damage. In the present study we evaluated the role of Tempol, a superoxide dismutase mimetic, on the effect of high fructose intake in apolipoprotein E-deficient (ApoE-KO) mice. Rodents were fed with fructose overload (FF, 10% w/v) for 8 weeks and treated with Tempol 1 mg/kg/day the latest 4 weeks. Tempol revert the pro-oxidant effects caused by FF, diminished lipid peroxidation and impaired vascular NADPH oxidase system through the downregulation of p47phox expression in the vascular wall. Tempol inhibited the expression of vascular adhesion molecule 1 (VCAM-1) in aorta and reduced the development of atheroma plaques. Our results indicate that tempol attenuates oxidative stress by interfering with the correct assembly of Nox2 oxidase complex in the vascular wall and is able to reduce atherosclerosis. Thus tempol represents a potential therapeutic target for preventing risk factors associated with metabolic syndrome.

Keywords: NADPH oxidase, atherosclerosis, oxidative stress, tempol

Abbreviations: ApoE-KO, ApoE-deficient mice; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; VCAM-1, vascular cell adhesion molecule-1

Introduction

Oxidative stress and associated vascular damage are mediators of vascular injury and inflammation in many cardiovascular diseases including atherosclerosis [1,2]. Extent data strongly support the hypothesis that oxidative stress, induced via activation of cell nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, plays a causative role in atherosclerosis [3–6]. Chronic administration of fructose fed (FF) is able to induce dyslipidemia, hyperglycemia, endothelial dysfunction [7] and oxidative damage [8], and previous results from our group showed that Tempol, a superoxide dismutase mimetic, administered to FF apolipoprotein E-deficient (ApoE-KO) mice decreases NADPH oxidase activity in the aortic wall [9]. NADPH oxidase is the major source of vascular reactive oxygen species (ROS) and is expressed in endothelial cells, vascular smooth muscle cells (VSMCs), fibroblasts, and monocyte/macrophages [10,11], and its activity is triggered by inflammatory mediators that induce the assembly of four cytosolic regulatory proteins (p40phox, p47phox, p67phox, and Rac2-GTPase) with the Nox2 core enzyme to stimulate superoxide formation [12]. ROS are able to regulate cellular growth (hyperplastic or hypertrophic), endothelial dysfunction, cell migration and inflammation [3], and are postulated to participate in the pathogenesis of atherosclerosis [13,14]. The role of endothelial inflammation in atherosclerosis is mediated by adhesion molecules, which are expressed by endothelial cells or circulating lymphocytes in response to several immunological stimuli [15], which mediate adhesion, migration, and accumulation of lymphocytes and monocytes [16]. Adhesion molecules, include vascular and intercellular adhesion, make up the inflammatory response [17]. Vascular cell adhesion molecule-1 (VCAM) is a cytokine-inducible member of the immunoglobulin superfamily that is expressed by arterial endothelial cells in regions predisposed to atherosclerosis and at the borders of atherosclerotic plaques [18–21]. VCAM-1 functions in combination with other adhesion molecules during chronic inflammation, activating NADPH oxidase [22]. Therefore the aim of the present study was to explore the effect of chronic administration of tempol on the molecular mechanisms involved in the activation of NADPH oxidase system and the development of atherosclerosis,

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in ApoE-KO mice fed with high-fructose diet. The expression of membrane and cytosolic NADPH oxidase subunits was quantified, and the effect of tempol on VCAM-1 expression and atherosclerotic plaque development was investigated.

Materials and methods

Ethical approval

All animals were cared for in accordance with the *Guiding Principles in the Care and Use of Animals* of the US National Institutes of Health (NIH). All procedures were approved by the Animal Research Committee of the Universidad Nacional de Cuyo (protocol approval #10089 CICUAL, School of Medical Science, Mendoza, Argentina). Male C57/BL6J ApoE-KO mice, 8 weeks of age (The Jackson Laboratories, Bar Harbor, ME) were maintained in a 22°C room with a 12-h light/dark cycle and received drinking water *ad libitum*, and were fed a standard commercial chow (GEPSA, Argentina) diet. Animals were randomly divided into two groups: control mice ($n = 12$), with free access to tap water; FF mice ($n = 12$) receiving 10% (w/v). fructose (Parafarm, Argentina) in their drinking water. After 4 weeks both group were randomized to no treatment or Tempol (1 mg/kg of body weight per day; Sigma Aldrich) in drinking water in light protected bottles, during 4 more weeks.

Biochemical determinations

After overnight fasting blood samples for glucose, triglycerides, and cholesterol determinations (GT Lab, Buenos Aires, Argentina) were taken from all animals, collected from cardiac puncture under anesthesia at the end of the experimental period. Insulin was determined by ELISA using a commercial kit (Crystal Chem, USA).

Markers of lipid peroxidation

Plasma Malondialdehyde (MDA) was determined as an indicator of lipid peroxidation in terms of thiobarbituric acid reactive substrates (TBARS) and expressed as mmol/L.

Dihydroethidium (DHE) assay

DHE or hydroethidine is a cell-permeable compound that, upon entering the cells, interacts with $O_2^{\bullet -}$ to form oxyethidium, which in turn interacts with nucleic acids to emit a bright red color detectable qualitatively by fluorescent microscope. In brief freshly isolated vascular segments from each treatment were embedded in tissue freezing medium and 6 uM criosections were mounted on cover slips. DHE in PBS to final concentration of 5 umol/l was added and incubated at 37°C in dark for 30 min. The excess of DHE was rinsed with PBS twice and mounted in cover slips using mounting media. Images were captured with a fluorescent microscope at excitation and emission wavelengths of 520 and 610 nm, respectively. For semi-quantitative analysis of ROS production, three to six images were acquired from three sections per aortic ring for each experimental condition. Images were analyzed with ImageJ 1.37v software (NIH) and changes in total fluorescence intensity were calculated relative to control.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated with the Trizol (Invitrogen) method from aortic tissue pools (6 aortas per group), 1 μg of total RNA was reverse transcribed, using random primer hexamers (Biodynamics, SRL) and M-MLV reverse transcriptase (Promega), according to the manufacturer's instructions. Real-time PCR was performed with the cDNA samples and EVA Green (GenBiTech, Argentina) by using a Rotor-Gene 6000 Series Software version 1.7 (Corbett). All samples were amplified in triplicate. The relative changes in the amount of transcripts in each sample were determined by normalizing with the actin RNA levels. The sequences of PCR primers (Invitrogen) used in this study are summarized in a (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.889295).

Western blotting

Western blot analysis was performed in aortic tissue homogenate from four animals per group using rabbit-anti VCAM antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit-anti-Nox2 (Gp91 phox, BD), mouse anti-p47phox (Santa Cruz Biotechnology, Santa Cruz, CA) and HRP-conjugated secondary antibodies (1:10 000 Jackson laboratory, US). Proteins were visualized by performing luminol-enhanced chemiluminescence. Loading of equal amounts of proteins was confirmed by reproving the membrane with an antibody against tubulin antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) or actin (1:100; Santa Cruz Biotechnology, Santa Cruz, CA). Protein expression was quantified using ImageJ 1.37v software (NIH).

Morfometric determination of atherosclerosis

Atherosclerotic plaques from six animals per group were visualized by Oil-red-O staining of lipid deposits. Quantification of atherosclerosis was performed in the aortic arch region up to the abdominal aorta by computer-assisted image analysis. Subsequently images of en face preparations of the whole mounted aorta were taken and the percentage of plaques in relation to the entire aortic surface calculated as plaque score in percent of total area using ImageJ 1.37v software (NIH).

Table I. Average plasma glucose and lipid levels in ApoE-KO mice that fed control diet or fructose diet with/without Tempol.

	Glucose (mmol/l; $n = 8$)	Insulin (nmol/l; $n = 8$)	Cholesterol (mg/dl; $n = 12$)	Triglycerides (mg/dl; $n = 12$)
Control Diet (CD)	5.1 ± 0.9	0.85 ± 0.01	321.6 ± 20.7	99.3 ± 21.5
Fructose Diet (FD)	12.2 ± 1.1***	1.3 ± 0.02***	406.0 ± 46.6	168.6 ± 20.2*
CD + Tempol	6.2 ± 1.2	1.1 ± 0.02	401.4 ± 20.6	116.8 ± 67.2
FD + Tempol	9.5 ± 0.5*	1.3 ± 0.03***	437.2 ± 53.6	174.8 ± 26.6*

Values are the mean ± SD. ***$P < 0.0001$, *$P < 0.01$ versus Control Diet

Data analysis

All values are expressed as means ± SEM. Comparisons across the four groups were analyzed using one-way ANOVA, followed by a Bonferroni's post-hoc test to determine significant differences between two groups using PrismV4 (GraphPad). Differences between groups with P value < 0.05 was considered statistically significant.

Results

Biochemical parameters

We measure fasting plasma metabolic parameters and lipid peroxidation in FF ApoE-KO mice treated with tempol. As shown in Table I, ApoE-KO mice fed on the high-fructose diet became hypertriglyceridemic, hyperinsulinemic and hyperglycemic, without affecting cholesterol levels.

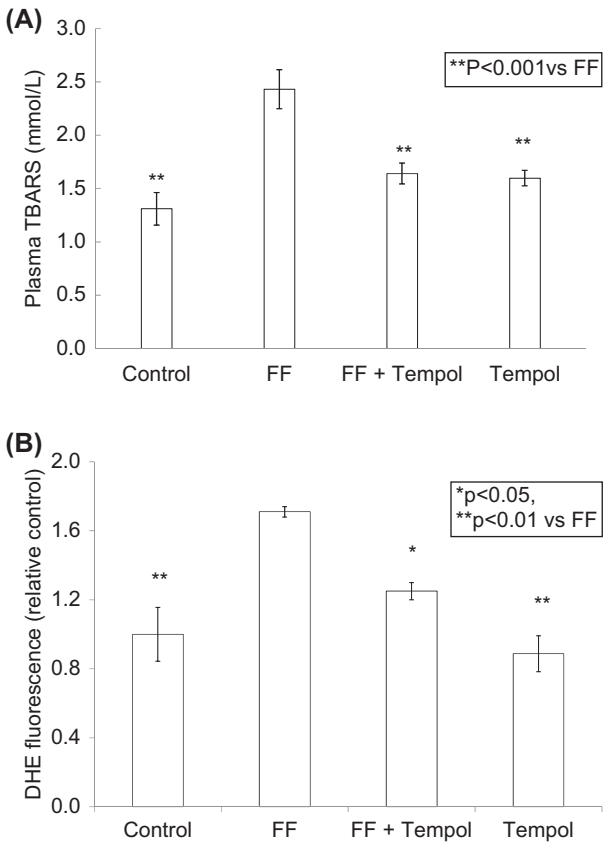

Figure 1. (A) Tempol prevents plasma lipid peroxidation. Plasma TBARs in ApoE-KO mice fed a control diet or high-fructose diet for 8 weeks treated with or without tempol. Values are mean ± SEM ($n = 8$), **$P < 0.001$ versus fructose-fed. (B) DHE staining is decreased by tempol. Fresh-frozen aortas were stained for 30 min with DHE. Results are typical of staining of sections from four different aortas. Digital scans of intimal regions of DHE stained aortas were quantified using Image J (NIH) software. Results shown are mean ± SEM. *$P < 0.05$, **$P < 0.01$ compared with high FF mice.

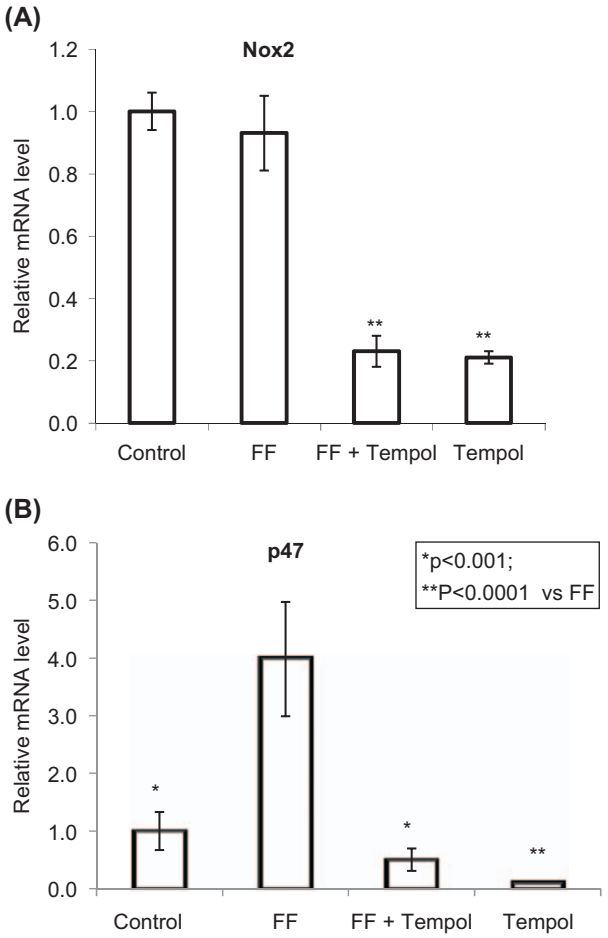

Figure 2. Tempol downregulates the expression of vascular NADPH oxidase subunits. (A) Quantitative real-time RT-PCR show decreased expression levels of Nox-2 in aortic tissue from ApoE-KO mice treated with tempol compared with FF ApoE-KO mice. (B) p47phox expression levels increase in FF ApoE-KO mice and this effect is prevented by tempol treatment. Results are means ± S.E.M ($n = 6$), from two independent experiments **$P < 0.0001$ versus FF; *$p < 0.001$ versus FF.

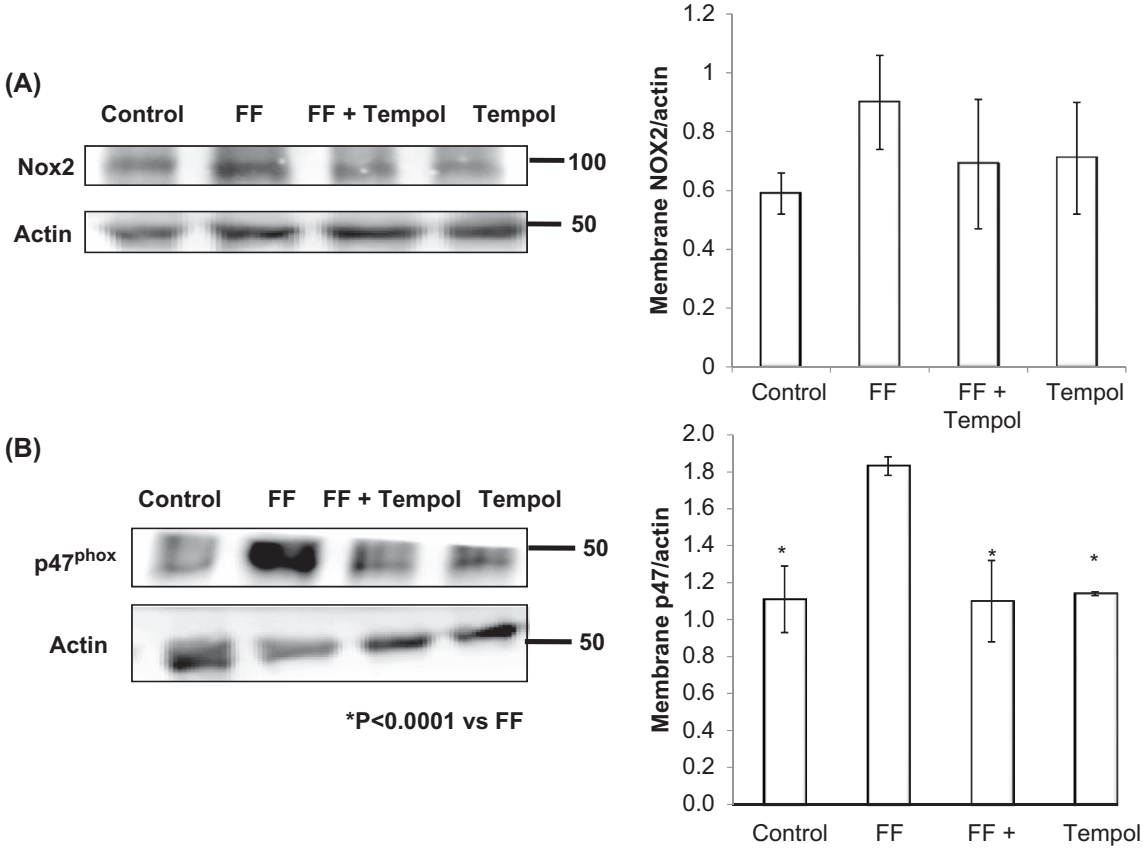

Figure 3. Expression of Nox2 (A) and p47phox (B) in membrane fractions of aortic tissue. Detection of Nox-2 and p47phox was determined by Western blot on membrane fraction from ApoE-KO mice fed with control diet (control), high-fructose diet (FF), FF treated with tempol (FF + tempol), or Tempol alone. Actin protein was used as control for relevant quantification. Results are means ± SEM, from two independent experiments (Density plot for $n = 2$ experiments). *$P < 0.05$ compared with FF.

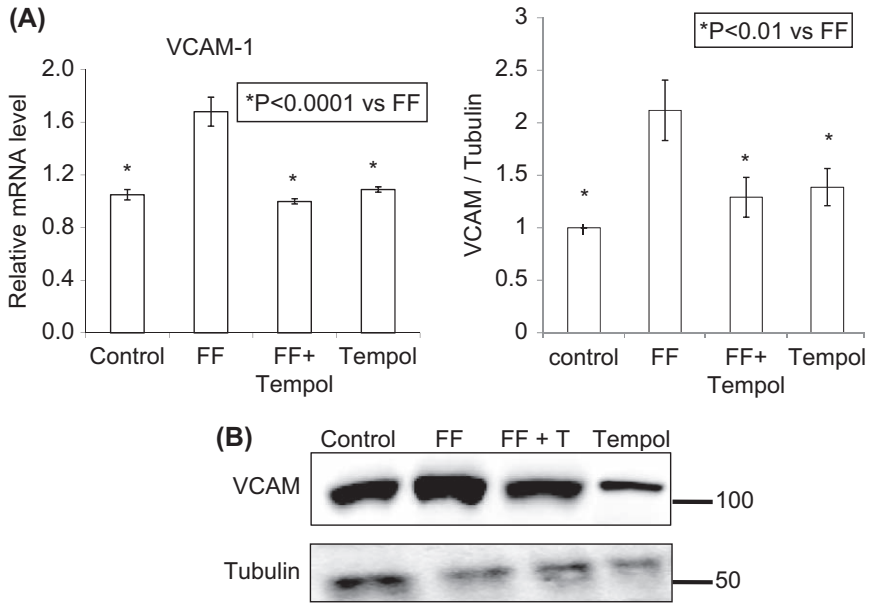

Figure 4. VCAM-1 expression on aortic tissue. Detection of VCAM-1 expression was determined by (A) western blot or (B) real time-PCR. (A) Protein samples are from total aortic tissue from ApoE-KO mice fed with control diet (control), high-fructose diet (FF), FF treated with tempol (FF + tempol), or tempol alone (tempol). Tubulin protein was used as the control for relevant quantification. Results are means ± S.E.M from three independent experiments (Density plot for $n = 3$ experiments). *$P < 0.001$ compared with FF. (B) Total mRNA was obtained from pools of four aortic vessels from each experimental group. Results are means ± SEM from two independent experiments (each experiment in triplicate). *$P < 0.0001$ compared with FF.

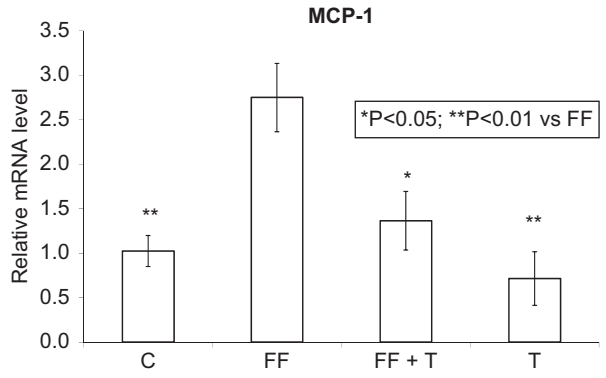

Figure 5. MCP-1 expression on aortic tissue. Comparison of MCP-1/Actin mRNA expression in aortic homogenates from ApoE-KO mice fed with control diet (control), high-fructose diet (FF), FF treated with tempol (FF + tempol), or tempol alone (tempol). Results are means ± SEM from two independent experiments (each experiment in triplicate). $*P < 0.05$; $**P < 0.01$ compared with FF.

Tempol treatment did not modify the increase of metabolic parameters induced by fructose diet.

Tempol decreases lipid peroxidation and oxidant production

To determine lipid peroxidation products, as indicative of oxidative stress, thiobarbituric acid-reacting substance (TBARS) were quantified. Fructose administration significantly increased plasma concentration of TBARS compared with control diet. Tempol significantly decreased TBARS levels, which would indicate that tempol decreases oxidative damage caused by high-fructose diet (Figure 1A).

Vascular oxidant generation was determined in situ in ApoE-KO mice aortic sections using the redox-sensitive dye, DHE. Figure 1B shows the semiquantitative analysis. Aortic sections of FF-ApoE-KO mice showed a 1.71 ± 0.03 fold increase in signal intensity compared with aortas from ApoE-KO mice in control diet. Tempol significantly inhibited high-fructose diet DHE signal-increase in the aortas of ApoE-KO mice ($P < 0.05$).

Tempol impairs Nox-2 oxidase system

To elucidate the molecular mechanisms involved in the reduction of NADPH oxidase activity mediated by tempol, we evaluated the mRNA levels of Nox-2 and p47phox in aortic tissue. We found that tempol significantly inhibited the mRNA expression of Nox2 regardless fructose diet (Figure 2A). We investigated the expression of others NADPH oxidase membrane isoforms, such as p22phox and Nox4, and we did not find any difference between experimental groups (Data not shown). The expression of p47phox in aortic tissue increased by fructose overload and tempol significantly inhibited this effect (Figure 2B).

The protein expression of NADPH oxidase subunits p47phox and Nox2, in cellular membrane extract, obtained from aortic tissue from each treated groups of animals, were evaluated by immunoblotting. We found that Nox2 expression in the membrane was not modifying by any of the treatments performed (Figure 3A). Instead, the expression of p47phox in the cell membrane extracts significantly augmented in FF ApoE-KO mice, compared with control diet, and tempol significantly inhibited p47phox increase induced by fructose diet (Figure 3B).

These results suggest that tempol is able to regulate the normal assembling of the subunits required for the activation of NADPH oxidase complex in the aortic wall.

Tempol diminishes fructose enhanced vascular inflammation and atherogenesis

Numerous molecules have been identified that play a critical role in several aspects of atherogenesis including vascular adhesion molecule 1 (VCAM-1) [23]. We aimed to determine the expression of VCAM-1 after 4 weeks with tempol treatment. Western blot quantification and real time-PCR revealed an upregulation of VCAM-1

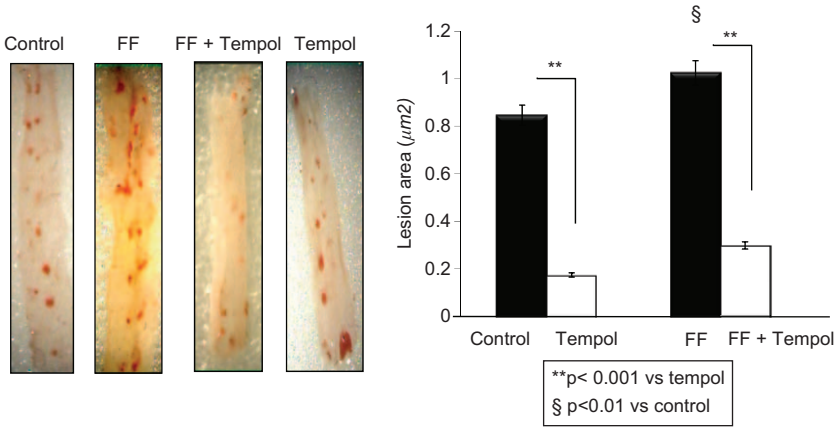

Figure 6. Tempol prevents atheroma development in FF ApoE-KO mice. Atheroma development in the whole aorta (aortic arch up to abdominal aorta) of ApoE-KO mice that fed control diet or fructose enriched diet (FF) treated with or without Tempol during 4 weeks was quantified by computerized morphometry. Arteries were stained with Oil-red O and results represent the area of lesion relative to total aortic surface ($n = 6$ mice per group). $^{\S}P < 0.05$ vs ApoE-KO control diet; $**P < 0.001$ vs Tempol treatment.

expression in aorta tissue from ApoE-KO mice fed with high-fructose diet, and tempol treatment significantly diminished this effect (Figure 4).

In order to assess if effect of tempol is associated with a shift in inflammatory state, we measured the expression of an inflammatory marker such as Monocyte Chemoattractant Protein-1 (MCP-1) in aortic homogenates by real time RT-PCR and we found that tempol inhibited the augmented expression of MCP-1 caused by fructose diet (Figure 5).

Finally, we performed studies to determine whether tempol could lead to a significant action on the development of atherosclerotic lesions in FF ApoE-KO mice. There was a significant reduction in total Oil red O-stained lesion area in mice treated with tempol compared with FF mice (Figure 6). Tempol treatment significantly decreased the severity of aortic atherosclerosis associated with metabolic syndrome.

Discussion

Oxidative stress and inflammation processes are key components of atherosclerosis, from fatty streak formation to plaque rupture and thrombosis [24,25]. This oxidative stress is characterized by overproduction of ROS and is dependent on the activation of NADPH oxidase [26]. Diets rich in fructose can alter cellular metabolism via several pathways, thereby accelerating oxidative stress. High-fructose feeding was associated with an early (1 week) increase in ROS production by aorta, heart and circulatory polymorphonuclear cells, in association with enhanced markers of oxidative stress [27]. Because tempol, a membrane permeable superoxide dismutase mimetic/free radical scavenger, has been found useful in treating oxidative stress-related vascular dysfunctions [28], we additionally demonstrated that tempol would prevent FF-induced inflammation and progression of atherosclerosis.

The membrane is a lipid-rich structure that upon oxidation generates peroxides (lipid peroxidation), which are decomposed into aldehydes such as 4-hydroxynonenal (4HNE) and MDA leading to degradation of the constituents of the cell membrane, loss of its structure, its permeability and function of its receptors. [29]. In this study we demonstrated that high-fructose diet in ApoE-KO mice significantly increases the plasma concentration of MDA, compared with control mice, and treatment with tempol significantly decreased lipid peroxidation. Also we confirm using DHE oxidant staining that tempol significantly inhibited ROS production on the vascular wall.

We investigated the expression of different NADPH oxidase subunits in aortic homogenates from FF ApoE-KO mice treated with or without tempol and found that this antioxidant decreased mRNA levels of Nox2 and p47phox. When we measured the protein expression of both NADPH subunits, we found no changes in Nox-2 protein expression in the membrane, but a significant decrease in p47phox expression suggesting that tempol impairs the activation of NADPH oxidase in aortic wall.

NADPH oxidases are found in virtually every tissue and are the major source of superoxide anion observed in the vasculature [30]. Multiple NADPH oxidase isoforms are constitutively expressed in each of the predominant cell types of the vascular wall. Endothelial cells express Nox1, Nox2, Nox4, and Nox5; vascular smooth muscle cells express Nox1, Nox4, and Nox5; and adventitial fibroblasts express Nox2 and Nox4 [12]. The expression of the vascular Nox2 subunit and/or its regulatory partners (e.g., p47phox or p22phox) is commonly increased by pro-inflammatory stimuli or in the presence of cardiovascular risk factors [31]. Nox2 seems to participate in the redox signaling implicated in initiation and progression of atherosclerosis [31]. Studies in transgenic and knockout animal models have shown the involvement of Nox1 and Nox2 and some of their regulatory subunits, such as p47phox, in the development of hypertension, atherosclerosis, and restenosis [12]. Barry-Lane reported many years ago that ApoE-KO mice lacking p47phox had a marked reduction of atherosclerosis in the descending aorta [32]. Although the diversity of Nox homologs was not known at the time, further support for a role of Nox2–NADPH oxidase in atherogenesis came from a subsequent finding that the protective effect of p47phox deficiency in an ApoE-KO background was due to both a decrease in Nox2–NADPH oxidase activity in vessel wall cells as well as in circulating macrophages [6]. Therefore, there is a strong rationale for therapeutically targeting NADPH oxidases in the arterial wall for the treatment of vascular disease.

Tempol has also been found useful in treating other oxidative stress-related vascular dysfunctions: Angiotensin II-induced hypertension in rats was prevented by orally-administered tempol [33]. Chronic treatment with tempol prevented vascular remodeling and progression of hypertension in spontaneously hypertensive rats [34,35]. Oral tempol given for 4 weeks to the FF rat model of type-2 Diabetes Miellitus prevented the accelerated neointimal proliferation and VSMC apoptosis in injured carotid arteries [36]. In our study we demonstrated that Tempol attenuates vascular inflammation and atherosclerosis development. Here we observed that both intimal VCAM-1 and MCP-1 expression were significantly increased in fructose-treated ApoE KO mice compared with control chow mice and tempol treatment significantly reduce the expression of these inflammatory markers within the arterial wall. Interestingly, we found a significant tempol-dependent reduction in the severity of aortic atherosclerosis compared with FF animals. Our results indicate that tempol decreases the activation of NADPH oxidase, reduces oxidative damage, attenuates vascular inflammation, and delays the development of atherosclerotic plaque, thus tempol would play an atheroprotective role in metabolic syndrome. We suggest that targeted therapies against reactive oxygen intermediates, which decrease the generation of ROS, could be useful in

minimizing vascular injury, delaying the formation of atherosclerotic plaque. Perhaps research for new drugs based on selective inhibitors of specific enzymes, involved in direct or indirect generation of ROS such as NADPH oxidase, could be directed for therapeutic use.

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Declaration of interest

All authors disclose no conflict of interest including any financial, personal, or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, their work.

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Supplementary material available online

Supplementary Table I.