Contribution of EDRF and EDHF to restoration of endothelial function following dietary restrictions in hypercholesterolemic rats

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The mechanisms underlying the impairment of endothelium-mediated vasorelaxation induced by dietary hypercholesterolemia and the mechanisms of restoration of endothelial function following reintroduction of low cholesterol diet were evaluated. Feeding rats with high cholesterol diet induced hypercholesterolemia and high blood pressure. This was associated with reduced vasorelaxation in response to acetylcholine, isoproterenol, and adenosine. At the same time, exaggerated contractile responses to serotonin and phenylephrine were observed. Reintroduction of a normal diet to cholesterol fed rats resulted in significant normalization of blood pressure, serum lipid profile, relaxation and contractile responses. The contributions of endothelial derived relaxing factors (EDRF), endothelial derived contractile factors (EDCFs)/prostanoids, and endothelial derived hyperpolarising factor (EDHF) to the vasorelaxation in each group of animals were assessed. EDCFs constricting activity was increased in both cholesterol fed groups as compared to the control group. EDRF and EDHF were found to be the primary factors involved in the regulation of endothelium-mediated responsiveness. In control animals, EDRF was responsible for 70-90% of relaxation, depending on the agonist used. In cholesterol fed animals, EDRF was significantly reduced while EDHF was maintained or enhanced showing that EDHF had a significant role in maintaining the endothelial responses. Importantly, the restoration of vasorelaxation following reintroduction of normal diet was mediated not only by improvement of EDRF-dependent relaxation, but also to a significant extent by a further increase in EDHF-mediated relaxation. Taken together, the data showed that EDRF was attenuated during hypercholesterolemia and dietary interventions with low fat content restored these responses. However, EDHF-mediated responses were not reduced by hypercholesterolemia and subsequently improved their function after application of low cholesterol diet. The results implicate EDHF-mediated relaxation is also an important mechanism for restoration of endothelial function upon application of dietary restrictions for reduction of serum cholesterol level.

Keywords: 5-Hydroxytryptamine, Acetylcholine, Adenosine, Endothelium, Hypercholesterolemia, Phenylephrine, Rat, Serotonin,

Hypertension and atherosclerosis are major risk factors leading to cardiovascular disabilities and therefore, remain a continuing challenge to public health. These disorders are modulated by various dietary factors. High dietary cholesterol intake induces hypercholesterolemia which is associated with impairment of endothelial functions and elevated blood pressure1,2. Endothelial dysfunction plays a critical role in the pathogenesis of atherosclerosis3,4. Endothelium participates in regulation of vascular function through its ability to synthesize and release different vasoactive substances including nitric oxide (NO), also referred to as endothelium derived relaxing factor (EDRF). Endothelium also generates contractile factors (EDCFs) like cyclooxygenase-derived arachidonic acid metabolites1,5. In addition to the EDRF and EDCFs, vascular responses are regulated by another endothelium-derived factor, which results in hyperpolarization of vascular smooth muscle. The exact nature and existence of endothelium derived hyperpolarizing factor (EDHF) remains to be elucidated6. It has been shown that EDHF-mediated responses are initiated by activation of K⁺ channels, which results in hyperpolarization of endothelial cells. This endothelial cell hyperpolarization spreads to the underlying smooth muscle layer through myoendothelial gap junctions7. The contribution of EDHFs to endothelium-dependent vasodilation is most apparent in resistance vessels8. Therefore, it is quite reasonable to speculate that any intervention leading to attenuation of synthesis or release of EDHF could critically affect the regulation

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of blood flow and may contribute to a pathophysiological state such as hypertension\textsuperscript{9}. Impaired vasorelaxation is one of the early manifestations of endothelial dysfunction under the hypercholesterolemic condition\textsuperscript{10}. This impairment could be attributed to various factors including a decrease in the release/availability of EDRF and/or EDHF and the inactivation of EDRF by oxygen derived free radicals. In addition, endothelium-dependent relaxation is counteracted by enhanced EDCFs production which could antagonize the relaxing action of EDRF and/or EDHF\textsuperscript{4,5}. Endothelial dysfunction is a reversible disorder\textsuperscript{11,12}. Interventions with lipid lowering drugs, hypertensive therapy, free radical scavengers and physical exercise have proven effective in restoring endothelial function and in slowing the progress of cardiovascular diseases\textsuperscript{13}. The effects of these therapies are mainly attributed to an enhanced production of EDRF. However, the significance and contribution of EDHF in vasoregulation has been the focus of fewer studies. Another important way to restore endothelial function is the dietary restriction of cholesterol consumption. Previous studies have shown the restoration of endothelial function using this approach with different animal models\textsuperscript{12,14}. However, the mechanism and pathways involved in the restoration of endothelial functions are yet to be clearly established.

In this communication, the blood vessel reactivity of healthy control animals, cholesterol fed animals, and cholesterol fed animals subjected to normal diet have been examined and role of endothelium-derived factors such as EDRF, EDHF, and EDCFs in the restoration of endothelial functions was determined.

**Materials and Methods**

*Animals*—All animal experiments were performed with ethical approval and within institutional biological research unit guidelines. Adult, healthy, albino Wistar rats of either sex, weighing between 200-300 g were used. All experimental animals were provided water and feed *ad libitum*. Animals were maintained on a balanced feed (Pranar Agro Industries Ltd, Delhi, India) and were divided into the following groups: normal control diet (group I), cholesterol (1% w/w) supplemented diet for 8 weeks (group II), and cholesterol supplemented diet for 8 weeks followed by control diet for 12 weeks (group IIA). The age of animals matched at the time of experiment to eliminate the influence of age on results. The ratio of male and female rats in each group was kept constant to minimize the gender influence on responses both in terms of magnitude and mechanism. The number of animals in group I, II and IIA were 10, 10 and 8, respectively.

**Lipid profile**—Serum total cholesterol, triglycerides, high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol were measured using enzymatic method employing commercial kits (Monozymes, India).

**Arterial blood pressure and heart rate**—Acute animal experiments were performed by previously described methods\textsuperscript{15}. Briefly, rats were anesthetized with chloralose (80 mg/kg, ip). Trachea was cannulated and animals were instrumented with catheters inserted into the femoral artery and vein (PE-50 tubing) for measuring arterial blood pressure and for intravenous administration of fluids. The arterial catheter was connected to a transbridge amplifier (TBM4, WPI, USA) and an oscilloscope (TDS 420A, Tektronix, USA) via a pressure transducer (Statham, P23 Db). An electrocardiogram (ECG) was recorded using bipolar silver electrodes. Heart rate was calculated from ECG tracings. Throughout the studies, the body temperature of the animal was maintained at 37\textdegree C with a water circulating pad.

**Vascular reactivity**—The rats were sacrificed with an overdose of pentobarbitone sodium (80 mg/kg body weight). Isometric tension of the thoracic aorta was measured as described earlier\textsuperscript{16}. The thoracic aorta was dissected, cut into small ring segments, mounted in a 10 ml organ bath containing Kreb’s buffer solution ([mM] NaCl -118.1, KCl -4.7, MgSO\textsubscript{4} -1.2, KH\textsubscript{2}PO\textsubscript{4} -1.2, CaCl\textsubscript{2} -0.5, NaHCO\textsubscript{3} -5.0, glucose -11.1) at 37\textdegree C with carbogen gas (95% O\textsubscript{2} + 5% CO\textsubscript{2}), and an initial pre-load of 2 g was applied. After 1 hr equilibration, vessels were precontracted with submaximal concentration of phenylephrine (PE) (10\textsuperscript{-6} M). Once contraction reached its plateau, agonists were added. In endothelium-denuded rings, endothelium was physically removed with a cotton swab and lack of responsiveness to acetylcholine was checked before studying concentration response.

**Experimental design**—Before the start of experimental protocols the responsiveness of endothelium was checked with ACh. For appropriate comparison the contraction produced by PE in all three group of animals were compared to study similar activation state of smooth muscle. The
concentration-response relationships were determined by cumulative addition of acetylcholine (ACh) (10⁻¹³-10⁻⁴ M), adenosine (10⁻¹³-10⁻⁴ M), isoproterenol (IP) (10⁻¹³-10⁻⁴ M), 5 hydroxytryptamine (5-HT) (10⁻¹³-10⁻⁴ M), sodium nitroprusside (SNP) (10⁻¹³-10⁻⁷ M) and PE (10⁻¹³-10⁻⁶ M) in aortic rings from all three groups of animals. Separate aortic rings were used to each agonist and the rings were thoroughly washed 3-4 times after each response curve to wash off the remnant effect. The concentration-response for ACh, IP and adenosine were obtained before and 30 min after treating the tissues with cyclooxygenase blocker, indomethacin (10⁻³ M), followed by indomethacin + nitric oxide synthase (NOS) inhibitor L-NMMA (100 μM). The extent of NO-mediated responses was confirmed by incubating the tissue with NOS substrate L-arginine (10⁻³ M). The concentration-response curves for ACh, IP and adenosine were also obtained after incubating the tissues with indomethacin + L-NMMA+KCl, which depolarizes endothelial cells and inhibits EDHF-mediated responses. In a parallel organ bath corresponding time control experiments were performed to eliminate time dependent variances on the results.

Drugs—The following drugs were used: Acetylcholine chloride, adenosine, isoproterenol, serotonin, sodium nitroprusside, L-arginine, phenylephrine hydrochloride, indomethacin and N⁴-monomethyl-L-arginine (L-NMMA) (Sigma Chemical Co USA). Indomethacin was dissolved in Na₂CO₃ (0.2 M) solution and all other drugs were dissolved in distilled water. Serial dilutions were made in triple distilled water.

Statistical analysis—The steady state contraction produced by 10⁻⁶ M PE was expressed as an increase in tension (g) from the resting level. The relaxant/contractile responses were expressed as a decrease/increase (percent) in steady state contraction; EC₅₀ was estimated graphically as the concentration causing 50% relaxation. Graph-pad Prism software was used for statistical analysis. The P value for statistical significance was set at 0.05. Student’s t-test for paired and unpaired data and analysis of variance (ANOVA) followed by Student-Neumen-Keul’s test were used wherever appropriate.

Results

Arterial blood pressure, heart rate, serum lipid profile—Since high cholesterol intake resulted in elevated arterial blood pressure, whether subsequent dietary restriction could improve cardiovascular function was tested first. High cholesterol diet led to an increased systolic, diastolic, and mean arterial pressure that was restored upon reintroduction of normal diet. A significant decrease in systolic pressure and mean arterial pressure was observed upon the withdrawal of high cholesterol diet (Table 1). No significant difference in heart rate among the different groups suggests that there was no cardiac abnormality in these animals. Concentrations of serum total cholesterol and triglycerides were significantly increased in cholesterol fed rats (Table 1). As anticipated cholesterol fed animals subjected to 12 weeks of normal diet showed significant improvements in their serum lipid profiles. However, it was still higher than control levels.

Vascular reactivity—Proatherogenic diets impair the endothelium-mediated responses to vasoactive substances. The hypothesis, that the withdrawal of high cholesterol from diet could restore the endothelial dependent vasorelaxation was tested and the mechanisms contributing to the restored vasorelaxation assessed. In order to address this question, the effects of major physiologically relevant vasoconstricting agents (e.g. ACh, IP, adenosine and SNP) using aortic rings preconstricted with PE as well as vasoconstricting agents (e.g. 5-HT and PE) were examined in the three experimental groups of animals. Contributions of EDRF, EDCFs, and EDHF were also assessed using specific inhibitors.
Response to acetylcholine—Acetylcholine produced a concentration-dependent relaxation in the control group (Fig. 1A). As anticipated, in endothelium-denuded aortic rings, the response to ACh was absent demonstrating that observed effects were endothelium-dependent (results not shown). A poor vasorelaxation was observed in cholesterol fed animals at all concentrations of ACh. Importantly, cholesterol fed animals subjected to normal diet showed a significant improvement in their response to ACh (Fig. 1A). These results demonstrate that lowering cholesterol through dietary restriction restores endothelial functions.

Next, the role of EDCF in this setting was assessed using the cyclooxygenase blocker, indomethacin. Indomethacin induced a significant increase in relaxation in all groups (Fig. 1B, 1C, 1D). However, indomethacin treatment was not able to relax the aortic rings from cholesterol fed animals to the level of the control, suggesting that the increased production of EDCF alone cannot explain the poor vasorelaxation in cholesterol fed group.

The contribution of EDRF to ACh-induced relaxation was assessed by treating the rings with L-NMMA, an inhibitor of NOS. L-NMMA caused a strong attenuation of ACh-induced relaxation in the vessels of all groups (Fig. 1B, 1C, 1D). In the control group, it inhibited 90% of ACh-induced relaxation. In contrast, L-NMMA inhibited only 50% of the ACh-induced relaxation in group II. These results suggest that the observed decrease in response to ACh in group II is due to a decrease in NO production, and proportionally, the non-NO dependent mechanisms are also important. In group IIa, the effect of L-NMMA was increased compared to control group, indicating the restoration of NO production upon cholesterol withdrawal. To test whether availability of L-arginine (the substrate for NOS) contributes to

![Fig. 1](image-url)
observed differences, the responses to ACh in the presence of excess of L-arginine was assessed. Vasorelaxation in group II was not completely restored, indicating that this was not the case.

Next, the contribution of EDHF was tested in this setting. KCl + L-NMMA completely inhibited ACh-induced relaxation meaning that relaxation can be mainly explained by the actions of EDHF and EDRF. However, in groups II and IIa the contribution of EDHF was higher as compared to control. These results implicate EDHF as important component acting to restore vasorelaxation upon cholesterol withdrawal.

Response to adenosine—Adenosine, a purinergic receptor agonist, exerts a vasodilatory effect and participates in the regulation of vascular tone in vivo. Adenosine produced a concentration-dependent biphasic response in aortic-rings from all groups (Fig. 2A). Lower concentrations produced weak contractions, whereas relaxation was observed at higher concentrations of adenosine. After removal of endothelium, relaxation was impaired, indicating partial endothelial dependence. In cholesterol fed animals, an attenuation of adenosine-induced relaxation was again noticed. However, reintroduction of normal diet to cholesterol fed animals resulted in a significant restoration of adenosine-induced vasorelaxation (Fig. 2A). These results demonstrated the advantageous effects of cholesterol withdrawal on purinergic receptor mediated-endothelial functions.

Indomethacin slightly increased adenosine-induced relaxation in all groups (Fig. 2B, 2C, 2D) revealing only a modest role of EDCFs in this setting. Next, the role of EDRF in adenosine-induced relaxation was examined (Fig. 2B, 2C, 2D). The attenuation of adenosine-induced relaxation by L-NMMA was much
stronger in control animals as compared to both groups of cholesterol-fed animals, indicating significantly and similarly reduced EDRF production in both groups. L-arginine reversed the effects of NO blockade with L-NMMA (Fig. 2B, 2C, 2D) confirming specificity of the effect of L-NMMA and indicating that attenuation of vasorelaxation in cholesterol-fed groups may not be caused by reduced availability of the substrate for NO.

Treatment of tissues with L-NMMA + KCl significantly abolished the adenosine-induced relaxation in all three groups of animals (Fig. 2B, 2C, 2D). Similar to results obtained with ACh, this revealed that the contribution of EDHF was much higher in cholesterol-fed animals as compared to the control. EDHF contribution was even further increased after reintroduction of cholesterol-free diet, demonstrating again a predominant role of EDHF in control of vasorelaxation in cholesterol-fed animals.

**Response to isoproterenol**—Beta-adrenergceptors play an important role in vasodilatation and help in maintenance of blood flow. Although IP is known to act mainly through endothelium-independent mechanism, cholesterol-fed animals showed lower relaxation in response to IP (Fig. 3A). This could be either due to damage of receptors on ECs or increase in contractile factors. Again, withdrawal of cholesterol from the diet led to a significant recovery in IP-induced relaxation. These results demonstrate a significant improvement in endothelial functions through β-adrenoceptor mediated signaling upon withdrawal of cholesterol.

Addition of EDCF inhibitor, indomethacin, enhanced the IP-induced relaxation in all three groups of animals (Fig. 3B, 3C, 3D). However, in cholesterol-fed animals the addition of indomethacin tended to have a more pronounced effect as compared to the control, suggesting that the production of EDCFs is significantly increased under hypercholesterolemic conditions.

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Fig. 3—Concentration response curve for isoproterenol: Cumulative concentration-response (10^{-14}-10^{-4} M) to isoproterenol in aortic rings contracted with phenylephrine (10^{-6} M). Percent increase in relaxation to precontracted endothelium–intact aortic rings from animals on normal diet (group I), on cholesterol supplemented diet for 8 weeks (group II) and on cholesterol supplemented diet for 8 weeks, followed by reintroduction of normal diet for 12 weeks (group IIa) [A]. Relaxant response to acetylcholine; control (•••), after incubation with indomethacin (10^{-5} M) (□), indomethacin+L-NMMA (100 μM) (○), indomethacin+L-NMMA+L-arginine (10^{-5} M) (■) and indomethacin+L-NMMA+KCl (10^{-6} M) (▲) in aortic rings, from rats on normal diet (group I) [B]; on cholesterol supplemented diet for 8 weeks (group II) [C], and on cholesterol supplemented diet for 8 weeks, followed by reintroduction of normal diet for 12 weeks (group IIa) [D]. Data represents mean ± SEM; 4 rings from each animal, *P<0.05 vs. group I, **P<0.05 vs. group II.
conditions. This tendency was also observed in ACh-induced vasorelaxation (Fig. 1).

The contribution of EDRF as assessed by attenuation of IP-induced relaxation with L-NMMA was stronger in the control as compared to cholesterol fed animals (Fig. 3B, 3C, 3D). These results demonstrate that in β-adrenoceptor mediated vasodilatation; EDRF is the main factor in control animals. However, under hypercholesterolemic conditions, the NO independent factor(s) also play an important role. Assessment of EDHF contribution demonstrated that in cholesterol fed animals, the EDHF component was larger than in the control group (Fig. 3B, 3C, 3D). The results demonstrated a distinct role of EDHF in restoration of β-adrenoceptor mediated vasodilatation with dietary modulation. This needs to be further confirmed in other vessel preparations.

Response to sodium nitroprusside—Nitroprussides are known to relax VSM through endothelium independent mechanisms. The response to sodium nitroprusside (SNP) was examined to find out whether relaxation due to VSM was preserved in cholesterol fed animals. The results demonstrated that SNP induced a strong and similar relaxant response in rings from all groups of animals (Fig. 4). The tissues relaxed completely even at lower concentrations of SNP (10^{-10} M). This result suggests that there was no damage to smooth muscle in cholesterol fed groups. It further suggests that impairment of vasorelaxation is caused by endothelial dysfunction.

Response to 5–hydroxytryptamine—Serotonin (5–hydroxytryptamine) plays an important role in modulation of vascular tone and activation of endothelial cells under stress. 5-HT produced a concentration-dependent vasoconstriction in all three groups of animals (Fig. 5). A strong augmentation of 5-HT-induced contraction was observed in cholesterol fed vessels as compared to control vessels (Fig. 5). Withdrawal of excess cholesterol (group IIa) resulted in an attenuation of 5-HT-induced contraction. A maximum of 111.6 ± 3.89% contraction was obtained with 10^{-4} M concentration of 5-HT in group IIa animals, as compared to a maximum in group II animals (130.91 ± 4.21%) (Fig. 5).

Response to phenylephrine—A concentration-dependent increase in tension was observed with PE in aortic rings, which was independent of endothelium. An enhanced contraction of aortic rings was seen in cholesterol fed animals as compared to animals on control diet (Fig. 6), suggesting the resetting of vascular tone upon hypercholesterolemia. However, reintroduction of normal diet resulted in an attenuation of PE-induced vasoconstriction. These results suggest that removal of cholesterol leads to the improvement of endothelial function not only by endothelium-mediated mechanisms, but also by reducing the contractile effects induced by underlying smooth muscle cells.
Discussion

Normalization of serum cholesterol level by dietary intervention results in restoration of endothelial function in animal models. In the present study, it was shown that restricting the cholesterol fed rats to low cholesterol diet restored the endothelial function impaired during hypercholesterolemia. The results demonstrate that EDRF plays a major role in the maintenance of the endothelium-mediated responsiveness of large blood vessels of rats in absence of hypercholesterolemia. In dietary-induced hypercholesterolemia, contribution of EDHF increases showing that both EDRF and EDHF play significant roles in regulation of vascular tone. Lowering the serum cholesterol levels by reducing dietary cholesterol intake induced the restoration of endothelial function and vasodilation response to nearly normal magnitudes. From this data it is hard to draw a conclusion that the changes in endothelial function attributed to changes in serum profile. The change in blood pressure may also contribute to the alteration in endothelial function. Improvement in vasorelaxation was mediated by increased EDRF production and, surprisingly, by a further increase in EDHF contribution. These results were similar when acetylcholine, adenosine or isoproterenol were used to induce vasodilation, indicating that pathways that mediate the observed effects are downstream of various receptors involved in vasodilation. The present results also suggest that removal of cholesterol leads to the improvement of endothelial function not only by endothelium-mediated mechanisms, but also by reducing the contractile effects induced by underlying smooth muscle cells.

The findings that dietary hypercholesterolemia greatly reduces EDRF production but does not inhibit or enhance EDHF–mediated vasorelaxation is in agreement with results from other groups. Kagota et al\textsuperscript{18} reported that excessive cholesterol intake in rats increases the release of EDHF-like factor and decreases that of NO\textsuperscript{18,19}. This result is not species and vessel type specific, since similar observations were made using rabbit renal artery\textsuperscript{19-21} and mesenteric arteries of apo E/− mice\textsuperscript{21,22}. Thus, it appears that EDHF production by EC is less sensitive to cholesterol and probably is even stimulated by hypercholesterolemia, compensating for reduced EDRF production and helping to maintain vasorelaxation. EDHF also plays a significant role in vasorelaxation in humans and compensates for deficient production of EDRF in such pathological states as hypoxia, heart failure, and glaucoma\textsuperscript{9,22,23}. The effect of hypercholesterolemia on EDHF in humans remains to be elucidated. A single study directly addressing this question reported that EDHF-mediated vasorelaxation is reduced in isolated gastroepiploic arteries from atherosclerotic patients\textsuperscript{23,24}. The difference between this result and the results of multiple animal studies may reflect that either the longer duration is needed for the development of hypercholesterolemia and/or the severity of atherosclerosis in humans.

The major findings of the present study are that EDHF contribution increased upon reintroduction of low cholesterol diet and that it plays a critical role in restoring vasorelaxation in conditions of mild hypercholesterolemia. Further work needs to be done to find out mechanisms of increased production of EDHF in hypercholesterolemia in general and its further increase after lowering of cholesterol level in particular. One potential explanation of the data is that moderately increased levels of serum cholesterol are optimal for EDHF production. Independent of the underlying mechanism, the present observation may have clinical significance, since dietary cholesterol restriction is a first clinical tool for reduction of serum cholesterol level. It appears that more attention should be directed at supporting EDHF production by EC. Interestingly, HMG-CoA reductase inhibitors that are commonly used for reduction of serum cholesterol

![Fig. 6](image-url)
and for prevention of atherosclerosis have beneficial effects on EDRF production even without lowering cholesterol level. However, they do not improve EDHF-mediated vasorelaxation in rabbit carotid or mesenteric arteries\textsuperscript{19,20,24,25} or even reduce EDHF in the rabbit renal artery\textsuperscript{20,21}. The EDCFs constricting activity was found to be more prominent in both groups of cholesterol fed animals as compared to the control group, probably contributing to impaired vasorelaxant response in these groups.

The rat has been considered a poor model for atherogenesis and it requires long exposure to a high cholesterol diet to develop atherosclerotic lesions. However, recent studies have suggested that rats could be more a appropriate model to study cardiovascular disease because aortic plaques that develop in hypercholesterolemic rats are very similar to those in humans\textsuperscript{26}. This model was chosen in the present study partially because we preferred to assess the effect of hypercholesterolemia on vascular tone when physical damage to the vascular wall is minimal. An intact endothelium with SEM was observed in cholesterol fed animals and a lack of accumulation of white blood cells in the subendothelial space as well as those associated with ECs. The findings suggest that the impairment of vascular responses in hypercholesterolemic animals is caused mainly by the functional damage to endothelium-mediated pathways and not by physical damage to endothelial cells. The nature of this functional damage is still unclear, but it is reflected in the decreased EDRF production.

Taken together, the present results indicate that EDHF has a prominent compensatory role in vasoregulation under hypercholesterolemic conditions where EDRF production is impaired. The restoration of endothelial function upon withdrawal of cholesterol from the diet is mediated through an increase in EDRF and EDHF production with shift of the equilibrium towards EDHF production/release. These results further support the growing appreciation for the role of EDHF in vasodilation under pathological conditions.

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