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Hypercholesterolemia-induced erectile dysfunction: endothelial nitric oxide synthase (eNOS) uncoupling in the mouse penis by NAD(P)H oxidase

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Abstract

INTRODUCTION—Hypercholesterolemia induces erectile dysfunction (ED) mostly by increasing oxidative stress and impairing endothelial function in the penis, but the mechanisms regulating reactive oxygen species (ROS) production in the penis are not understood.

AIMS—We evaluated whether hypercholesterolemia activates nicotinamide adenine dinucleotide phosphate (NAD[P]H) oxidase in the penis, providing an initial source of ROS to induce endothelial nitric oxide synthase (eNOS) uncoupling and endothelial dysfunction resulting in ED.

METHODS—Low-density-lipoprotein receptor (LDLR)–null mice were fed Western diet for 4 weeks to induce early-stage hyperlipidemia. Wild type (WT) mice fed regular chow served as controls. Mice received NAD(P)H oxidase inhibitor apocynin (10 mM in drinking water) or vehicle. Erectile function was assessed in response to cavernous nerve electrical stimulation. Markers of endothelial function (phospho [P]-vasodilator-stimulated-protein [VASP]-Ser-239), oxidative stress (4-hydroxy-2-nonenal [HNE]), sources of ROS (eNOS uncoupling and NAD[P]H oxidase subunits p67^{phox}, p47^{phox}, and gp91^{phox}), P-eNOS-Ser-1177, and eNOS were measured by Western blot in penes.

MAIN OUTCOME MEASURES—Molecular mechanisms of ROS generation and endothelial dysfunction in hypercholesterolemia-induced ED.

RESULTS—Erectile response was significantly ($P < 0.05$) reduced in hypercholesterolemic LDLR-null mice compared to WT mice. Relative to WT mice, hypercholesterolemia increased ($P < 0.05$) protein expressions of NAD(P)H oxidase subunits p67^{phox}, p47^{phox} and gp91^{phox}, eNOS uncoupling, and 4-HNE-modified proteins, and reduced ($P < 0.05$) P-VASP-Ser-239 expression in the penis. Apocynin treatment of LDLR-null mice preserved ($P < 0.05$) maximal intracavernosal pressure, and reversed ($P < 0.05$) the abnormalities in protein expressions of gp67^{phox} and gp47^{phox}, 4-HNE, P-VASP-Ser-239, and eNOS uncoupling in the penis. Apocynin treatment of WT mice did not affect any of these parameters. Protein expressions of P-eNOS-Ser-1177 and total eNOS were unaffected by hypercholesterolemia.

CONCLUSION—Activated NAD(P)H oxidase in the penis is an initial source of oxidative stress resulting in eNOS uncoupling, thus providing a mechanism of eNOS uncoupling and endothelial dysfunction in hypercholesterolemia-induced ED.

INTRODUCTION

Hypercholesterolemia resulting in atherosclerosis of the penile vasculature is one of the leading causes of vasculogenic erectile dysfunction (ED). Hypercholesterolemia in men and in cholesterol-fed animal models impairs endothelium-dependent^{1–6} and endothelium-independent relaxations^{1, 5} of the corpus cavernosum, decreases the cavernosal content of endothelial cells, alters the function of smooth muscle cells, and increases collagen content.^{1, 7–9} Reduced NO signaling in the hypercholesterolemic penis has been attributed to impaired endothelial and neuronal nitric oxide synthase (eNOS and nNOS) expressions,^{6, 10–12} reduced eNOS activity and nitric oxide (NO) production,^{10–16} increased caveolin-1 expression¹⁷ and its interaction with eNOS,¹⁸ and deranged cGMP signal transduction pathways.¹⁹

Impaired eNOS function and endothelial NO availability in the hypercholesterolemic vasculature have been mostly attributed to increased vascular superoxide production. Oxidative stress seems to contribute significantly to hypercholesterolemia-associated ED.^{2, 19} The corpus cavernosal tissue of cholesterol-fed animals exhibits increased production of ROS,^{2, 18, 19} which has been attributed to the activation of nicotinamide adenine dinucleotide phosphate (NAD[P]H) oxidase¹⁹ and eNOS uncoupling.¹⁸

NAD(P)H oxidase is one of the most important enzymes responsible for ROS formation in the vasculature. Experimental and clinical studies suggest a role for this enzyme in initiation and progression of atherosclerotic disease. NAD(P)H oxidase is an inducible electron transport system present in endothelial cells, smooth muscle cells, fibroblasts, and phagocytic mononuclear cells, and generates superoxide through the assembly of a multi-subunit protein complex utilizing NADH or NADPH as the electron donor. The enzyme is composed of 2 transmembrane subunits (p22^{phox} and gp91^{phox}, or its homologues Nox 1–5, Duox1, or Duox2, which together form the cytochrome b₅₅₈) and 4 cytosolic subunits (p47^{phox}, p67^{phox}, p40^{phox}, and a GTPase Rac1 or Rac2), which assemble in the membrane upon activation by proatherosclerotic stimuli such as angiotensin II, mechanical stretch, and proinflammatory cytokines.²⁰ Superoxide anion may directly inactivate NO and decrease its bioavailability. Moreover, the reaction of superoxide anion and NO results in the formation of reactive nitrogen species such as the highly toxic molecule peroxynitrite. Peroxynitrite may cause oxidative damage to DNA, proteins and lipids, eNOS uncoupling, promote release of vasoconstrictors, increase apoptosis, and cause tissue injury and inflammation.²¹ Increased oxidative stress may promote atherosclerotic disease through the oxidation of LDL, the major carrier of plasma cholesterol. Vascular cells take up oxidized LDL via scavenger receptors, and oxidized LDL itself increases superoxide anion generation via the induction of NAD(P)H oxidase, xanthine oxidase, mitochondrial electron transport chain, and uncoupled eNOS, leading to further ROS generation. These early events in the pathogenesis of atherosclerosis are followed by plaque propagation, which involves vascular smooth muscle cell proliferation, apoptosis and necrosis, resulting in calcification, plaque rupture and formation of thrombus.²² While several studies demonstrated that NAD(P)H oxidase is present in the penis and upregulated in ED,^{19, 23–27} the significance of NAD(P)H oxidase in the penis as it relates to hypercholesterolemia-associated erectile pathophysiology in vivo is not known.

Another major mechanism for ROS production in vascular diseases is uncoupled eNOS. eNOS uncoupling is associated with increased superoxide anion formation from the enzyme, as well as reduced NO production, predisposing to atherosclerosis.²⁸ The underlying mechanisms of eNOS uncoupling have been attributed to decreased availability of the eNOS substrate L-arginine, deficiency in an eNOS cofactor tetrahydrobiopterin (BH₄), and oxidation and nitrosylation of the zinc thiolate cluster of eNOS.²⁸ While several studies in

the vasculature,^{29–32} including that of the penis,¹⁸ have confirmed that eNOS uncoupling occurs in vivo contributing to oxidative stress and endothelial dysfunction associated with hypercholesterolemia, the physiologic significance of eNOS uncoupling to penile erection, and the mechanisms underlying eNOS uncoupling in the penis, and specifically in hypercholesterolemia-induced ED, are unknown.

AIMS

Our hypothesis in the present study was that hypercholesterolemia activates NAD(P)H oxidase in the penis, providing an initial source of ROS to induce eNOS uncoupling and endothelial dysfunction resulting in ED. For this investigation, we used a well-established LDL receptor (LDLR)-null mouse as an animal model of homozygous familial hypercholesterolemia, characterized by an absence of functional LDL receptors.³³ These mice show delayed clearance of triglyceride-rich lipoproteins and elevated circulating cholesterol levels (similar to levels in humans), and develop human-like atherosclerotic lesions upon being fed a high cholesterol diet.³⁴ Importantly, this model resembles the human condition of dysfunctional high-density lipoprotein (HDL) being unable to protect LDL against oxidation, which may be more relevant than elevated HDL cholesterol levels in the development of atherosclerotic lesions.³⁵

MATERIALS AND METHODS

Animals

2-month-old male LDLR-null (LDL^{tm1Her}) mice on a C57BL/6 background were obtained from Jackson Laboratory (Bar Harbor, Maine). LDLR-null mice were fed Western diet (Tekland, Madison, WI, diet No. 88137, containing 0.15% cholesterol) for 4 weeks to induce early-stage hyperlipidemia.³⁶ A group of LDLR-null mice and WT mice were treated with an NAD(P)H oxidase inhibitor apocynin (10 mM, 8.3 mg/day in drinking water,²⁷ Calbiochem, San Diego, CA) during the 4-week feeding period. Wild type mice fed regular chow diet served as controls. All experiments were conducted in accordance with the ethical standards of the Johns Hopkins University School of Medicine Guidelines for the Care and Use of Animals.

Physiologic Erection Studies

To monitor intracavernosal pressure (ICP), animals were anesthetized with intraperitoneal (i.p.) injection of 50 mg/kg Ketamine/5 mg/kg Xylazine. The shaft of the penis was denuded of skin and fascia, and the left corpus cavernosum was perforated with a 25-gauge needle connected via PE-50 tubing to a pressure transducer (DI-190; Dataq Instruments, Akron, OH). For electrically stimulated penile erections, a bipolar electrode attached to a Grass Instruments S48 stimulator (Quincy, MA) was placed around the cavernous nerve as described previously.³⁷ Stimulation parameters were 2V at a frequency of 16 Hz with square-wave duration of 5 msec for 1 min. Submaximal stimulation parameters were chosen because maximal stimulation may obscure the erectogenic effect of different stimulatory factors. Response parameters (maximal ICP above baseline and ICP area above baseline) were calculated using MATLAB software (Mathworks, Natick, MA). Because of concern about the viability of mice with extensive procedures, cannulations of the right carotid artery to monitor systemic blood pressure (mean arterial pressure [MAP]) were performed in a separate set of animals.

Western blot analysis

Minced penile tissue was homogenized and partially purified for NOS as described.³⁸ Purified NOS samples (for phospho [P]-eNOS-Ser-1177 and eNOS uncoupling) or penile

homogenates (for other signals) were resolved on 4–20% Tris gels and transferred to polyvinylidene difluoride membrane. Membranes were probed with polyclonal rabbit anti-P-eNOS (Ser-1177) antibody at 1:450, rabbit anti-P-vasodilator-stimulated-protein (VASP) (Ser-239) antibody (both from Cell Signaling Technology, Beverly, MA) at 1:500, rabbit anti-4-hydroxy-2-nonenal (HNE) antibody at 1:2,000 (Alpha Diagnostic International, San Antonio, TX), rabbit anti-p47^{phox} antibody at 1:1,000 (Upstate Biotechnology inc, Lake Placid, NY), mouse anti-p67^{phox} antibody at 1:600, mouse anti-gp91^{phox} antibody at 1:1,000, or mouse anti-eNOS antibody (all from BD Transduction Laboratories, San Diego, CA, USA) at 1:1,000 dilutions. Membranes used for P-eNOS and P-VASP analyses were then stripped and probed with antibodies for the index proteins, mouse anti-eNOS (BD Transduction Laboratories) or rabbit anti-VASP (Cell Signaling Technology), respectively, at 1:1,000 dilutions. Other signals were standardized to β -actin. For the immunoblot analysis of the dimeric and monomeric forms of eNOS, purified samples in Laemmli buffer were not heated, and the temperature of the gel was maintained below 15°C during electrophoresis (low-temperature SDS gel electrophoresis).¹⁸ Membranes were then probed with rabbit anti-eNOS antibody (BD Transduction Laboratories) at 1:1,000 dilution. Bands were detected by horseradish peroxidase conjugated anti-mouse or anti-rabbit antibodies (GE Healthcare, UK), and analyzed using National Institutes of Health Image software. eNOS uncoupling was represented inversely as a ratio of active eNOS dimers to inactive eNOS monomers. Results were expressed relative to WT mouse treated with vehicle.

Statistical Evaluation

Statistical analysis was performed by using the t-test for testing hypotheses for significant differences in mean values for a variety of variables between pairs of groups. The data were expressed as the mean $\pm$ standard error of the mean (SEM). A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Blood lipid concentrations and blood pressure

Four weeks of Western diet significantly ($P < 0.05$) increased plasma cholesterol, triglycerides, and LDL in LDLR-null mice compared to WT mice, with apocynin having no effect on blood lipid concentrations in WT or hypercholesterolemic LDLR-null mice (Table 1). Blood pressure was not different in LDLR-null mice with and without apocynin treatment compared to WT mice (Table 1).

Effect of hypercholesterolemia and apocynin treatment on erectile response

At submaximal voltage (2V), erectile response, expressed as maximal ICP and total ICP (ICP area under the curve), was significantly ($P < 0.05$) reduced in LDLR-null mice fed a high cholesterol diet for 4 weeks compared to control WT mice. Concomitant treatment with apocynin, an NA(D)PH oxidase inhibitor, significantly ($P < 0.05$) increased maximal ICP in LDLR-null mice to the levels found in WT mice, and slightly, but not significantly, increased total ICP in these mice. Apocynin did not affect erectile response in WT mice fed regular chow (Table 2).

Effect of hypercholesterolemia and apocynin treatment on protein expressions of NADPH oxidase subunits

Protein expressions of p67^{phox}, p47^{phox}, and gp91^{phox} were significantly ($P < 0.05$) increased in penes of LDLR-null mice fed a high cholesterol diet compared to WT mice on regular chow. Treatment of LDLR-null mice during high cholesterol feeding with apocynin, which inhibits the assembly of the subunits within the membrane NAD(P)H oxidase complex, and/

or decreases protein expressions of NAD(P)H oxidase subunits,^{39, 40} significantly ($P<0.05$) decreased protein expressions of p67^{phox} and p47^{phox}, but not gp91^{phox} (Figure 1). Apocynin treatment of WT mice on normal-fat diet did not affect protein expression of any of the NAD(P)H oxidase subunits.

Effect of hypercholesterolemia and apocynin treatment on eNOS uncoupling

The ratio of eNOS dimer (functional eNOS)/monomer (non-functional eNOS), inversely related to eNOS uncoupling, was significantly ($P<0.05$) decreased in penes of LDLR-null mice fed a Western diet relative to levels found in WT mouse penes (Figure 2). Apocynin treatment significantly ($P<0.05$) increased the dimer/monomer ratio in penes of LDLR-null mice to levels which were significantly ($P<0.05$) higher than that found in WT mice. Apocynin treatment of WT mice on normal-fat diet did not affect this ratio.

Effect of hypercholesterolemia and apocynin treatment on 4-HNE-modified proteins

4-HNE is a major product of lipid peroxidation of polyunsaturated fatty acids, which covalently binds to cysteine, lysine, and histidine residues of proteins, causing the modification and malfunction of proteins.⁴¹ Thus, the expression of 4-HNE adducts is an index of protein peroxidation and a biomarker for oxidative damage. Compared with the pattern in WT mice, the amount of 4-HNE-modified proteins was significantly ($P<0.05$) increased in penes of LDLR-null mice fed a Western diet (Figure 3). Apocynin significantly ($P<0.05$) decreased 4-HNE expression in the penis of LDLR-null mice fed a high cholesterol diet, and had no effect on 4-HNE expression in penes of WT mice on regular chow.

Effect of hypercholesterolemia and apocynin treatment on NO/cGMP signaling pathway

The phosphorylation state of a VASP on Ser-239, a substrate of protein kinase G-I, is a surrogate parameter of the integrity of the NO/cGMP signaling pathway, shown previously to closely follow changes in endothelial function and vascular oxidative stress.^{42, 43} The levels of P-VASP were significantly ($P<0.05$) decreased in penes of LDLR-null mice fed a Western diet (Figure 4). Apocynin treatment significantly ($P<0.05$) increased P-VASP expression in penes of LDLR-null mice fed a high cholesterol diet, and had no effect on P-VASP expression in WT mice on regular chow.

Effect of hypercholesterolemia on eNOS and P-eNOS (Ser-1177) expressions

P-eNOS (Ser-1177) and eNOS protein expressions in the penis of LDLR-null mice fed a Western diet did not differ from the values in WT mice on normal chow (Figure 5).

DISCUSSION

The present study demonstrated that hypercholesterolemia increased protein expression of NAD(P)H oxidase subunits p47^{phox}, p67^{phox}, and gp91^{phox}, and induced eNOS uncoupling in the penis, resulting in increased oxidative stress, increased markers of endothelial dysfunction, and ED. These findings provide evidence that oxidative stress generated by both NAD(P)H oxidase and eNOS uncoupling underlies ED associated with hypercholesterolemia. Furthermore, pharmacologic inhibition of NAD(P)H oxidase by apocynin both preserved endothelial function by preventing eNOS uncoupling and decreasing oxidative stress in the hypercholesterolemic penis and preserved maximal ICP, a measure of maximal extent of erectile response, although not ICP area, in the face of induced hypercholesterolemia. These findings indicate that upregulated NAD(P)H oxidase is the initial source of oxidative stress resulting in eNOS uncoupling, thus providing a mechanism of eNOS uncoupling in the penis in hypercholesterolemia-induced ED. The lack of reversal of ICP area by apocynin implies that other mechanisms, besides NAD(P)H

oxidase-derived oxidative stress and NO-dependent endothelial dysfunction, also contribute to ED associated with hypercholesterolemia. Such mechanisms may include increased vasoconstriction,¹¹ reduced production of growth factors,^{12, 14, 17, 44} decreased cavernosal content of smooth muscle cells, endothelial cells, and elastic fibers,^{1, 7–9} increased oxidative stress by sources other than NAD(P)H oxidase, or derangements in endothelial cell-to-cell junctions.⁴⁵

NAD(P)H oxidases, uncoupled eNOS, and mitochondrial respiratory chain, are thought to be the major sources of superoxide production in the hypertensive, atherosclerotic, and diabetic vasculature.^{20, 28} Increased activation of vascular NAD(P)H oxidases and/or increased expression of its subunits has been implicated in the initiation and progression of atherosclerosis in animal models and hypercholesterolemic patients. mRNA and protein expressions of gp91^{phox} and p22^{phox} were increased in atherosclerotic human arteries compared to nonatherosclerotic arteries, and correlated with the severity of atherosclerosis.^{46, 47} In hypercholesterolemic patients reduced endothelial vasorelaxation of saphenous vein and increased vascular NAD(P)H oxidase activity were associated with increased clinical risk factors for atherosclerosis.⁴⁸ The expression of p47^{phox} was increased in the atherosclerotic aorta of hyperlipidemic ApoE-null mice,⁴⁹ while a loss of p47^{phox} in apolipoprotein (apo) E-null mice resulted in a reduction in aortic lesion formation and oxidative stress.^{50, 51} In addition, lower NADPH production in glucose-6-phosphate dehydrogenase deficiency reduced NAD(P)H oxidase-derived superoxide anions and reduced growth of aortic lesion in high-fat fed ApoE-null mice.⁵² Corpus cavernosal tissue of cholesterol-fed animals exhibited increased production of superoxide anion, which was reversed by NAD(P)H oxidase inhibitors, indicating NAD(P)H oxidase as a possible source of ROS.^{2, 19} In the present study we demonstrated increased expression of the cytosolic subunits p47^{phox} and p67^{phox} and the membrane subunit gp91^{phox} of the NAD(P)H oxidase in the penis of hypercholesterolemic LDLR-null mice, expanding these previous findings. Furthermore, we demonstrated that upregulation of NAD(P)H oxidase is directly associated with ED in the context of hypercholesterolemia.

In accordance with our previous findings that high-fat diet induced eNOS uncoupling in the pig penis,¹⁸ we now show that eNOS is uncoupled in the penis of hypercholesterolemic LDLR-null mice. We further extended these observations by providing data from functional studies showing that eNOS uncoupling in the penis is associated with ED in the context of hypercholesterolemia. Apparently, activation of both NAD(P)H oxidase and eNOS uncoupling contributes to endothelial dysfunction (downregulation of P-VASP) and increased oxidative stress (upregulation of 4-HNE-modified proteins) in the penis, and ED. The lack of effect of hypercholesterolemia on protein expressions of total eNOS in the penis of hypercholesterolemic LDLR-null mice confirms previous findings^{12, 14, 15, 18} that eNOS dysfunction is not due to downregulation of the enzyme's abundance. The lack of effect of hypercholesterolemia on eNOS phosphorylation on Ser-1177 in the penis of hypercholesterolemic LDLR-null is in agreement with our previous studies in high-fat fed pigs,¹⁸ as well as with in vitro studies in human umbilical vein and bovine aortic endothelial cells,^{53, 54} which demonstrated that the impairment of eNOS is due to downregulation of functional activity, but not necessarily catalytic activity of the enzyme. We contrast these findings with those of Ryu et al.,^{14, 15} Park K et al.,¹¹ and Xie et al.,¹² who found decreased eNOS phosphorylation at Ser-1177 in the penes of cholesterol fed rats and rabbits. This disparity may pertain to different stages of atherogenesis progression and use of different animal models.

A major cause of eNOS uncoupling involves deficiency of the eNOS cofactor BH₄.⁵⁵ In vascular disease states superoxide derived from NAD(P)H oxidase or xanthine oxidase (so-called "kindling radicals") may combine with NO formed by a still functional eNOS,

leading to an increased formation of peroxynitrite. Peroxynitrite may oxidize BH₄ resulting in eNOS dimer destabilization and electron transfer to molecular oxygen instead of L-arginine with consequent production of superoxide (so-called “bonfire radical”), thus perpetuating a cycle of oxidative stress and sustaining endothelial dysfunction.⁵⁶

In order to determine whether eNOS uncoupling in the hypercholesterolemic mouse penis may be due to NAD(P)H oxidase-derived ROS, we treated these mice with apocynin during high-cholesterol feeding. Apocynin is a selective NAD(P)H oxidase inhibitor, which hampers the assembly of the p47^{phox} and p67^{phox} subunits within the membrane NAD(P)H oxidase, and/or decreases protein expression of NAD(P)H oxidase subunits.^{39, 40} We found that apocynin normalized endothelial function in the penis and preserved erectile response by decreasing protein expression of NAD(P)H oxidase subunits p47^{phox} and p67^{phox}, preventing eNOS uncoupling, and decreasing oxidative stress. These data imply that the source of ROS for eNOS uncoupling in the penis is NAD(P)H oxidase, providing a molecular mechanism of vascular ROS production and impairment of eNOS function in hypercholesterolemia-induced ED. Future studies will determine whether hypercholesterolemia oxidizes BH₄ thus shifting the BH₄/BH₂ ratio towards decreased BH₄ availability in the penis. The fact that apocynin decreased protein expressions of NAD(P)H subunits p47^{phox} and p67^{phox}, but not gp91^{phox}, implies that specific NAD(P)H subunits may modulate endothelial dysfunction in specific vascular beds. Interestingly, apocynin not only prevented eNOS uncoupling in the penis of hypercholesterolemic mouse, but actually increased “coupled” eNOS function above the level found in WT, intact mice. While the reason for this is unclear, it may be due to non-specific actions of apocynin resulting in decreased ROS production from sources other than NAD(P)H oxidase which are activated by hypercholesterolemia. Inhibition of NAD(P)H oxidase with apocynin did not affect responses in WT mice, implying that NAD(P)H oxidase is not upregulated in the penis of healthy WT animals.

LDLR-null mice fed a high cholesterol diet for 4 weeks exhibit very high blood lipid levels, as shown in this study and other studies.⁵⁷ While these levels are much higher than in hypercholesterolemic men, no animal model precisely mimics the human condition. Similar serum lipid levels have been reported previously in other mouse models of hypercholesterolemia-induced ED and endothelial dysfunction.^{58, 59}

CONCLUSIONS

Hypercholesterolemia increased protein expression of NAD(P)H oxidase subunits and induced eNOS uncoupling in the penis, resulting in increased oxidative stress, endothelial dysfunction, and ED. Apocynin exerted a protective effect on endothelial function in the penis and erectile function through inhibition of NAD(P)H oxidase-dependent eNOS uncoupling.

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Figure 1.

Effect of hypercholesterolemia and apocynin treatment on protein expressions of NAD(P)H oxidase subunits in the penis. A, D, and G are representative Western immunoblots of the corresponding NAD(P)H oxidase subunits in penes of WT, LDLR-null (LDLR^{-/-}), and LDLR-null mice treated with apocynin. B, E, and H are representative Western immunoblots of the corresponding NAD(P)H subunits in penes of WT mice and WT mice treated with apocynin. Lower panels (C, F, I), represent quantitative analysis of NAD(P)H oxidase subunits p67^{phox}, p47^{phox}, and gp91^{phox} in penes of WT, WT treated with apocynin, LDLR-null, and LDLR-null mice treated with apocynin. Hypercholesterolemic LDLR-null mouse penes exhibited increased protein expressions of p67^{phox}, p47^{phox}, and gp91^{phox} compared to WT mouse pig penes. Apocynin prevented increase in p67^{phox} and p47^{phox}, but not gp91^{phox} protein expression. a, P < 0.05 vs. WT; N = 4–8.

Figure 2.

Effect of hypercholesterolemia and apocynin treatment on eNOS dimers/monomers in the penis. A is a representative Western immunoblot of the dimers/monomers in penes of WT, LDLR-null (LDLR^{-/-}), and LDLR-null mice treated with apocynin. B is a representative Western immunoblot of the dimers/monomers in penes of WT and WT mice treated with apocynin. C represents quantitative analysis of eNOS dimers/monomers in penes of WT, WT treated with apocynin, LDLR-null, and LDLR-null mice treated with apocynin. The ratio of eNOS dimers to monomers was reduced (thus eNOS uncoupling increased) in hypercholesterolemic LDLR-null mouse penes compared to WT mouse penes, while apocynin increased “coupled” eNOS function in the penis of hypercholesterolemic mice above the levels found in WT mice. a, $P < 0.05$ vs. WT; $N = 4-8$.

Figure 3.

Effect of hypercholesterolemia and apocynin on 4-HNE-modified proteins in the penis. (A) is a representative Western immunoblot of 4-HNE in penes of WT, WT treated with apocynin, LDLR-null (LDLR^{-/-}), and LDLR-null mice treated with apocynin. (B) is a quantitative analysis of 4-HNE-modified proteins in penes of WT, WT treated with apocynin, LDLR-null, and LDLR-null mice treated with apocynin. The analysis of 4-HNE is a densitometric composite of all proteins in each lane. 4-HNE expression was increased in hypercholesterolemic LDLR-null mouse penes compared to WT mouse penes, and this was prevented by apocynin. a, $P < 0.05$ vs. WT; $N = 4-5$.

Figure 4.

Effect of hypercholesterolemia and apocynin on P-VASP in the penis. (A) is a representative Western immunoblot of P-VASP in penes of WT, WT treated with apocynin, LDLR-null (LDLR^{-/-}), and LDLR-null mice treated with apocynin. (B) represents quantitative analysis of P-VASP in penes of WT, WT treated with apocynin, LDLR-null, and LDLR-null mice treated with apocynin. P-VASP was reduced in hypercholesterolemic LDLR-null mouse penes compared to WT mouse penes, and this was prevented by apocynin. a, $P < 0.05$ vs. WT; $N = 5-7$.

Figure 5.

Effect of hypercholesterolemia on P-eNOS (Ser-1177) and total eNOS in the penis. Upper panels are representative Western blot data, and lower panels represent quantitative analysis of P-eNOS (Ser-1177; A) and eNOS (B) in penes of WT and LDLR-null (LDLR^{-/-}) mice. No statistically significant differences were found in the treatment group. N = 7.

Table 1

Plasma cholesterol, triglycerides, and total LDL levels, and blood pressure (BP) in WT (WT-Veh), WT treated with apocynin (WT-Apoc), LDLR-null (LDLR^{-/-}-Veh), and LDLR-null mice treated with apocynin (LDLR^{-/-}-Apoc). n=5–10

	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	LDL (mg/dl)	BP (mmHg)
WT-Veh	81.8 ± 2.2	81.0 ± 9.0	25.8 ± 2.1	83.5 ± 7.5
WT-Apoc	99.0 ± 3.7	114.0 ± 11.3	38.3 ± 3.5	ND
LDLR ^{-/-} -Veh	763.5 ± 128.7*	443.1 ± 50.9*	664.9 ± 122.8*	90.8 ± 5.8
LDLR ^{-/-} -Apoc	809.8 ± 197.2*	390.7 ± 81.2*	913.3 ± 121.3*	104.7 ± 7.7

* P<0.05 vs. WT-Veh

ND, not determined

Table 2

Effect of hypercholesterolemia and apocynin treatment on erectile response to electrical stimulation of the cavernous nerve at submaximal (2V) parameters. Erectile response was reduced in hypercholesterolemic LDLR-null mice (LDLR^{-/-}) compared to WT mice; the decrease in ICP (but not ICP area) was prevented by apocynin. N = 5–7.

	Maximal ICP (mmHg)	ICP area
WT-Veh	36.4 ± 2.3	1405.2 ± 36.5
WT-Apoc	40.8 ± 11.8	1314.7 ± 280.6
LDLR ^{-/-} -Veh	24.2 ± 3.1 *	969.1 ± 127.7 *
LDLR ^{-/-} -Apoc	34.2 ± 2.9 **	1160.3 ± 78.8

* P<0.05 vs. WT-Veh

** P<0.05 vs. LDLR^{-/-}-Veh