ELSEVIER

Atherosclerosis 188 (2006) 301–308

ATHEROSCLEROSIS

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Dietary reversal of experimental hypercholesterolemia improves endothelial dysfunction of epicardial arteries but not of small coronary vessels in pigs

Katherine J.E. Sattler [a], Offer Galili [a], Martin Rodriguez-Porcel [a], James D. Krier [b], Lilach O. Lerman [a,b], Amir Lerman [a,*]

[a] *Division of Cardiovascular Diseases, Mayo Clinic College of Medicine, Rochester, MN, USA*
[b] *Division of Nephrology and Hypertension, Rochester, MN, USA*

Received 18 August 2005; received in revised form 2 November 2005; accepted 3 November 2005
Available online 15 December 2005

Abstract

Endothelial dysfunction is characterized by impaired vasodilation, increase of oxidative stress and inflammation. The current study was designed to test the hypothesis that reversal of hypercholesterolemic diet alone does not normalize all the parameters of endothelial dysfunction. After 10 weeks on a high-cholesterol diet, female juvenile pigs were randomized to normal diet ($n=5$, "Reversals") or continued on the same diet ($n=6$, "HC") for another 6 weeks. A control group of 11 pigs received a normal diet ("C"). Coronary epicardial and arteriolar endothelial function was tested in vitro. NFκB and p47phox expression was analyzed in epicardial arteries and myocardium, respectively. P47phox localization in coronary arteries was demonstrated with immunohistochemistry. Lipid levels normalized in Reversal pigs. Epicardial arteries of Reversals showed a normalized relaxation and NFκB expression compared to HC ($p<0.05$). Small vessel relaxation remained attenuated, and expression of p47phox in myocardial tissue was elevated in Reversals compared to C ($p<0.05$). Dietary lowering of serum cholesterol and LDL improves vascular function of epicardial arteries but neither of small vessels nor vascular oxidative stress within this time frame. Hence, dietary normalization of serum lipid levels alone may not be synonymous to normalization of the components of endothelial dysfunction.

Keywords: Cholesterol; Vasorelaxation; Nuclear factor κB; Small vessels

1. Introduction

Early coronary atherosclerosis is characterized by endothelial dysfunction, consisting of both impaired vasorelaxation of the epicardial arteries and the microcirculation as well as an increase in vascular inflammation [1]. The clinical significance of endothelial dysfunction is underscored by the observations that it is an independent predictor of cardiovascular events in patients with and without manifest coronary artery disease [2,3]. Previously we have demonstrated that experimental hypercholesterolemia is associated with endothelial dysfunction of coronary epicardial arteries, as well as small vessels, and an increase of inflammatory markers and oxidative stress [4]. Activated nuclear factor κB (NFκB), an important mediator of inflammation, is expressed in porcine coronary arteries exposed to hypercholesterolemia [5]. NFκB activation is directly related to hypercholesterolemia-induced increase of oxidative stress, which results in enhanced ubiquitination of cellular proteins [6]. Among the proteins targeted by ubiquitination for degradation is IκB, the inhibitory subunit of nuclear factor κB, thus, ubiquitination of IκB increases NFκB activity during

* Corresponding author at: Division of Cardiovascular Diseases, Mayo Clinic College of Medicine, 200 First Street S.W., Rochester, MN 55905, USA. Tel.: +1 507 255 4152; fax: +1 507 255 2550.
E-mail address: lerman.amir@mayo.edu (A. Lerman).

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doi:10.1016/j.atherosclerosis.2005.11.009

hypercholesterolemia [7]. Imbalance of the redox state during hypercholesterolemia is, furthermore, demonstrated by down-regulation of counteracting enzymes such as superoxide dismutase both in tissue [8] and in the circulation [9]. Additionally, elevated serum cholesterol-levels decrease bioavailability of NO, leading to attenuated endothelial-dependent vasodilation [10] despite of up-regulation of cyclic GMP in the vessel wall [11].

In previous studies, we demonstrated reversal of inflammation [12], oxidative stress [13] and subsequently vasodilation [13] in hypercholesterolemic animals by treatment with simvastatin independent of cholesterol-lowering.

Based on these results, we hypothesized that hypercholesterolemia initiates adverse effects that may not be entirely dependent on serum cholesterol-levels.

To test this hypothesis, pigs were exposed to hypercholesterolemia for ten weeks and afterwards randomized to a normal diet. Effects of the diet change on vasorelaxation of large and small coronary arteries, inflammatory markers (NFκB), nitric oxide pathway integrity (cGMP expression) and oxidative stress (SOD-1, NAD(P)H-oxidase subunit p47phox) were analyzed subsequently.

2. Methods

2.1. Animals

The study was reviewed and approved by the Mayo Foundation Institutional Animal Care and Use Committee and conforms to 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). Eleven female juvenile pigs (12 weeks of age) received a high-cholesterol diet containing 2% cholesterol and 15% lard by weight (TD 93296, Harlan Teklad, Iowa) over a period of 10 weeks, as described previously [11]. Subsequently, the pigs were randomized to receive a normal diet ($n = 5$, "Reversals") or to continue with the same high-cholesterol diet as before ($n = 6$, "HC") for another 6 weeks. A control group consisting of 11 pigs (12 weeks of age) received a normal diet for 16 weeks ("C"). Serum total cholesterol, HDL and LDL levels and plasma levels of superoxide dismutase were measured at diet change and again at 16 weeks. At 16 weeks, blood pressure was measured in all animals with a previously described method [12]. After 16 weeks the animals were sacrificed (sodium pentobarbital (Sleepaway®), 20 ml i.v., Fort Dodge Laboratories, Iowa) and the epicardial arteries and small coronary arteries immediately harvested.

2.2. Vascular endothelial function

2.2.1. Epicardial arteries

Samples were prepared and tested following a previously described protocol [11]. In short, rings of epicardial coronary arteries (2–3 mm long) were placed in an organ chamber filled with 25 ml of modified Krebs solution [5], and oxygenated with 94% O_2 and 6% CO_2. The rings were suspended between two stirrups and connected to a strain gauge for recording changes of isometric tension. After 1 h at resting tension, viability of the vessels was assessed by response to 20 mmol/l KCl at baseline, 2, 4 and 6 g of tension for 4 min each, each time after the KCl had been washed out with Krebs solution. At 6 g, all vessels were subsequently exposed to substance P (10^{-6} mol/l, Sigma, St. Louis, MO, USA) to verify the functional integrity of the vascular endothelium. All following experiments were performed at a resting tension of 6 g (baseline tension). Vessels were pre-contracted with endothelin-1 (10^{-7} mol/l, Phoenix Pharmaceuticals, California) and endothelium-independent vasodilatation was tested with increasing concentrations of sodium-nitroprusside (10^{-9} to $10^{-4.5}$ mol/l, Sigma). In additional vascular rings from each group, the response to the endothelium-dependent vasodilator bradykinin (10^{-11} to 10^{-7} mol/l, Sigma) and to the non-receptor mediated endothelium-dependent vasodilator calcium ionophore A23187 (10^{-11} to 10^{-6} mol/l, Sigma) was obtained.

2.2.2. Small vessels

Endothelial function of small vessels was assessed according to an established protocol from our laboratory [11]. Vascular rings, corresponding to the secondary branch of the left circumflex artery with diameters <500 μm, were placed in an arteriograph and mounted onto microcannulas (Living System Instrumentation). The arteriograph was placed on a microscope (Diaphot-TMD, Nikon) which was connected to a video camera. Rings were pressurized to 50 mmHg with Krebs solution. Luminal diameter and wall thickness were recorded and measured electronically. Pre-contraction was performed with endothelin-1 (10^{-8} mol/l, Phoenix Pharmaceuticals, California) and vasorelaxation was tested with an increasing concentration of bradykinin (10^{-11} to $10^{-6.5}$ mol/l, Sigma).

2.2.3. cGMP production

cGMP production was measured in coronary smooth muscle cells in response to the exogenous NO donator diethylamine according to a previously published protocol [14]. Endothelium was removed from rings from coronary arteries of each group, and the rings were placed in an organ chamber filled with Krebs solution. After 1 h of incubation, cAMP phosphodiesterase was inhibited by adding 10^{-4} molar 3-isobuthyl-1-methyl-xanthine (Sigma) and production of prostaglandins was blocked by adding 10^{-5} M indomethacin (Sigma) for 30 min. Afterwards, samples were randomized to treatment with diethylamine (DEA; 10^{-6} mol/l, Cayman Chemical Company, Michigan) for 1 min or to an untreated control group. All samples were then shock-frozen in liquid nitrogen. After homogenization of one gram tissue of each sample in acidic ethanol (1 ml