High Methionine and Cholesterol Diet Abolishes Endothelial Relaxation

Anthony Zulli, Robert E. Widdop, David L. Hare, Brian F. Buxton, M. Jane Black

Objective—High plasma cholesterol or homocysteine is a risk factor for atherosclerosis. Cholesterol and methionine, the precursor of homocysteine, are rarely eaten separately. Thus, the aims of this study were to determine neointima formation, aortic reactivity, and factors involved in endothelial function in rabbits fed high dietary cholesterol, methionine, or a combination of the two for 12 weeks.

Methods and Results—Rabbit dietary groups were randomized into the following: control (Con), 0.5% cholesterol (Chol), 1% methionine (Meth), and 1% methionine + 0.5% cholesterol (MethChol). Aortic reactivity was studied by isometric tension techniques, aortic volumetric analysis was determined by stereological techniques, and immunohistochemistry was used to localize endothelial and inducible NO synthases, superoxide dismutase, macrophages, and nitrotyrosine. Atherosclerosis was present in the Chol and MethChol groups. Endothelium-dependent relaxation was virtually abolished in the MethChol group compared with control. Such decrease in relaxation was not attributable to a vascular smooth muscle cell defect or to a decrease in endothelial NO synthase or superoxide dismutase content. Macrophages and inducible NO synthase immunoreactivity were present in Chol and MetChol groups.

Conclusions—The combination of high dietary cholesterol plus methionine virtually abolishes endothelium-dependent relaxation, underscoring the importance of multiple risk factors in the development of cardiovascular disease. (Arterioscler Thromb Vasc Biol. 2003;23:1358-1363.)

Key Words: cholesterol ■ homocysteine ■ endothelial function ■ plaque ■ nitric oxide

High plasma cholesterol1 or homocysteine2 are risk factors for cardiovascular disease. They can be brought about by genetic defects in cholesterol or homocysteine metabolism or through dietary regimens. Cholesterol and methionine, the precursor of homocysteine, are rarely ingested separately, because animal products such as eggs, meat, and milk contain both cholesterol and methionine. In fact, it is estimated that 30% of the population in the United States may have high plasma levels of homocysteine2 and more than 50% have high plasma levels of cholesterol.3 Thus, it is likely that there are individuals with high plasma levels of both risk factors.

Experimental,4–5 human,6–8 and murine genetic knockout studies (cystathionine β-synthase-deficient mice)9,10 in hypercholesterolemia and hyperhomocysteinemia show an impairment in endothelial-dependent relaxation to agonist stimulation in both conduit and resistance arteries, which has been partly linked to a decrease in the bioavailability of a major endothelium-derived relaxing factor, nitric oxide (NO). Because NO can inhibit the early events of atherogenesis,11 a decrease in the bioavailability of NO is thought to promote atherosclerosis. The mechanisms whereby cholesterol and homocysteine decrease NO bioavailability are not well understood; however, it is popular opinion that an excess availability of the oxygen free radical $O_2^-$ (oxygen stress) can scavenge NO, thus decreasing its bioavailability.12 Furthermore, the reaction of excess $O_2^-$ with excess NO, which may arise from the increased expression of inducible NO synthase (iNOS)13 from macrophages and smooth muscle cells, forms peroxynitrite (ONOO$^-$), which can nitrate the tyrosine residue of proteins forming nitrotyrosine.14 Thus, the identification of nitrotyrosine in the aortic wall by immunohistochemistry is a marker of nitrosative stress.15 This is evidenced by a study from Eberhardt et al,9 who have shown that high plasma levels of homocysteine in genetically modified mice leads to increased $O_2^-$ production, nitrotyrosine formation, and endothelial dysfunction. As well, a decrease in the $O_2^-$ scavenger superoxide dismutase (SOD) may also increase $O_2^-$ levels.16

In the present study, we tested the hypothesis that the combination of high dietary cholesterol and methionine would exacerbate the development of atherosclerosis and endothelial dysfunction compared with either agent alone. To this end, a stereological approach was used to quantitate the volume of the intima and media within a defined anatomical site of the abdominal aorta. This approach was used to avoid sampling errors attributable to plaque inhomogeneity. In
The combination of cholesterol and methionine, whereas hyperhomocystinemia was only present in the Meth group. Interestingly, the combination of cholesterol and methionine did not significantly increase plasma homocysteine levels compared with control.

Methods

Animals

Male New Zealand White rabbits at 3 months of age were purchased from Animal Services, Monash University, Gippsland Campus, Victoria, Australia, and were randomly allocated into 4 groups and fed their respective diet for 12 weeks (n=8/group). Group 1 (control) was fed a normal rabbit chow diet; group 2 (Chol) received a normal rabbit chow diet supplemented with 0.5% cholesterol; group 3 (Meth) received a normal rabbit chow diet supplemented with 1% methionine; and group 4 (MethChol) received a normal rabbit chow diet supplemented with 1% methionine+0.5% cholesterol. The animals were housed in individual cages and maintained at a constant temperature of approximately 21°C. Food and water was supplied ad libitum. The experiments were approved by the Monash University, Department of Anatomy Ethics Committee and were carried out according to the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (6th Edition, 1997).

For information on blood sampling and analysis, tissue collection, quantification of plaque, functional reactivity studies, immunohistochemistry, and data analysis, please see the online supplement at http://atvb.ahajournals.org.

Results

Plasma Cholesterol and Homocysteine Levels

The total plasma homocysteine, cholesterol, cholesterol/HDL ratio, and triglyceride levels are shown in the Table. Hypercholesterolemia was present in the Chol and MethChol groups, whereas hyperhomocystinemia was only present in the Meth group. Interestingly, the combination of cholesterol plus methionine did not significantly increase plasma homocysteine levels compared with control.

Body Weights

Please see the online data supplement.

Atherosclerosis, Intima, and Media Volumes

Sections of abdominal aorta from the Chol and MethChol groups showed a large amount of plaque compared with control and the Meth groups. The Meth group did not show plaque formation throughout the section of abdominal aorta studied; however, intimal thickening was prevalent throughout the vessel (Figure 1). The total volume of the intima was significantly increased in the cholesterol group (7.54±1.00 mm³, P<0.01) compared with control (1.32±0.21 mm³) and additionally increased in the combination group (8.42±1.99 mm³, P<0.001) compared with control. There was no significant difference in total intimal volume between the control and Meth groups (2.42±0.23 mm³) nor between the Chol and MethChol groups. The media volume of abdominal aortic sections showed no significant difference between groups, although there seemed to be an increase in the media volumes of Chol (13.71±1.23 mm³) and MethChol groups (11.06±0.92 mm³) compared with control (7.56±1.06 mm³) and Meth groups (9.03±1.07 mm³).

Intima to Media Ratio

The intima to media ratio in the animals fed high dietary cholesterol was significantly increased compared with control (0.54±0.05 versus 0.18±0.02, respectively, P<0.05), and
Endothelium-Dependent Relaxation

The maximum endothelium-dependent relaxation to acetylcholine in the control group was 70.0±5.0%, with a −logEC50 value of 7.21±0.11. In both the Chol and Meth groups, there was a decreased sensitivity to acetylcholine compared with control, as well as a trend for a reduction in the maximum relaxation evoked by acetylcholine. Strikingly, the MethChol group showed virtually no endothelium-dependent relaxation to acetylcholine compared with control, and in fact, some of the aorta showed contraction to acetylcholine rather than relaxation. Importantly, there were no differences between the groups in the magnitude of initial precontraction evoked by phenylephrine (Figure 2A). All −logEC50 and maximal responses are shown in online Table I, available at http://atvb.ahajournals.org.

Endothelium-Independent Relaxation

Endothelium-independent aortic relaxation was assessed by constructing a concentration response curve to the NO donor sodium nitroprusside (Figure 2B). The −logEC50 values indicate that the smooth muscle cell sensitivity to the vasodilator sodium nitroprusside was not affected by any of the treatments, although there was a trend for increased sensitivity and maximum responses in the MethChol group compared with control. All −logEC50 and maximal responses are shown in online Table I.

Contractile Function

To determine whether the dietary regimens affected smooth muscle cell sensitivity to vasoconstrictors, aortic contraction was assessed by constructing a concentration response curve to the α1-adrenergic receptor agonist phenylephrine. All −logEC50 and maximal responses are shown in online Table I. Maximal contractions to phenylephrine were not significantly different between groups, although phenylephrine was less sensitive in both the Meth and MethChol groups compared with control, indicating parallel rightward shifts of the CR curves to phenylephrine in these groups (Figure 2C). Thus, these results indicate that there is a decrease in the smooth muscle cell sensitivity to the vasoconstrictor phenylephrine.

For information on the immunolocalization of eNOS, iNOS, SOD, nitrotyrosine, and macrophages, please see the online data supplement.

Discussion

The main findings of the present study are that the combination of high dietary cholesterol plus methionine virtually abolished endothelium-dependent relaxation, which was not attributable to a decrease in eNOS protein or SOD protein, and that the combination of high dietary cholesterol plus methionine did not additionally exacerbate the level of atherosclerosis compared with each dietary supplement alone.

In this study, high dietary methionine feeding for 12 weeks did not lead to atherosclerosis formation in the abdominal aorta; however, intimal thickening was pronounced throughout the vessel, which may indicate the early pathological changes in the atherosclerotic cascade. These results are in accordance with a previous study by Toborek et al.17 where aortic intimal thickening was the only pathological change in 8 of 10 rabbits fed a 0.5% methionine diet for 6 or 9 months,
whereas atherosclerosis was present in the other 2 rabbits studied.

There are many studies that have reported the effect of high dietary cholesterol on the development of atherosclerosis. However, in these earlier studies, the effects of high plasma cholesterol on the development of aortic atherosclerosis, as described in this study, have been previously investigated using alternate approaches, such as the oil-red O technique.18,19 However, these en face preparations of tissue do not allow for the volumetric quantification of plaque or the encroachment on the lumen, nor do they detect intimal proliferation. Other investigators have quantitated atherosclerosis formation in paraffin-embedded sections, although this method of processing causes variable degrees of tissue shrinkage and thus complicates the interpretation of the end results. The present study is novel because we chose a stereological approach to quantitate atherosclerosis along a section of the abdominal aorta between 2 anatomically defined sites, and to minimize tissue shrinkage, all tissue samples were processed and embedded in glycolmethacrylate. Because the formation of plaque is heterogenous along the artery wall, using a stereological approach, we were able to prevent either an overestimation or underestimation of plaque volume when only arterial cross-sectional measurements were made. The degree of atherosclerosis in the blood vessel wall was not significantly different between the rabbits fed the high cholesterol diet and the combined cholesterol and methionine diet, suggesting that high dietary methionine may not additionally exacerbate plaque development, at least at the doses used in the present study. However, we cannot exclude the fact that atheroprotective enzymes might be highly expressed in the aortae of the combination group, which could mask the detrimental effects of the combination diet on plaque content. Indeed, recent evidence suggests that the potent antioxidant heme oxygenase-1 is highly expressed in human and animal plaques.20 If high levels of heme oxygenase-1 are found in the plaques of the combination group, this would inhibit additional atherosclerosis formation.

In these same vessels, we examined endothelial function. As expected, there was modest endothelial dysfunction in the cholesterol-fed group as seen by the rightward shift in the concentration response curve to acetylcholine compared with control. Our findings support previous studies of hypercholesterolemia4,7 on endothelial function. Likewise, a similar impairment was observed in the methionine-fed group despite unconfirmed atherosclerosis, and this is supported by other animal5 and human studies.6 In contrast to the modest endothelial impairment of each diet alone, we have shown for the first time the combination of high dietary cholesterol plus methionine administered abolished endothelium-dependent relaxation to acetylcholine in the abdominal aorta. Such dysfunction seemed to be at the endothelial cell layer rather than a direct effect on smooth muscle cell reactivity, because the response to the NO donor, sodium nitroprusside, was not impaired. As well, both high dietary methionine and the combination of high dietary methionine plus cholesterol decreased the sensitivity of the smooth muscle cells to α1-adrenergic stimulation, represented by the increase in the EC50 to phenylephrine, but not the maximal response, evoked by phenylephrine compared with control. In support of these results, a study by Alexandre et al21 showed that smooth muscle cell contractility to phenylephrine was decreased when NO bioavailability was reduced by long-term NOS inhibition in the rat. Interestingly, aortic contractile responses to other vasoconstrictors such as angiotensin II22 and thromboxane A2 analogs23 are not significantly altered by hypercholesterolemia. The mechanisms whereby reduced NO bioavailability differentially affects vasoconstriction is not clear.

The vasoconstriction to acetylcholine observed in the abdominal aorta of the rabbits fed the combination of high dietary cholesterol plus methionine is similar to the vasoconstriction described in arteries from human subjects with coronary artery disease.8 For example, in an investigation by Ludmer et al,8 vasoconstriction rather than vasorelaxation was induced by acetylcholine in all human atherosclerotic coronary arteries examined. These studies indicate that complete endothelial dysfunction can also occur in humans.

However, it is unlikely that the decrease in endothelium-dependent relaxation seen in the abdominal aorta of animals fed high dietary cholesterol or methionine is attributable to a decrease in eNOS protein or SOD enzyme. In this study, high levels of eNOS protein were evident immunohistochemically throughout the endothelial layer of the abdominal aorta in both regions free of plaque and those complicated with plaque, including evidence of eNOS within the plaque itself. Interestingly, eNOS does seem to be abundant in the rabbit model of atherosclerosis24 but decreased in human vessels with atherosclerosis25 and in human endothelial cells incubated with oxidized LDL.26 The reason for the observed discrepancy between eNOS immunoreactivity in these human studies and those described in the rabbit remains unclear. It may relate to differences in the time course of the disease. It is possible that eNOS is present in the early stages of the disease, but in the long term (many decades of life in the human), levels of eNOS may become diminished.

In addition, there was no evidence to suggest that the decrease in endothelial function observed in the experimental groups was attributable to a decrease in SOD enzyme, because SOD immunoreactivity was intense throughout the endothelial layer and within plaques of the abdominal aorta, which has also been previously reported by others.27 Interestingly, it has been suggested that the SOD enzyme in plaques may be inactive28; however, other studies have shown an increase in aortic SOD activity after high dietary cholesterol29 or methionine.30 Moreover, Miller et al31 failed to improve aortic endothelium-dependent relaxation in hyperlipidemic rabbits when endothelial O2·− levels were reduced by 130% by the gene transfer of CuZn SOD or extracellular SOD. Taken together, these studies indicate that decreased endothelium-dependent relaxation does not seem to be attributable to a lack of the SOD enzyme.

Given that we have no evidence to suggest that eNOS or SOD proteins are decreased in this model, why is aortic relaxation impaired in animals exposed to high dietary cholesterol or methionine and virtually abolished when both diets are combined? The answer to this question is no doubt complex and may relate to recent evidence suggesting that
eNOS may produce $O_2^−$ rather than NO in a stressful milieu.\textsuperscript{32} In addition, others have suggested that the eNOS enzyme itself may be dysfunctional,\textsuperscript{33} and additional evidence for a dysfunctional eNOS protein comes from gene transfer studies, whereby the genetic transfer of eNOS to atherosclerotic vessels significantly improved vasodilation\textsuperscript{14} whereas genetic transfer of SOD to atherosclerotic vessels had no effect.\textsuperscript{31}

As previously mentioned, the reaction of excess $O_2^−$ with excess NO forms peroxynitrite (ONOO$^−$), which can nitrate the tyrosine residue of proteins forming nitrotyrosine.\textsuperscript{14} Thus, the identification of nitrotyrosine in the aortic wall by immunohistochemistry is a marker of nitrative stress.\textsuperscript{15} Because the formation of nitrotyrosine in atherosclerotic plaques is not solely attributable to the reaction of the tyrosine residue in proteins with ONOO$^−$, but also by the reaction of tyrosyl radicals, NO$_2^−$, NO$_3^−$, NO$_2$Cl, and HOCl with tyrosine,\textsuperscript{15} the formation of nitrotyrosine is an accurate marker of nitrative stress. Thus, nitrative stress seemed to be present in the aortae of animals fed high dietary cholesterol, methionine, or a combination of the two, which is in accordance with previous reports in humans\textsuperscript{89} but not with other subjects.\textsuperscript{36} Whether the nitrotyrosine present in the vessels in this study is attributable to excess ONOO$^−$ or other nitrative compounds remains uncertain.

In support of the results presented in this study, Eberhardt et al\textsuperscript{10} have shown that in a murine model of mild hyperhomocystinemia, the mice exhibited impaired endothelial relaxation to methacholine with no visible decrease in eNOS immunoreactivity, increased nitrotyrosine immunoreactivity, and increased $O_2^−$ generation in the aorta compared with wild-type mice. Furthermore, Lentz et al\textsuperscript{10} have shown in the same animal model that low dietary folate also raises plasma homocysteine levels and this leads to endothelial dysfunction.

It has been shown that macrophages within plaques produce cytokines that can stimulate iNOS production, $O_2^−$ production, and a variety of biologically active substances into their local milieu.\textsuperscript{37} Our results support this idea, in that macrophages are localized in plaques in conjunction with iNOS, eNOS, SOD, and nitrotyrosine. As well, the identification of iNOS in the atherosclerotic plaques indicates that there might be high levels of NO formed within these areas.\textsuperscript{13} Indeed, such high levels of NO can lead to the formation of nitrotyrosine, which has also been identified in these plaques. It is conceivable that these toxic, cytolytic effects of excess NO may contribute to cell death and tissue necrosis commonly observed within advanced atherosclerotic lesions.\textsuperscript{38} In this regard, the excess production of NO within atherosclerotic lesions may be deleterious rather than beneficial to the function of the vessel wall. Taken together, our results and others indicate that endothelial dysfunction observed in hyperhomocysteinemia or hypercholesterolemia is a multifactorial process, and additional investigations into the mechanisms of endothelial dysfunction are warranted.

An interesting finding in this study was that the plasma homocysteine level was elevated with high dietary methionine, but when both diets were combined (in the MethChol group), the plasma homocysteine levels in these animals were less than those fed with high dietary methionine alone, and although slightly elevated compared with control, these levels were not significantly different. This may represent a novel pathway of homocysteine metabolism. Indeed, cholesterol and homocysteine metabolism may be interrelated, because in vitro studies have shown that homocysteine stimulates cholesterol production in hepatocytes\textsuperscript{89} and a study in humans has shown that plasma homocysteine levels were correlated with plasma cholesterol levels.\textsuperscript{90} Taken together with our results, it seems that in vivo in the rabbit, elevations in plasma cholesterol may be affecting homocysteine levels. Although we cannot exclude the possibility that plasma homocysteine levels may be elevated in the combination group at the early stages of dietary intervention, additional studies are needed to validate this hypothesis.

In conclusion, our results show that the combination of high dietary cholesterol plus methionine administered to rabbits for 12 weeks virtually abolished endothelial function. These results suggest that the combination of high dietary cholesterol and methionine may exacerbate the onset of atherosclerosis and underscores the importance of multiple risk factors in the progression of cardiovascular disease.

References


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A High Methionine and Cholesterol Diet Abolishes Endothelial Relaxation.
Submission Type: Original Contribution
**Blood sampling and analysis**

Prior to commencing the dietary regime and at the termination of the 12 week diet, blood samples were collected from the marginal ear vein to determine the baseline levels of plasma cholesterol and homocysteine. One ml of non-fasting blood was collected and immediately processed as previously described\(^1^7\). Total plasma cholesterol, triglycerides, HDL and Total cholesterol:HDL ratio was measured with a Hitachi 917 Automatic Analyzer (Roche Diagnostics). The plasma total cholesterol level / HDL levels were used as a more accurate indicator of cardiovascular disease risk. Homocysteine was measured using high performance liquid chromatography using a previously published method\(^1^8\).

**Tissue collection**

Under anesthesia (3mg/kg xylazine + 10mg/kg ketamine), the abdominal aorta was exposed and ligated midway between the diaphragm and bifurcation. The abdominal aorta was excised distally from the ligature to the bifurcation, flushed with oxygenated Krebs buffer and immediately used for endothelial function studies (see below). The animals were then perfusion fixed at 100mmHg via a catheter located proximal to the aortic ligature with a solution of 1% formaldehyde + 0.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) after the administration of heparin sodium (5000 IU) to inhibit coagulation and papaverine hydrochloride (12mg) to promote vasodilatation. The remaining aorta was then excised and cleaned of fat and connective tissue and stored at +4°C in 0.1M cacodylate buffer (pH 7.4).
Quantification of plaque

The aortae were excised between the celiac and superior mesenteric arteries and placed in plastic processing cassettes to avoid warping of tissue. The samples were dehydrated through a series of graded alcohols (20 – 80%), and infiltrated and embedded in glycolmethacrylate Technovit 7100 resin (Heraeus Kulzer, Germany).

To quantify plaque by stereological means, approximately 15 evenly spaced sections of the abdominal aortic segment was sampled. To do this, the segment of each aorta was measured (in µm) and divided by 15. This value was then rounded off to the lowest multiple of ten, and this number was used as the sampling fraction (n). The blocks were exhaustively sectioned at 10 µm using a Leica RM2165 Supercut Microtome (Germany).

Every $n^{th}$ section was sampled for the abdominal aorta, the first being chosen using randomly generated numbers (i.e. between one and n, respectively). The sections were mounted on glass slides, dried on a hotplate at 40°C and incubated at 60°C in an oven for 4 days. Sections were then stained with haematoxylin and eosin.

Using a microscope equipped for projection (Olympus BX50F4, Japan), the sampled sections of the aortae were projected onto a table top at 59X magnification. An orthogonal grid (2cm x 2cm) was superimposed over the projected image, and the number of grid points overlying the intima and media of the abdominal aorta were counted. The volume of the aortic intima and media was estimated using the Cavalieri Principle:

$$\text{Volume} = \Sigma_{\text{points}} \times t \times f \times a(p)$$
Where $\Sigma_{\text{points}}$ is the sum of the grid points counted, $t$ is the thickness of the section, $f$ is the inverse of the sampling fraction and $a(p)$ is the area associated with each grid point.

**Functional reactivity studies**

Freshly excised abdominal aortae were cut into four aortic rings (4 mm lengths) that were then mounted between two metal hooks in organ baths attached to force displacement transducers (Grass FT03). The baths were filled with Krebs of the following composition (mmol/L): NaCl 118.4, KCl 4.7, NaHCO$_3$ 1.2, MgSO$_4$ 1.2, CaCl$_2$ 2.5, glucose 11.1 and kept at a constant temperature of 37 °C and continuously bubbled with 95% O$_2$/5% CO$_2$.

After a 1-hour equilibration period, viability of ring preparations were tested by addition of high potassium physiological salt solution (KPSS, of the following composition (mmol/L): KCl 123.7, MgSO$_4$ 1.17, KH$_2$PO$_4$ 1.18, NaHCO$_3$ 25, glucose 5.5).

*Endothelium-dependent relaxation to acetylcholine:* To assess endothelial dependent relaxation mediated by acetylcholine, aortic rings were precontracted with phenylephrine to approximately 30% of maximal contraction to KPSS. After the contraction reached a plateau, a cumulative concentration-response (CR) curve to acetylcholine-induced relaxation was obtained. The rings were then repeatedly flushed with fresh Krebs every 10 minutes and allowed to equilibrate once more for 1 hour. In a subset of preparations, NO-mediated mechanisms causing relaxation were examined using the NOS inhibitor, $\text{N}^\text{G}$-nitro-L-arginine (NOLA), and the
cyclooxygenase inhibitor, indomethacin. Preincubation of aortic rings with 0.1 mmol/L NOLA completely abolished endothelium dependent relaxation to acetylcholine whereas the preincubation of aortic rings with 0.1 mmol/L indomethacin had no significant effect on endothelium dependent relaxation to acetylcholine.

Vascular smooth muscle cell relaxation to a NO donor: To assess smooth muscle function, aortic rings were precontracted with phenylephrine to approximately 30% of maximal contraction to KPSS. After the contraction reached a plateau, a CR curve to sodium nitroprusside-induced relaxation was then obtained.

Vascular smooth muscle contractile function
Concentration response curves were also constructed to the \( \alpha_1 \)-adrenergic receptor agonist, phenylephrine. Aortic rings were subjected to a cumulative concentration-response curve to phenylephrine (0.01 \( \times \) 10\(^{-3} \) mmol/L – 3 \( \times \) 10\(^{-3} \) mmol/L, at 0.5 log unit increments), and the phenylephrine was added at intervals of between 2-5 minutes depending on the time taken to reach a plateau.

Immunohistochemistry
The remaining parts of the abdominal aortae were cut into two mm segments and processed for paraffin to be used for the immunohistochemical detection of eNOS, SOD and nitrotyrosine. Immunohistochemistry was performed with the Envision kit system using the monocye/macrophage monoclonal antibody (DAKO Corporation, Carpentaria, USA), the eNOS antibody and iNOS (mouse monoclonal IgG1, Transduction Laboratories, USA), the Cu-Zn-SOD antibody (mouse monoclonal IgG1, Clone no.SD-G6, Sigma Aldrich Chemical Company) and the nitrotyrosine antibody (mouse monoclonal IgG1, Zymed Laboratories, USA).
**Data analysis**

All pre-experimental baseline cholesterol, homocysteine, triglyceride and total cholesterol/HDL measurements were compared to their respective post-experimental values by a two tailed Students ‘t’ test.

Responses to phenylephrine were measured as increases in force (g) and expressed as a percentage of the initial KPSS response. Concentrations of phenylephrine that caused 50% of the maximal contractile response (pEC$_{50}$ value) and maximum responses ($E_{\text{max}}$) were determined from each individual CR curve (using Graphpad Prism). These data are presented as –log EC$_{50}$ values (pEC$_{50}$). CR curves obtained from individual tissues were then pooled and plotted as mean log CR curves.

Relaxation responses to acetylcholine and sodium nitroprusside were calculated in an analogous manner, except that data are presented as a percentage relaxation of the sub maximal phenylephrine-induced pre-contraction.

All values shown are expressed as mean ± standard error of the mean (SEM) of $n$, which equals the number of animals. For the KPSS and the first CR curve to acetylcholine that was performed in all tissues, the responses for each animal represent the mean of 4 separate aortic rings. All -logEC$_{50}$ values, maximal contractile or relaxant responses, and volumes were compared between the four experimental (dietary) groups using a one way analysis of variance (ANOVA), followed by a Tukey’s post hoc test to determine statistical difference between individual groups. In all cases, significance was accepted at a level of $p<0.05$. 
Body weights

At the termination of the dietary regime, there was no significant difference between the final body weights between groups. As well, the total weight gain of the rabbits over the experimental period was not significantly different between any of the groups (results not shown).

Localization of eNOS, iNOS, SOD, nitrotyrosine and macrophages

eNOS immunoreactivity was present throughout the aortic endothelial cell layer in the control, Chol, Meth and MethChol groups. eNOS immunoreactivity was also present within the aortic plaques of the Chol and MethChol groups (Figure I). SOD immunoreactivity was visible throughout the endothelial cell layer of the abdominal aorta in rabbits from the control, Chol, Meth and MethChol groups. In addition, strong SOD immunoreactivity was present within the plaques of the Chol and MethChol groups (Figure II). Inducible NOS and macrophages were immunolocalised within all plaques of Chol and MethChol groups, but were not present in control or Meth groups. Figure III shows a representative cross-section of an atherosclerotic plaque obtained from the MethChol group identifying iNOS, macrophages, nitrotyrosine, eNOS and SOD immunoreactivity. Plaques from the Chol group showed similar results (figures not shown). Nitrotyrosine immunoreactivity, a maker for nitrative stress, was present in the endothelial layer of the abdominal aortae in animals from the Chol, Meth and MethChol groups, but not in the control group. Nitrotyrosine immunoreactivity was also present within the plaques of the Chol and MethChol groups (Figure IV) “please see http://atvb.ahajournals.org”. No immunostaining was present in the negative controls (Figure V) “please see http://atvb.ahajournals.org”.
Table I: Table of $-\log EC_{50}$ and maximal response (Max. resp.) evoked by acetylcholine, sodium nitroprusside and phenylephrine in aortic rings obtained from the experimental groups.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Control</th>
<th>Cholesterol</th>
<th>Methionine</th>
<th>MethChol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$-\log EC_{50}$</td>
<td>7.21 ± 0.10</td>
<td>6.67 ± 0.14 *</td>
<td>6.71 ± 0.12 *</td>
</tr>
<tr>
<td>Ach</td>
<td>Max. resp.</td>
<td>70 ± 5.0%</td>
<td>47 ± 9.0%</td>
<td>46 ± 6.0%</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>$-\log EC_{50}$</td>
<td>6.50 ± 0.19</td>
<td>6.47 ± 0.35</td>
<td>6.94 ± 0.29</td>
</tr>
<tr>
<td>SNP</td>
<td>Max. resp.</td>
<td>87 ± 3.7%</td>
<td>77 ± 11%</td>
<td>88 ± 2.2%</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>$-\log EC_{50}$</td>
<td>6.74 ± 0.14</td>
<td>6.23 ± 0.12 †</td>
<td>5.84 ± 0.07 †</td>
</tr>
<tr>
<td>PE</td>
<td>Max. resp.</td>
<td>124 ± 4.0%</td>
<td>118 ± 10.5%</td>
<td>120 ± 7.1%</td>
</tr>
<tr>
<td>n</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

* p<0.05, † p< 0.01, ‡ p< 0.001 compared to control, § p< 0.001 compared to cholesterol and methionine group. ″/n=2 for EC$_{50}$ since CR curve could only be fitted to data from 2 animals because of markedly impaired responses. n= number of animals.
Figure I  Representative cross-section through the abdominal aorta of the control, Meth Chol and MethChol group showing eNOS protein. Note the presence of immunoreactivity in the endothelial layer and plaque (x400).

Figure II Representative cross-section through the abdominal aorta of the control, Meth Chol and MethChol group showing SOD protein. Note the presence of immunoreactivity in the endothelial layer and plaque (x400).

Figure III  Representative cross-section through the abdominal aorta of the MethChol group showing iNOS, macrophage, nitrotyrosine, eNOS and SOD immunoreactivity (x200).

Figure IV  Representative cross-section through the abdominal aorta of the control, Meth Chol and MethChol group showing nitrotyrosine immunoreactivity. Note the presence of immunoreactivity in the endothelial layer and plaque (x400).

Figure V  Representative cross-section through the abdominal aorta of the control, Meth Chol and MethChol group showing no immunoreactivity (negative control). Note the absence of immunoreactivity in the endothelial layer and plaque (x400).