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Antiatherogenic effects of *S*-nitroso-*N*-acetylcysteine in hypercholesterolemic LDL receptor knockout mice

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Abstract

Background: The pathophysiology of the NO/NO synthase system and dysfunctional changes in the endothelium in the early phases of the atherogenic process are incompletely understood. In this study, we investigated the effects of the nitrosothiol NO donor *S*-nitroso-*N*-acetylcysteine (SNAC) in the early prevention of plaque development in the hypercholesterolemic LDLr−/− mice as well as the changes in endothelium-dependent relaxation and NO synthase expression.

Methods and results: LDLr−/− mice were fed a 1.25% cholesterol-enriched diet for 15 days. Plasma cholesterol/triglyceride levels increased and this increase was accompanied by the development of aortic root lesions. Aortic vasorelaxation to acetylcholine was increased, although endothelium-independent relaxation in response to sodium nitroprusside did not change, which suggest stimulated NO release enhanced. This dysfunction was associated with enhanced aortic superoxide production and with increased levels of constitutive NOS isoform expression, particularly neuronal NOS. SNAC (*S*-nitroso-*N*-acetylcysteine) administration (0.51 μmol/kg/day i.p. for 15 days) decreased the extent of the plaque by 55% in hypercholesterolemic mice, but had no effects on vasomotor changes. It did, however, lead to a decrease in constitutive NOS expression. The SNAC induced only minor changes in plasma lipid profile.

Conclusion: The present study has shown that, in early stages of plaque development in LDLr−/− mice, specific changes in NO/NO synthase system develop, that are characterized by increased endothelium-dependent vasorelaxation and increased constitutive NOS expression. Since the development of plaque and the indicator of endothelial cell dysfunction were prevented by SNAC, such treatment may constitute a novel strategy for the halting of progression of early plaque.

Keywords: Nitric oxide; Atherosclerosis; Nitric oxide synthases; Endothelial dysfunction; Oxidative stress; Deficient receptor LDL knockout mice; Nitrosothiol

Endothelial cell dysfunction, which is intimately linked to reduction of the nitric oxide (NO) bioavailability, is an early event in the pathogenesis of atherosclerosis [1]. Endothelial function depends on redox signaling, which is mediated by the bioavailability of NO. Since a reduction in the acetylcholine-induced vasorelaxation in the aorta can be used to identify the dysfunction of the endothelium, most experimental models of hypercholesterolemia/atherosclerosis, include it as an important mechanism; however, in the aortas of ApoE knockout mice (apolipoprotein E knockout mice) [2], this reduc-

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tion fails to occur. Moreover, decreased endothelium-dependent dilation is uniformly observed in systemic vessels from patients with risk factors for atherosclerosis [3], as well as in diseased coronary arteries [4]. The decrease in NO bioavailability is associated with eventual plaque development, since it is known that in initial phases of the disease, it causes LDL oxidation and inflammatory cell activation. However, the mechanisms involved in this reduction in the later stages of this disease, often accompanied by a decrease in eNOS, are due to increased superoxide production by sources such as NAD(P)H oxidase [5], xanthine oxidase [6] or uncoupled NO synthase(s) [7]. However, the pathophysiology of early alteration in endothelial function remains incompletely understood. In particular, the natural evolution of vasoreactivity is unknown, and the link between hypercholesterolemia and an increased NOS output/activity, as well as enhanced oxidative stress and NOS uncoupling has still not yet been fully elucidated [8]. Early endothelial dysfunction is associated with cell activation and oxidative stress [9], adhesion molecule expression [10], macrophage infiltration, and the uptake of modified LDL particles [11], but these effects are likely to be counteracted by an increase in bioactive nitric oxide [12], although they are worsened by reactive byproducts of the nitric oxide–superoxide reaction [13].

Cell surface LDL receptors (LDLr) play a fundamental role regulating plasma cholesterol levels by mediating cellular uptake of LDL and intermediate-density lipoproteins (IDLs). Defects in the LDLr gene are the best documented genetic cause of premature atherosclerosis in humans. LDLr−/− (LDL receptor knockout mice) on a normal chow diet have only mild pathological lesions, with a slightly elevated plasma cholesterol (IDL/LDL fraction), which is a phenomenon attributed to the presence of an alternative ApoB-II pathway for LDL clearance in the mouse. However, when fed an atherogenic diet, these mice develop significant fatty streak lesions with a lipid-filled necrotic core [14]. McCaffrey et al. [15] have demonstrated that such lesions begin to develop in the aortic arch and near the ostia after only 4 weeks on the diet. But it is important to study earlier events of the atherogenic process to investigate possible therapeutic intervention, which is possible with the hypercholesterolemic LDLr−/− mouse model [14].

Recently, *S*-nitrosothiols have been identified as potential NO reservoirs, which is in line with the role of thiol nitrosylation as an effector mechanism of NO signaling [16]. Various nitrosothiols, both naturally occurring and chemically synthesized, have been used in clinical application [17], but SNAC has as yet been unexplored. However, it has been shown hypotensive effects in normal or hypertensive conscious rats [18].

The present study was designed to investigate the changes in endothelial cell function and the pathophysiology of the NO/NO synthase system in a very early phase of the atherogenic process in hypercholesterolemic LDLr−/− mice. Especially, identify how these processes are affected by exogenous administration of the NO donor, SNAC.

Materials and methods

Protocols

LDLr−/− mice were obtained from Jackson Laboratory (Bar Harbor, ME) by homologous recombination, as described by Ishibashi et al. [19]. In this study, three-month-old male LDLr−/− mice weighing 25 ± 3 g were used. The animals were kept on cycles of 12 h of light and 12 h of darkness, at controlled temperature (22 ± 2 °C). Animals were handled under protocols (number 375-1) approved by the Institutional Committee for Ethics in Animal Research (Campinas State University—UNICAMP, Brazil).

LDLr−/− mice were distributed into three groups that received diet and water ad libitum. (1) Control mice (C, daily dose of 0.2 μl PBS/ i.p.), which received commercial diet (Nuvital CR1); (2) hypercholesterolemic mice (HC, daily dose of 0.2 μl PBS/ i.p.) received a diet containing 20% fat, 1.25% cholesterol, 0.5% cholic acid (Diets, Bethlehem); (3) Similarly handled hypercholesterolemic mice given SNAC (SNAC + HC; daily dose of 0.51 μmol/kg i.p.). After 15 days, mice were anesthetized with Ketamine (Bayer, 40 mg/kg i.p.) and Xylazine (Parke–Davis, 6 mg/kg i.p.) for blood collection and subsequently killed by exsanguination followed by tissue harvesting. SNAC was synthesized as described by Santos et al. [18].

Vascular reactivity

Mice were anesthetized and, after thoracotomy, the descending thoracic aortas were collected and dissected. Rings ± 2 mm long were obtained from each aorta, were connected to a hook and kept in an organ bath containing Krebs–Henseleit buffer with the following composition (mM): NaCl 115.0; KCl 4.6; CaCl 2.5; KH_2PO_4 1.2; $MgSO_4$ 2.4; $NaHCO_3$ 25; glucose 11.0 and ascorbic acid 0.1, pH 7.4, 37 °C, aerated with 95% O_2 and 5% CO_2. The rings remained under 5 mN tension during 1 h for stabilization. Tension cumulative was measured by an isometric transducer (F-60, Narco Biosystems) connected to a Narco Bio-System polygraph (Model DMP-4). Cumulative concentration relaxation curves [20] to acetylcholine (ACh[1]; 10^{-10}–10^{-4} M; Sigma Chemical) or to

[1] *Abbreviations used:* ACh, acetylcholine; RSNOs, reactive species nitrogen oxide; SMC, smooth muscle cell; SNAC, *S*-nitroso-*N*-acetylcysteine; SNP, sodium nitroprusside; PHE, phenylephrine; ko, knockout.