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Hypercholesterolemia increases myocardial oxidative and nitrosative stress thereby leading to cardiac dysfunction in apoB-100 transgenic mice

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Abstract

Objective: We have previously shown that cholesterol diet-induced hyperlipidemia (marked hypertriglyceridemia and moderate hypercholesterolemia) increases cardiac formation of peroxynitrite and results in a moderate cardiac dysfunction in rats. Here our aim was to further clarify the mechanism of hyperlipidemia-induced nitrosative stress in a transgenic mouse model and to test if high cholesterol or high triglyceride is responsible for the hyperlipidemia-induced cardiac dysfunction.
Methods and results: To determine the effect of cholesterol-enriched diet on cardiac performance and oxidative/nitrosative stress, wildtype and human apoB100 transgenic mice were fed a 2% cholesterol-enriched or a normal diet for 18 weeks. Serum cholesterol and LDL-cholesterol levels were significantly elevated only in the cholesterol-fed apoB100 transgenic mice, while serum triglycerides were increased in the transgenic mice fed a normal diet. Cholesterol-enriched diet significantly increased cardiac superoxide generation and NADPH oxidase expression and activity in apoB100 mice but not in wildtypes. Cardiac NO content and NO synthase activity did not change in either group. As assessed in isolated working hearts, aortic flow was significantly decreased only in apoB100 transgenic mice fed a cholesterol-enriched diet. The peroxynitrite decomposition catalyst FeTPPS attenuated the decrease in aortic flow in cholesterol-fed apoB100 mice. Immunohistochemistry showed elevated nitrotyrosine in the hearts of apoB100 mice fed the cholesterol-enriched diet.
Conclusions: We conclude that hypercholesterolemia but not hypertriglyceridemia leads to increased formation of superoxide and peroxynitrite, and thereby results in cardiac dysfunction in hearts of human apoB100 transgenic mice.
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1. Introduction

Atherosclerosis results in coronary heart disease (CHD), one of the major cause of morbidity and mortality in civilized societies. The link between elevated cholesterol and CHD has been clearly established and the National Cholesterol Education Program clinical guidelines for the treatment of hypercholesterolemia identify low-density lipoprotein (LDL) cholesterol as the primary treatment target [1]. Nevertheless, recent research activities identify novel and independent risk factors for CHD. Hypertriglyceridemia

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increase the risk of acute coronary events, and some clinical trials found high serum triglycerides to be an independent risk factor for cardiovascular disease [2–7].

Although the focus of research so far has been mainly on the vascular effects of hyperlipidemia, i.e. arteriosclerosis, it is now quite evident that hyperlipidemia exerts direct effects on the myocardium in addition to the development of atherosclerosis [8,9]. Intracellular lipid accumulation in cardiomyocytes and several alterations in the structural and functional properties of the myocardium have been observed in response to cholesterol diet [10–13]. We and others have previously shown that hyperlipidemia attenuates the cardioprotective effect of ischemic preconditioning via a mechanism independent from atherosclerosis and other vascular effects of hyperlipidemia [8,9,14,15]. Furthermore, we have recently shown that a moderate hypercholesterolemia combined with a marked hypertriglyceridemia leads to a moderate contractile dysfunction in isolated rat hearts [12], and to marked alterations in the expression of several genes of various functional clusters in the myocardium [13]. These results show that hyperlipidemia exerts complex effects on the myocardium.

Hyperlipidemia is often linked to oxidative/nitrosative stress in the vasculature and in the myocardium, although the exact mechanism of such a relationship is yet to be clarified [9,12,13,16,17]. We have previously shown an increased formation of peroxynitrite, a toxic reaction product of superoxide and nitric oxide, in the rat myocardium in cholesterol-enriched diet-induced hyperlipidemia [12]. Peroxynitrite has been reported to induce DNA damage, to increase lipid peroxidation, and to cause post-translational modification on proteins (e.g. nitration, oxidation of thiol groups), thereby activating (e.g. poly-ADP-ribose polimerase, matrix metalloproteinases) or inhibiting (e.g. aconitase, superoxide dismutase) certain enzymes [18]. These cellular effects of peroxynitrite may contribute to the development of contractile dysfunction seen in hyperlipidemic rats, however, the precise mechanisms leading to increased peroxynitrite remain to be investigated, and further studies are required to explore whether hypercholesterolemia or hypertriglyceridemia is responsible for the peroxynitrite-mediated detrimental effect of experimental hyperlipidemia.

Recent advances in the development of genetically engineered mouse models of hypercholesterolemia may allow for a more appropriate control for the effect of species-dependent genetic differences in lipid transport and metabolism. Transgenic mice expressing human apolipoprotein B-100 showed slightly elevated total cholesterol and markedly elevated triglycerides when compared to wildtype mice fed normal diet [19]. However, when these human apoB-100 transgenic mice were fed a diet high in fat and cholesterol serum levels of non-HDL and total cholesterol were further increased and serum triglycerides were returned to normal levels [19]. Thus, these mice seem suitable for investigation of the differential effect of hypercholesterolemia or hypertriglyceridemia on cardiac oxidative stress and contractile function. In addition, the lipoprotein profile of apoB-100 transgenic mice, especially when fed a high-fat diet is similar to that seen in humans, i.e. higher proportions of VLDL and LDL fractions compared to HDL [19–21].

Therefore, in order to investigate whether hypercholesterolemia per se induces oxidative stress and thereby leads to cardiac dysfunction in the heart we have used human apoB-100 transgenic or wildtype mice fed either a cholesterol-enriched or normal diet. To further clarify the mechanism of hyperlipidemia-induced oxidative and nitrosative stress we have systematically analyzed serum lipid profile, myocardial levels of superoxide and nitric oxide, the activity and expression of their enzymatic sources, cardiac function, and the effect of pharmacologic decomposition of peroxynitrite on cardiac function.

2. Methods

This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and was approved by the local ethics committee.

2.1. Animals, diets, experimental groups

Transgenic mice overexpressing human apoB-100 protein were generated as described earlier [22]. Briefly, fertilized oocytes of C57/B6×CBA F1 females were collected and injected with the purified P1-phagemid DNA containing the entire 43 kb human apoB-100 gene, the 19 kb of the 5′ and the 14 kb of the 3′ flanking genomic sequences [20] in a concentration of 1 ng/ml, according to a standard technique [23]. Tail DNA of 10-day-old pups was purified [22], and integrated transgenes were detected by PCR, using primers from the 5′ promoter region of human apoB gene [20]. The expression of human apoB-100 transgene was detected by using real-time RT-PCR and Western blot analysis. The best-expressing line (line 485) was selected for further studies.

ApoB-100 transgenic F1 and control wildtype F1 mice were backcrossed twice with C57/B6 inbred mice in order to get closer to the atherosclerosis susceptible C57/B6 genetic background [24,25]. Wildtype and homozygous apoB-100 transgenic mice of either sex, housed in a room maintained at 12 h light/dark cycles and a constant temperature of 22± 2 °C, were fed either a laboratory chow enriched with 2% cholesterol or a standard chow for 17–19 weeks. At the end of the diet period, hearts were isolated for measurement of cardiac function and biochemical parameters, and aortas were removed for lipid staining. Some tissue was embedded in OCT for immunohistochemistry and histology studies (for details see Supplementary materials). Blood samples were collected from separate animals after 30 min fasting and stored at −70 °C until assayed for serum lipids, glucose, and malondialdehyde.

In order to check if peroxynitrite causes cellular damage and myocardial dysfunction due to hypercholesterolemia, separate groups of transgenic mice fed cholesterol-enriched or normal diet were injected twice intraperitoneally with

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2×20 mg/kg FeTPPS (5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III]), a peroxynitrite decomposition catalyst, 24 h and 1 h prior to isolation of the hearts [12].

2.2. Determination of serum lipids and glucose

Serum lipids including total cholesterol, triglycerides, LDL and HDL cholesterol, as well as glucose levels were measured in triplicate, using commercially available colorimetric assay kits applying enzymatic determinations (Diagnosticum Ltd., Budapest, Hungary) adapted to 96-well plates. Accuracy of the assays was monitored by using Standard Lipid Controls (Sentinel, Milan, Italy). Results are expressed as mmol/l of serum.

To analyze the ratios of α, pre-β, and β-lipoproteins in the serum of wildtype and transgenic mice fed cholesterol-enriched or normal diet, lipoproteins were separated on agarose gel, using Paragon Electrophoresis System Lipoprotein Electrophoresis Kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions.

2.3. Measurement of serum malondialdehyde levels

In order to measure the level of systemic lipid peroxidation serum malondialdehyde was assayed spectrophotometrically at 535 nm as described previously [12]. Results are expressed as nmol MDA/mL serum.

2.4. Measurement of superoxide production

To assess if hypercholesterolemia or hypertriglyceridemia leads to increased formation of cardiac superoxide, in separate experiments, superoxide production in freshly minced ventricles was assessed by lucigenin-enhanced chemiluminescence in wildtype and apoB-100 transgenic mice fed either normal or cholesterol-enriched diet as described previously [12,26]. Approximately 50 mg cardiac tissue was placed in Krebs–Henseleit buffer containing 5 μmol/L lucigenin. Chemiluminescence was measured at room temperature in a liquid scintillation counter (Tri-Carb 2100TR, Packard Instrument Company, Meriden, CT). Cardiac superoxide production was expressed as counts per min/mg wet tissue weight.

2.5. In situ detection of superoxide

In situ detection of superoxide was performed by confocal laser scanning microscopy using the oxidative fluorescent dye dihydroethidium. Dihydroethidium is freely permeable to cell membranes and fluorescing red when oxidized to ethidium by superoxide. Fresh frozen heart sections (30 μm) were incubated with 10^{-6} mol/L dihydroethidium (Sigma) in PBS (pH 7.4; 37 °C; 30 min) in a dark humidified container. Fluorescence in heart sections was then detected by an LSM 410 confocal microscope with a 590-nm long-pass filter. Images of sections treated with saline (negative control) were measured first.

2.6. Measurement of cardiac NO by electron spin resonance spectroscopy (ESR)

To measure cardiac NO content directly, in vivo spin-trapping of NO was applied, followed by ESR analysis of ventricular tissue samples as described [27,28]. The spin trap diethyl-dithio-carbamate (DETC, 500 mg/kg), 50 mg/kg $FeSO_4$, and 200 mg/kg sodium citrate were administered i.p. under ether anesthesia. Thirty minutes after injections of DETC and a mixture of $FeSO_4$ and citrate, ventricular samples were placed into quartz ESR tubes, frozen in liquid nitrogen until assayed for ESR spectra of the NO–Fe^{2+}(DETC)$_2$ complex. Fe^{2+}(DETC)$_2$ has high affinity for NO while forming NO–Fe^{2+}(DETC)$_2$. ESR spectra was recorded with a Bruker ECS106 (Rheinstetten, Germany) spectrometer at a temperature of 160 K as described [27,28].

2.7. Isolated working mouse heart

Cardiac performance was assessed in isolated working mouse hearts, as described [29]. Heparinized mice (100 U i.p.) were anesthetized with diethyl ether. Hearts were then isolated, the aorta was cannulated and initially perfused in Langendorff mode (at a constant pressure of 60 mmHg, 37 °C) with Krebs–Henseleit buffer containing (in mmol/L) 118 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.75 $CaCl_2$, and 11.1 glucose, oxygenated with 95% O_2–5% CO_2 (pH=7.4). Meanwhile the left atrium was cannulated and the perfusion was then switched to a recirculating working mode (10 min, 37 °C, preload 15 mmHg, afterload 60 mmHg). Coronary flow, aortic flow, and left ventricular pressure were measured at the end of working perfusion. Cardiac work was calculated as cardiac output×peak left ventricular systolic pressure. At the end of perfusion the hearts were snap frozen in liquid nitrogen and stored at −70 °C until they were used for biochemical assays.

2.8. Measurement of cardiac activities and expression of NAD(P)H oxidase, superoxide dismutase (SOD), and NOS

The frozen ventricles were ground at the temperature of liquid nitrogen and homogenized as described [29]. Protein concentration was measured in the homogenates by the bicinchoninic acid method using bovine serum albumin as a reference standard (Sigma St. Louis, MO).

NAD(P)H-stimulated superoxide production in freshly prepared ventricular homogenates was assessed by lucigenin-enhanced luminescence as described [29] with some modifications. To estimate background levels of superoxide formation ventricular homogenate (30 μL) was added to 1 mL Krebs–Henseleit–HEPES buffer containing 5 μmol/L lucigenin. Chemiluminescence was measured with liquid scintillation counter every 15 s for 5 min and the last 6 readings were averaged. To measure NAD(P)H-stimulated superoxide generation, either 100 μmol/L NADH or 100 μmol/L NADPH were added to the tubes and changes

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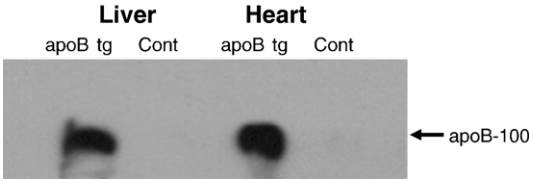

Fig. 1. Expression of human apoB-100 protein in the liver and heart of transgenic and wildtype mice.

in luminescence after each addition were measured. The background luminescence was subtracted from the readings with NADH or NADPH. Values were standardized to the amount of protein present and expressed as counts per minute/mg protein. NADH or NADPH alone did not evoke lucigenin chemiluminescence in the absence of homogenate.

To monitor gene expression changes of Nox1, Nox2 and Nox4, quantitative real-time PCR (QRT-PCR) reactions [30] and Western blot analysis were done (for details see Supplementary materials).

To estimate the level of the main enzymatic defense mechanism against superoxide myocardial SOD activity was determined [26]. Total activity of SOD in ventricular homogenate was measured by a spectrophotometric assay using a kit (Randox Laboratories, UK) according to the instructions of the manufacturer.

NOS activities in cardiac homogenate of frozen ventricular tissue were determined from the rate of conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline as described [26]. Samples were incubated for 60 min at 37 °C in the presence or absence of EGTA (1 mmol/L) plus NG-monomethyl-L-arginine (1 mmol/L) to determine the level of total NOS activities expressed in pmol/min/mg protein.

2.9. Statistical analysis

Data were expressed as means±SEM and analysed with one-way ANOVA followed by Tukey's post-hoc test. $p<0.05$ was used as the criterium for indicating a statistically significant difference.

3. Results

3.1. Validation of human apoB-100 expression

Expression of human apoprotein B-100 was validated in the liver and heart of both wildtype and human apoB-100 transgenic mice. Human apoprotein B-100 showed a marked

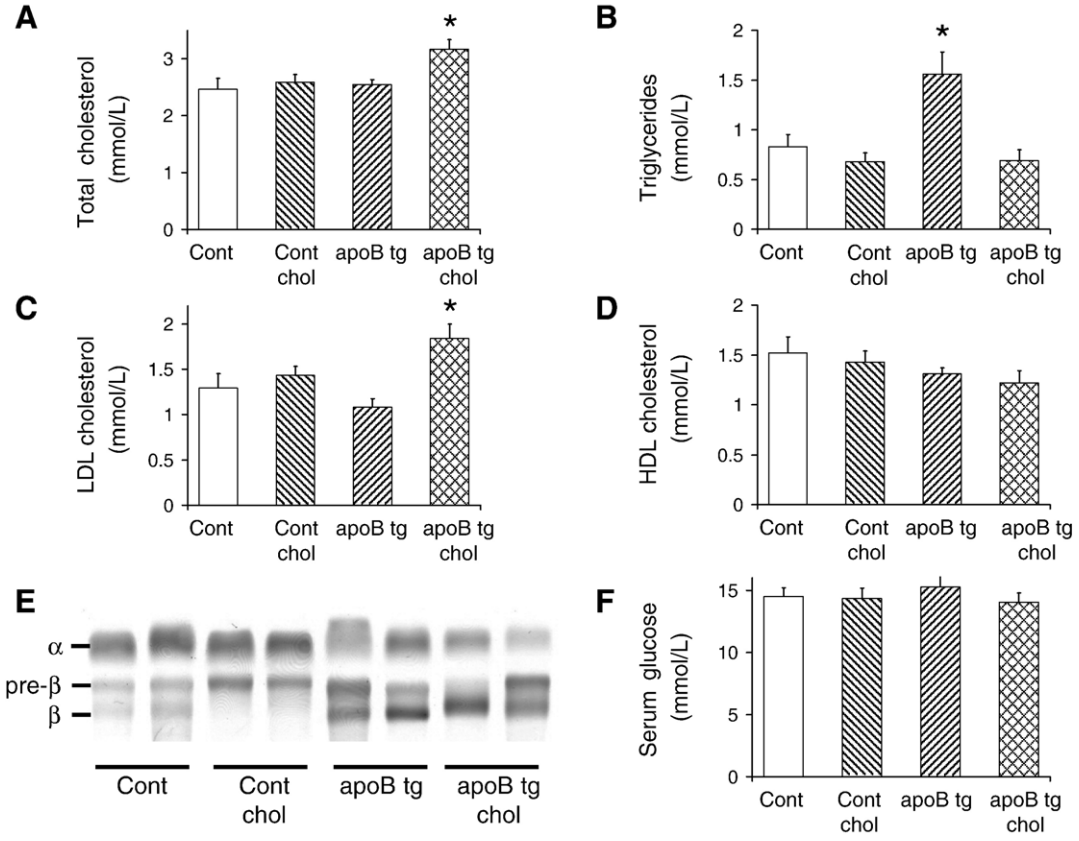

Fig. 2. Serum total cholesterol (A), serum triglycerides (B), LDL-cholesterol (C), HDL-cholesterol (D), lipoprotein electrophoresis (E), and serum glucose (F) levels in apoB100 and WT (Cont) mice fed either cholesterol-enriched (chol) or normal diet. Results are means±SEM ($n=5$–8 in each group). *$P<0.05$ vs. controls.

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Table 1
Distribution of lipoproteins in the sera of wildtype (Cont) and apoB100 transgenic mice fed normal or cholesterol-enriched diet

	Control	Control cholesterol	apoB tg	apoB tg cholesterol
α	67±5%	63±3%	35±4%*	40±3%*
pre-β	24±5%	37±3%	31±5%	34±6%
β	10±3%	1±1%	31±4%*	26±6%*

*$p<0.05$ vs. corresponding wildtype controls; $n=7–8$.

expression in both the liver and the heart of transgenic animals, without being expressed in wildtype mice (Fig. 1).

3.2. Serum lipids

Neither cholesterol-enriched diet, nor apoB-100 transgene affected serum total cholesterol when compared to normal diet-fed wildtype mice (Fig. 2A). However, serum cholesterol was increased significantly due to cholesterol-enriched diet in the apoB-100 transgenic animals (Fig. 2A, $n=7–8$ in each group). Similarly, the level of LDL cholesterol was increased significantly only in the apoB-100 transgenic mice fed cholesterol-enriched diet (Fig. 2C, $n=5–6$ in each group). On the other hand, neither of the treatments affected HDL cholesterol levels significantly (Fig. 2D, $n=5–6$ in each group). ApoB-100 transgene markedly increased the level of triglycerides in the serum compared to normal diet-fed wildtypes, however, serum triglycerides were reduced significantly by cholesterol in transgenic mice (Fig. 2B, $n=7–8$ in each group).

To determine the lipoprotein distribution in the transgenic or wildtype mice fed normal or cholesterol-enriched diet, plasma samples were analyzed by gel electrophoresis (Fig. 2E, Table 1). In the plasma of the apoB-100 transgenic mice, the amount of β-migrating lipoproteins increased dramatically with a parallel decrease in the α fraction ($p<0.05$, $n=7–8$ in each group). Cholesterol feeding slightly but not significantly increased preβ-migrating lipoproteins in wildtypes (Table 1), while caused only a slight shift of the β-fraction towards the preβ-fraction in the transgenic animals (Fig. 2E) without affecting the level of β-fraction.

To determine if cholesterol-enriched diet influenced carbohydrate metabolism, serum glucose levels were measured. No significant alterations in serum glucose concentration were detected in either groups (Fig. 2F).

3.3. Systemic oxidative stress

As a marker of systemic lipid peroxidation due to oxidative stress, serum malondialdehyde levels were determined in apoB-100 transgenic and wildtype mice fed either

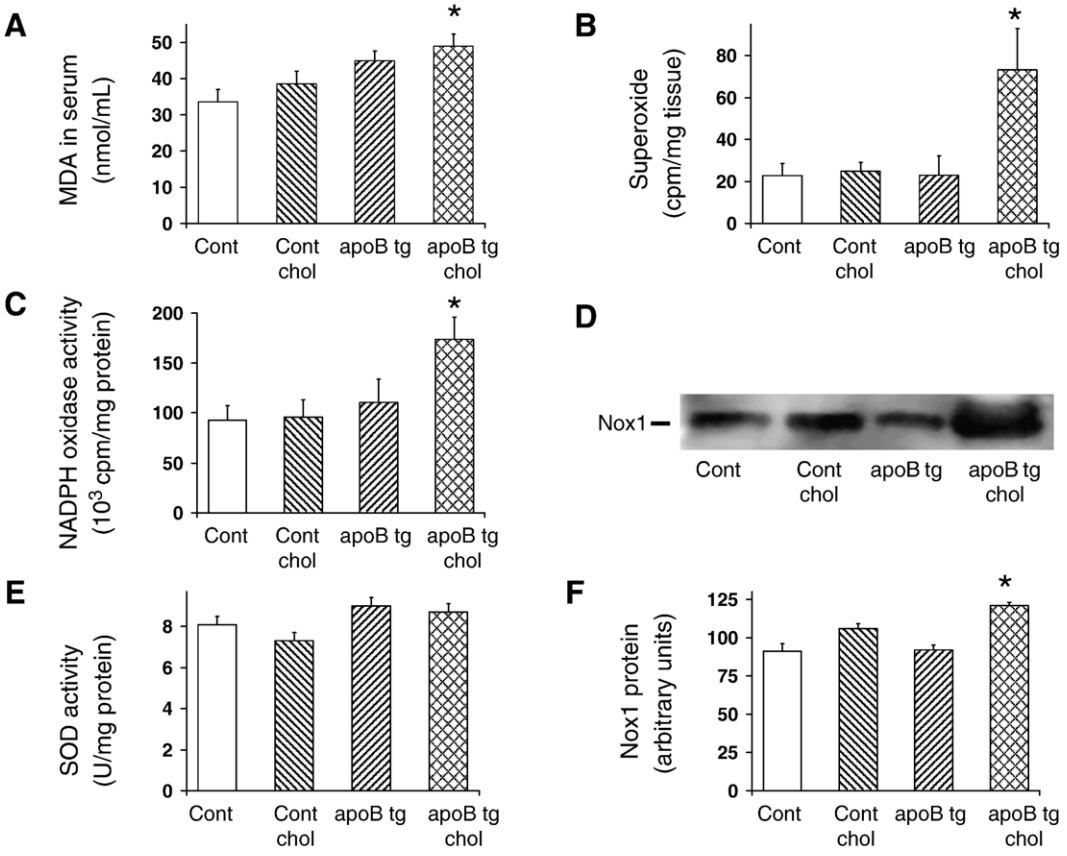

Fig. 3. Serum malondialdehyde levels (MDA) as indicators of lipid peroxidation (A), cardiac superoxide production (B), myocardial NADPH oxidase activity (C), myocardial superoxide dismutase (SOD) activity (E), representative western blot of cardiac NADPH oxidase 1 (Nox1) (D), and cardiac Nox 1 (F) in apoB100 and WT (Cont) mice fed either cholesterol-enriched (chol) or normal diet. Results are means±SEM ($n=3–8$). *$P<0.05$ vs. controls.

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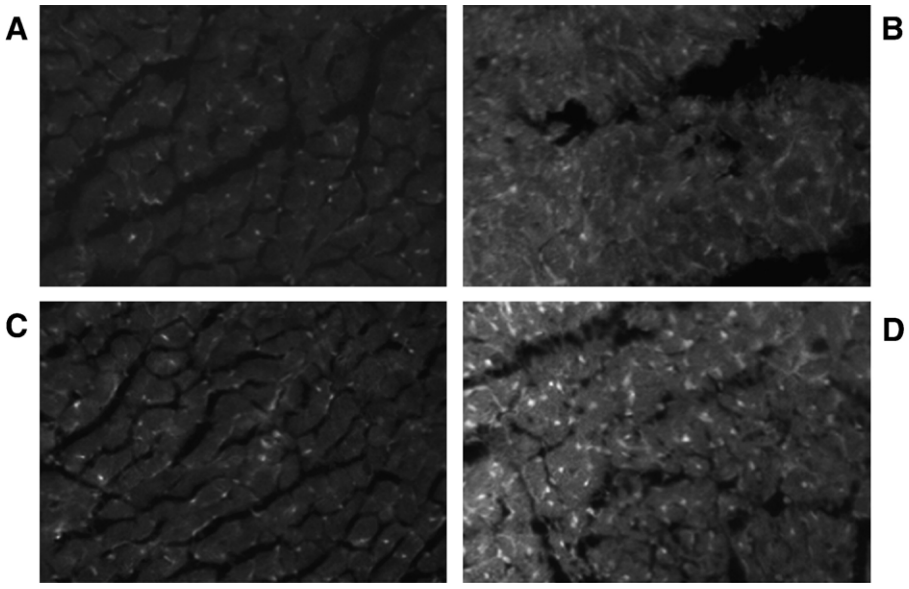

Fig. 4. Images of cardiac slides from wildtype (A, B) and human apoB100 transgenic mice (C, D) fed either normal (A, C) or cholesterol-enriched (B, D) diet following treatment with dihydroethidium (1 μM).

cholesterol-enriched or normal diet (n=5–6 in each group). Malondialdehyde levels were significantly increased in cholesterol-fed transgenic animals when compared to wild-type ones fed normal diet (Fig. 3A).

3.4. Cardiac superoxide, NADPH oxidase and superoxide dismutase activity

Cardiac superoxide formation was estimated in freshly minced heart tissue by lucigenin-enhanced chemiluminescence. Cardiac superoxide was markedly increased by cholesterol-enriched diet in the apoB-100 transgenic mice, but not in wildtypes (Fig. 3B). ApoB transgene alone did not change cardiac superoxide in mice fed normal diet (Fig. 3B). In order to confirm these data, we have detected superoxide in situ. Dihydroethidium staining of heart slices showed increased fluorescence due to cholesterol-enriched diet, especially in the apoB-100 transgenic animals (Fig. 4). The transgene alone did not affect superoxide-induced cardiac dihydroethidium fluorescence (Fig. 4).

To determine possible sources of cardiac superoxide, the activity of NAD(P)H oxidase and XOR was measured from heart homogenates. Cholesterol-enriched diet significantly increased the activity of NADPH oxidase in human apoB-100 transgenic mice without having an effect in wildtype ones (Fig. 3C, n=6–8 in each group). Activities of neither NADH oxidase nor XOR have changed significantly in any of the treatment groups (n=5–6 in each group, data not shown). Since we found increased cardiac activity of NADPH oxidase in the cholesterol-fed apoB-100 transgenic mice, we have further looked at the expression of NADPH oxidase 1 (Nox1), Nox2 and Nox4 by quantitative real-time PCR (n=4 in each group). Cholesterol-enriched diet significantly increased 1.75 times the cardiac expression of NADPH oxidase 1 mRNA in the apoB-100 transgenic mice while having no effect in wildtype ones (data not shown). The expression of Nox 2 and Nox4 did not change significantly in either group (data not shown). Western blot analysis of cardiac homogenates showed increased Nox1 protein level in cholesterol-fed apoB100 mice (Fig. 3D, F, n=3 in each group).

To estimate the activity of the most important enzymatic antioxidant mechanism against superoxide, total cardiac superoxide dismutase activity was determined. Neither apoB-100 transgene, nor cholesterol-enriched diet affected

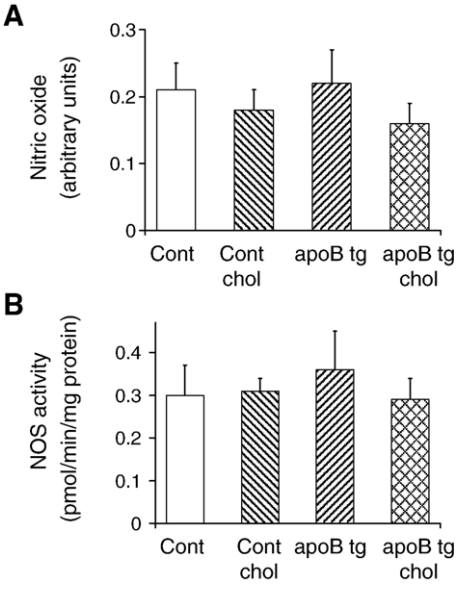

Fig. 5. Myocardial NO content (A), and NOS activity (B) in apoB100 and WT (Cont) mice fed either cholesterol-enriched (chol) or normal diet. Results are means±SEM (n=5–7).

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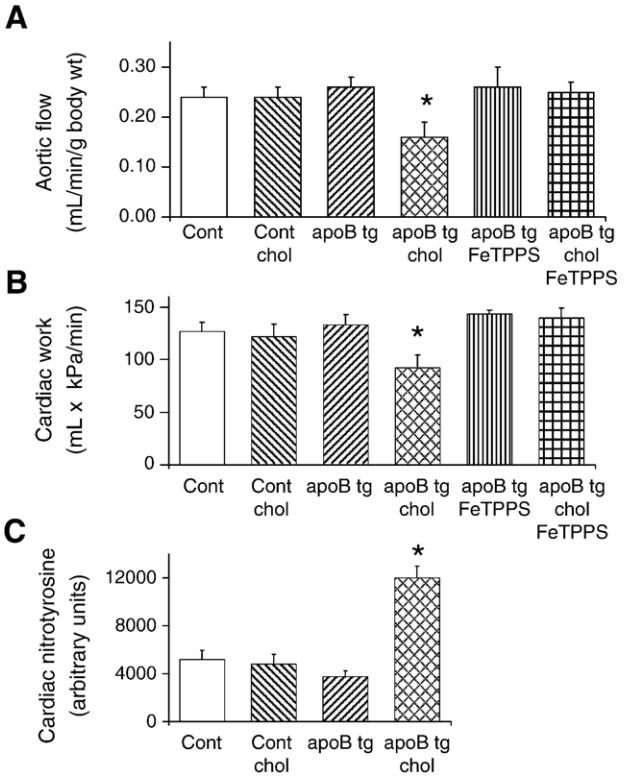

Fig. 6. Aortic flow (A) and cardiac work (B) in isolated working hearts of apoB100 transgenic and WT (Cont) mice fed either cholesterol-enriched (chol) or normal diet when pretreated or not with the peroxynitrite decomposition catalyst FeTPPS. Cardiac nitrotyrosine level in wildtype and apoB100 transgenic mice fed normal or cholesterol-enriched diet as assessed by immunohistochemistry (C) Results are means±SEM (n=5–7). *P<0.05 vs. controls.

total superoxide dismutase activity in the mouse heart (Fig. 3E, n=6–8 in each group).

3.5. Cardiac nitric oxide content, NOS activity

To study if cholesterol-enriched diet or human apoB-100 transgene affects nitric oxide content in mouse hearts, electron spin resonance spectroscopy following in vivo spin trapping was applied (n=5–6 in each groups). Cardiac nitric oxide content did not change due to apoB-100 transgene or cholesterol-enriched diet in either groups (Fig. 5A). Moreover, cardiac activity of NOS, the enzyme responsible for the endogenous synthesis of NO, was not altered in either groups (Fig. 5B, n=5–7 in each groups).

3.6. Myocardial dysfunction and peroxynitrite

Parameters of cardiac performance are shown on Fig. 6. and Table 2. Neither the presence or absence of the apoB-100 gene, nor the cholesterol content of the diet had any significant effect on heart rate, coronary flow, left ventricular peak systolic pressure, or $\pm dp/dt_{max}$ (Table 2). Aortic flow and cardiac work were determined to estimate basal pumping capacity of the heart. The apoB-100 transgene did not alter aortic flow and cardiac work when the animals received normal diet (Fig. 6). However, cholesterol-enriched diet significantly deteriorated aortic flow and cardiac work in the human apoB-100 transgenic mice without having an effect in wildetypes (Fig. 6, n=7–9 in each group).

To test if peroxynitrite is involved in cardiac dysfunction induced by cholesterol-enriched diet in apoB-100 transgenic mice, a peroxynitrite decomposition catalyst, FeTPPS, was injected to transgenic mice 24 and one hour prior to isolation of the hearts and assessment of cardiac performance. FeTPPS-pretreatment significantly improved aortic flow deteriorated by cholesterol-enriched diet in apoB-100 transgenic mice (Fig. 6, n=5–7 in both groups).

To further prove that cholesterol-enriched diet leads to increased nitrosative stress in apoB100 transgenic mice we have looked at nitrotyrosine immunostaining of heart sections, and found elevated tissue level of nitrotyrosine in the cholesterol fed transgenic mice (Fig. 6, n=3 in each groups).

Hematoxylin-eosin and Crossman's trichrome staining for histology did not reveal any pathological changes in either groups (data not shown).

4. Discussion

In this study, we have systematically analyzed the plasma lipid, lipoprotein, and MDA levels, as well as the cellular mechanisms of cardiac oxidative and nitrosative stress, along with the estimation of myocardial contractile function in human apoB100 transgenic and WT mice fed normal or high-cholesterol diet. We have shown here that in apoB100 transgenic mice hypercholesterolemia induced by cholesterol-enriched diet increases cardiac oxidative/nitrosative stress

Table 2
Hemodynamic data in isolated working mouse hearts

	Control	Control cholesterol	apoB tg	apoB tg cholesterol	apoB tg FeTPPS	apoB tg cholesterol FeTPPS
Body weight (g)	26.8±1.7	26.5±1.8	24.1±0.6	28.7±1.3	24.8±2.0	27.0±1.3
Heart rate (bpm)	327±18	334±17	325±13	334±18	312±22	331±17
Coronary flow (ml/min/g bw)	0.12±0.01	0.12±0.01	0.14±0.02	0.13±0.01	0.14±0.02	0.14±0.02
Cardiac output (ml/min/g bw)	0.37±0.03	0.37±0.03	0.42±0.03	0.25±0.03*	0.43±0.06	0.41±0.03
LVPSP (kPa)	13.2±0.2	12.6±0.4	13.6±0.4	12.7±0.5	14.4±0.4	13.4±0.3
$+dp/dt_{max}$ (kPa/s)	614±33	661±32	665±26	617±32	663±18	648±27
$-dp/dt_{max}$ (kPa/s)	413±17	463±28	487±22	455±33	513±29	506±30

*p<0.05 vs. corresponding wildtype and apoB transgenic controls; n=7–9.

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thereby leading to cardiac dysfunction, which can be prevented by pharmacologic attenuation of cardiac nitrosative stress. Furthermore, this is the first demonstration that hypercholesterolemia but not hypertriglyceridemia is responsible for the hyperlipidemia-induced, peroxynitrite-mediated cardiac dysfunction and that increased cardiac expression of NADPH oxidase is the major source of increased oxidative stress in hypercholesterolemia.

In our present studies, cholesterol-enriched diet resulted in an increase in serum total cholesterol and LDL cholesterol levels in the human apoB-100 transgenic mice without having an effect in wildtypes. HDL cholesterol did not change significantly between the groups. Similarly to previous findings [19,21], serum triglycerides were markedly elevated in apoB-100 transgenics fed normal diet compared to wildtype values. Cholesterol-enriched diet did not have an effect on serum triglycerides in wildtype mice, however, it did reduce elevated triglycerides back to normal values in apoB-100 transgenic animals. This unique lipid profile makes our transgenic model a suitable tool for the investigation of the distinct effects of hypercholesterolemia (in cholesterol-fed apoB-100 transgenic mice) and hypertriglyceridemia (in apoB-100 transgenic mice fed normal diet) on the heart. Another useful feature of this model is the more "human-like" distribution of lipoproteins in the cholesterol-fed transgenic mice than seen in wildtypes. In accordance with previous studies [19,31] we have found an increased LDL cholesterol/HDL cholesterol ratio in the cholesterol-fed apoB-100 mice that is more characteristic for the human rather than the murine lipid profile.

It is well known that hyperlipidemia increases vascular oxidative stress [32,33]. Here we have found that oxidative/nitrosative stress in the heart was increased in cholesterol-fed apoB-100 mice compared to wildtypes. In order to assess cardiac oxidative/nitrosative stress, we have systematically analyzed myocardial formation of superoxide (measured by lucigenin chemiluminescence and dihydroethidium staining), NO, and peroxynitrite (by nitrotyrosine immunohistochemistry), as well as the activities and expression of major enzymatic sources of these species (xanthin oxidoreductase, NAD(P)H oxidase, and NOS), and the most relevant antioxidant enzyme activities (SOD). In agreement with our previous study in a rat model of hyperlipidemia [12], we have shown here an increase in cardiac superoxide production. This is the first demonstration that both the expression and activity of NADPH oxidase is responsibe, at least in part, for the increased superoxide production seen in cholesterol-fed apoB100 transgenic mice. We have further shown that increased oxidative stress was associated with a significant myocardial dysfunction in the transgenic mice fed cholesterol. The mechanism by which superoxide has contributed to the cardiac dysfunction likely involves the formation of the cytotoxic species, peroxynitrite. Pathological conditions resulting in increased production of NO, superoxide, or both often leads to increased formation of peroxynitrite, which causes cellular damage [34–36]. We have previously reported that hyperlipidemia (marked hypertriglyceridemia and moderate hypercholesterolemia) induced by cholesterol-enriched diet resulted in enhanced formation of peroxynitrite in rat hearts [12]. Our present study shows that cholesterol-enriched diet in apoB-100 transgenic mice leads to increased myocardial oxidative stress and cardiac dysfunction. We have further shown here that the peroxynitrite decomposition catalyst FeTPPS improved cardiac contractile function deteriorated by cholesterol-enriched diet in apoB-100 transgenic mice, indicating that formation of peroxynitrite in these mice exerts a deleterious effect on cardiac function. Our data also indicates that the increased superoxide formation is responsible for the increased peroxynitrite in these hearts, since neither the activity of NOS, nor cardiac NO content showed any change among the treatment groups.

In this study we have also determined if the development of myocardial oxidative/nitrosative stress and cardiac dysfunction is affected by either hypercholesterolemia or hypertriglyceridemia. Cardiac formation of superoxide as well as the activity and expression of NADPH oxidase increased parallel with serum cholesterol but independent of serum triglyceride levels. Similarly, in our study, hypercholesterolemia but not hypertriglyceridemia seemed to be associated with cardiac dysfunction. This likely means that hypertriglyceridemia per se does not affect the myocardium directly. In our study, we have not found an association between the level of serum cholesterol or triglycerides and changes in the activities of XOR, NADH oxidase, or SOD. Thus, it seems a plausible explanation that cholesterol-enriched diet-induced hypercholesterolemia induces NADPH oxidase and increases cardiac superoxide, thereby leading to increased peroxynitrite production, which results in cardiac dysfunction. Increased NAD(P)H oxidase-mediated superoxide generation seen in vessels of hypercholesterolemic subjects [2,37–39] further supports our present results.

Although the direct link between serum cholesterol and the activation of NADPH oxidase is not entirely clear in the myocardium one possible mechanism has been described for the activation of the phagocyte NADPH oxidase [40]. This paper suggest that cholesterol-enriched microdomains in the membrane act to recruit and/or organize the cytosolic NADPH oxidase factors in the assembly of the active NADPH oxidase.

In summary, cardiac oxidative/nitrosative stress induced by hypercholesterolemia but not hypertriglyceridemia leads to myocardial dysfunction. Consequently, lowering of serum cholesterol or attenuation of oxidative/nitrosative stress by pharmacological tools may be effective potential targets to protect the heart in hypercholesterolemia.

It is plausible to speculate that lowering of serum cholesterol by pharmacological tools may protect the heart against oxidative and nitrosative stress in hypercholesterolemia, however, it needs further investigations. Some statins have been shown to decrease vascular oxidative and nitrosative stress, and we have previously found that e.g. inhibition of the mevalonate pathway did not influence

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cardiac nitric oxide synthesis [41]. Nevertheless, a number of clinical trials and animal experiments have shown the beneficial effects of cholesterol-lowering drugs in the prevention of ischemic heart diseases [42,43].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cardiores.2007.06.006.

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