Atorvastatin Restores Endothelial Function in Offspring of Protein-Restricted Rats in a Cholesterol-Independent Manner

Christopher Torrens, Christopher J. Kelsall, Laura A. Hopkins, Frederick W. Anthony, Nick P. Curzen, Mark A. Hanson

Abstract—Maternal protein restriction in rats leads to endothelial dysfunction and decreased NO bioavailability in the offspring. Statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) are recognized to have pleiotropic actions including increasing NO bioavailability and reducing inflammation and oxidative damage. This study assessed statin treatment on vascular function in a model of endothelial dysfunction, which is independent of dyslipidemia. Wistar rats were fed a control (18% casein) or protein-restricted (9% casein) diet throughout pregnancy. At weaning, a subset of the protein-restricted group was given atorvastatin (10 mg/kg per day) in the drinking water. At 145 days of age, offspring were euthanized by CO₂ inhalation. Plasma samples were collected for markers of inflammation, vascular reactivity of the thoracic aorta, and small mesenteric arteries were assessed on the wire myograph, and tissues were snap frozen for molecular biology analysis. Thoracic aorta endothelial-dependent vasodilatation was attenuated in the male offspring from both protein-restricted groups compared with controls (P<0.05) but was similar in females (P value not significant). Endothelial-dependent dilatation of mesenteric arteries was attenuated in male and female protein-restricted offspring (P<0.05) and was corrected by atorvastatin. Maternal protein restriction increased plasma inflammatory markers granulocyte chemotactic protein, lipocalin-2, and β₂-microglobulin in male and C-reactive protein in female offspring (P<0.05). Atorvastatin had no effect on inflammatory markers in the males but restored C-reactive protein to control levels in the females (P<0.05). Aortic and mesenteric artery mRNA levels of endothelial NO synthase, superoxide dismutase 1, and tumor necrosis factor-α were unchanged. These data suggest that atorvastatin can restore endothelial function in this model, but its effects are gender specific and dependent on the vascular bed. (Hypertension. 2009;53:661-667.)

Key Words: statins (atorvastatin) ■ experimental models ■ fetal programming ■ vascular biology ■ endothelium ■ nitric oxide ■ inflammation

There is considerable evidence that the etiology of cardiovascular and metabolic diseases has origins partly in the developmental environment.¹ A widely used animal model to study this phenomenon is the rat, in which the restriction of dietary protein during pregnancy leads to raised blood pressure and endothelial dysfunction in the offspring.²⁻⁴ Increasingly, evidence from this model points to a disruption to the NO pathway as a key component of the underlying pathophysiology.⁵⁻⁶

The healthy vascular endothelium plays an important role in maintaining vascular tone through the release of factors including NO, prostacyclin, and endothelial-derived hyperpolarizing factor.⁶ The importance of a healthy endothelium is seen in cardiovascular and metabolic diseases, where endothelial dysfunction is associated with atherosclerosis, hypertension, and the metabolic syndrome.⁷⁻⁹ Important in the development of such endothelial dysfunction are oxidative stress¹⁰,¹¹ and inflammation,¹²,¹³ both of which are common in the pathogenesis of cardiovascular diseases.

Statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) are used in the management of dyslipidemia but are considerably more effective at influencing cardiovascular end points than other cholesterol-lowering therapies.¹⁴ This may be because statins have a number of pleiotropic, cholesterol-independent actions, including increasing NO bioavailability and reduction of oxidative damage.¹⁴⁻¹⁷ The present study was designed to test whether atorvastatin would correct the endothelial dysfunction induced by maternal protein restriction, because this model is independent of dyslipidemia.

Methods

All of the animal procedures were in accordance with the regulations of the British Home Office Animals (Scientific Procedures) Act, 1986, and were approved by the local ethical review committee.
A more detailed Methods section is available online at http://hyper.ahajournals.org.

**Animals and Dietary Protein Restriction**

Wistar rats (Harlan) were fed either control (18% casein; n=6) or an isocaloric protein-restricted diet (PR; 9% casein; n=14) throughout pregnancy, as described previously.3 Dams were allowed to deliver naturally and immediately postpartum returned to standard laboratory chow; pups remained on standard chow until the end of the experiment. At weaning (21 days), a subgroup of PR litters (PRS; n=7) was given atorvastatin (10 mg/kg per day; Pfizer) in the drinking water. Atorvastatin was continued from weaning until the end of the experiment. At 145 days of age, male and female offspring were randomly selected from each litter and euthanized by CO2 inhalation and cervical dislocation.

**Vascular Protocol**

Thoracic aorta (TA) and mesenteric arteries (MAs; ID \( \approx 300 \) µm) were dissected and mounted on a wire myograph (Danish Myo Technology A/S), as described previously.3,4,18 Cumulative concentration response curves were constructed to phenylephrine (PE). Segments were preconstricted with a submaximal dose of PE and concentration response curves to acetylcholine (ACh) and sodium nitroprusside (SNP) performed. In TA, responses to ACh were repeated in the presence of the NO synthase inhibitor N\( \mathrm{\text{\textendash}}} \)nitro-L-arginine methyl ester (L-NAME; 10 \( \mu \)mol/L) alone, whereas in MA this was in combination with the cyclooxygenase inhibitor indomethacin (INDO; 10 \( \mu \)mol/L; MA). Inhibitors were incubated for 30 minutes before experiments. All of the drugs were obtained from Sigma unless otherwise stated.

**Determination of Plasma Markers**

Plasma was snap frozen in liquid nitrogen. Plasma levels of (non-fasting) glucose, triglycerides (TG), and cholesterol were analyzed using standard reagents (Kone). Plasma levels of the nonesterified fatty acids (NEFAs) were analyzed by the WAKO reagent kit. Levels of inflammatory biomarkers were analyzed using Rules Based Medicine rodent Multi Analyte Profiles.

**Hepatic Endothelial NO Synthase Activity Assay**

Total hepatic NO synthase activity was determined in liver homogenates by measuring the conversion of \( \left[ ^{14} \mathrm{C} \right] \)arginine to \( \left[ ^{14} \mathrm{C} \right] \)citrulline using the commercially available kit and instructions (NOSdetect Assay kit, ALX-850 to 006-K101, Alexis Biochemicals), as previously described.19 Activity is expressed as counts per minute.

**Determination of Protein Carbonyl Concentration**

Hepatic protein carbonyl concentration was determined as described previously20 using the commercially available protein carbonyl enzyme immunoassay (Zentech Protein Carbonyl ELISA kit 850-312-K101). The concentration of protein carbonyls in the liver samples is expressed per milligram of protein as determined by protein assay (Pierce Coomassie Plus 1856210).

**Analysis of mRNA levels**

Expression of endothelial NO synthase (eNOS), superoxide dismutase 1 (SOD1), tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)), glutathione S-transferase a4 (GSTa4), NAPDH oxidase, and 3-hydroxy-3-methylglutaryl-coenzyme A reductase mRNA levels in liver, as well as TA and MAs, were determined using real-time PCR relative to \( \beta \)-actin (TaqMan, Applied Biosystems).

**Calculations and Statistical Analysis**

All of the data are expressed as means±SEMs. Constrictor responses were calculated as the percentage of maximum contraction induced by 125 mmol/L of KPPS and relaxant responses as the percentage reversal of PE-induced contraction. Cumulative concentration response curves to agonists were analyzed by fitting to a 4-parameter logistic equation using nonlinear regression to obtain the effective concentration equal to 50% of maximum (pEC\(_{50}\)) and a maximal response, which were compared by 1-way ANOVA (Prism 3.0, GraphPAD Software Inc). Significance was accepted if \( P \) value was <0.05. At all points, the investigator was blinded to the dietary group.

**Results**

**Growth and Development at 145 Days**

Neither maternal weight gain during pregnancy (grams: control [C], 104.7±6.5, n=6; PR, 92.8±4.1, n=14; \( P \) value not significant [NS]) nor birth weight in males (g: C, 5.87±0.21, n=6; PR, 5.43±0.29, n=14) or females (g: C, 5.71±0.18, n=6; PR, 5.38±0.29, n=14) were significantly different between the groups. Postnatal growth was similar between the groups, and at postmortem, body weight was similar between the groups, as was the weight of the heart, lungs, liver, and right or left kidney. This was true for both male and female offspring and whether weights were expressed as a gross weight in grams or as a percentage of body weight (data not shown).

**Vascular Reactivity**

**Vasoconstriction**

PE produced a concentration-dependent vasoconstriction in both male and female offspring in all of the artery segments. In male TA, PRS exhibited a greater constriction compared with both PR and C animals (% max response: C, 138.3±4.0, n=7; PR, 160.6±5.4, n=8; PRS, 190.3±12.7, n=5, \( P<0.05 \)). This was not seen in TA from female offspring (% maxresponse: C, 142.2±8.5, n=6; PR, 148.8±18.2, n=6; PRS, 137.8±9.7, n=6; \( P=\text{NS} \)), nor was it seen in either male MA (percentage of maximum: C, 80.6±2.7, n=5; PR, 85.1±2.4, n=6; PRS, 86.9±1.9, n=5; \( P=\text{NS} \)) or female MA (percentage of maximum: C, 80.9±4.2, n=5; PR, 86.0±1.8,
n=6; PRS, 82.6±3.4, n=5; P=NS), where responses to PE were similar between the groups.

**Endothelial-Dependent Vasodilatation**

In all of the TA segments, the endothelium-dependent vasodilator, ACh, produced a concentration-dependent relaxation of PE-induced tone, which was completely abolished by the NO synthase inhibitor l-NAME (100 μmol/L; data not shown). In TA from male offspring, vasodilatation to ACh was significantly blunted in both the PR and PRS groups compared with controls (P<0.05; Figure 1A), yet in females the responses were similar between the groups (P=NS; Figure 1B).

In MA segments from male offspring, ACh-induced vasodilatation was significantly attenuated in the PR group compared with controls and was partially restored by atorvastatin (P<0.05; Figure 2A). Responses to ACh were sensitive to blockade with l-NAME (100 μmol/L) and INDO (10 μmol/L) in both the PRS group (P<0.05) and controls (P<0.01). In contrast, ACh responses in the PR group were insensitive to l-NAME and INDO blockade (P=NS; Figure 2B). As with the males, MA from female offspring showed an attenuated dilatation to ACh in the PR group, which was partially corrected by statin (P<0.05; Figure 3A). Again, ACh responses in the PR group were insensitive to l-NAME and INDO (P=NS), unlike the responses in the control (P<0.05) and PRS groups (P<0.05; Figure 3B).

**Endothelial-Independent SNP Vasodilatation**

The NO donor SNP produced a concentration-dependent vasodilatation of PE-induced tone in TA segments from both male (pEC_{50}: C, 8.53±0.06, n=7; PR, 8.39±0.07, n=8; PRS, 8.24±0.10, n=5; P=NS) and female (pEC_{50}: C, 8.71±0.05, n=5; PR, 8.99±0.05, n=6; PRS, 8.70±0.05, n=5; P=NS) offspring, which were similar between the groups. The same was in seen in MA, where responses to SNP were similar between the groups in both male (pEC_{50}: C, 7.02±0.18, n=5; PR, 7.06±0.13, n=6; PRS, 7.24±0.23, n=5; P=NS) and female offspring (pEC_{50}: C, 7.89±0.20, n=5; PR, 8.08±0.21, n=5; PRS, 7.60±0.20, n=4; P=NS).

**Hepatic eNOS Activity and Protein Carbonyls**

Total hepatic NO synthase activity was determined in liver homogenates by measuring the conversion of [1^{14}C]citrulline to [1^{14}C]arginine, expressed as counts per minute. There was no significant difference in hepatic NO synthase activity between the groups in either the male (cpm: C, 19 120.0±597.0, n=5; PR, 18 550.0±339.3, n=7; PRS, 19 810.0±891.9, n=6; P=NS) or female offspring (cpm: C, 22 550.0±2008.0, n=6; PR, 21 750.0±1731.0, n=6; PRS, 23 350.0±2995.0, n=5; P=NS). Levels of hepatic protein carbonyls were similar in all of the groups in both males (C, 0.23±0.03, n=4; PR, 0.33±0.04, n=7; PRS, 0.48±0.11, n=6; P=NS) and females (C, 0.57±0.11, n=6; PR, 0.42±0.05, n=6; PRS, 0.54±0.11, n=5; P=NS).

**Plasma Analysis**

Confirmation of the presence of atorvastatin in the circulation was provided by detectable levels of both atorvastatin and metabolites in the plasma of the PR group (Wickham Laboratories; data not shown). Levels of cholesterol, TG, NEFAs, glucose, insulin, and leptin were similar among all of the groups in both males and females (P=NS; Table 1). Inflammatory markers β₂-microglobulin (P<0.05; Figure 4A), granulocyte chemotactic protein (GCP) (P<0.05; Figure 4B), and lipocalin-2 (P<0.05; Figure 4C) were increased in both the PR and PRS groups compared with control in male plasma but were similar between the groups in females.
Conversely, levels of C-reactive protein (CRP) were similar in males and were elevated in the PR females compared with controls and PRS (P<0.05; Figure 4D).

Analysis of mRNA Levels
The presence of atorvastatin in the PRS group was further supported by the increased hepatic expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase mRNA in the PRS group compared with both the PR and controls (C, 0.012±0.002, n=5; PR, 0.014±0.002, n=7; PRS, 0.034±0.006, n=6; P<0.01).21 MA mRNA levels of eNOS (Figure 4E), SOD1 (Figure 4F), GSTa4, NADPH oxidase, and TNF-α were similar between groups (Table 2). TA mRNA levels of eNOS and SOD1 were similar between the groups (data can be found in Table S2).

Discussion
It is now clear that the early life environment has a significant role to play in the etiology of cardiovascular disease.1 Previously, we and others have demonstrated in the rat that maternal protein restriction in pregnancy leads to raised systolic blood pressure and endothelial dysfunction in the offspring.2–4 In the current study we have used this model that, unlike high-fat models, is not associated with dyslipidemia to evaluate the effect of early statin treatment on such vascular dysfunction in a cholesterol-independent manner.

Endothelial dysfunction is a common phenomenon in cardiovascular disease, occurring in the metabolic syndrome7 and hypertension,8 and is known to be the primary event in atherosclerosis.9 As such, attenuating endothelial dysfunction has particular significance for future therapeutic interventions. Endothelial dysfunction is also a common phenotype in a number of rodent models of developmental origins of disease, including the maternal high-fat model22 and models of total maternal nutrient restriction.23,24 It is also observed in the offspring of protein-restricted dams, in which more detailed investigations suggest a disruption in the NO pathway,3,4 a finding that is supported by the current study. Vasodilatation to ACh is mediated by the release of NO, prostacyclin, and endothelial-derived hyperpolarizing factor,6 but in the PR group, responses to ACh were both attenuated and insensitive to eNOS blockade by L-NAME (100 μmol/L), suggesting that the NO pathway is absent in this group. An important observation of the present study is that we demonstrate that atorvastatin can restore both endothelial function and the NO component of the ACh response in
offspring of protein-restricted rats. This effect is independent of effects on cholesterol and appears to be specific to individual vascular beds, a finding that will require future investigation.

A decrease in eNOS expression in the MAs may provide a possible explanation for these data. Mice lacking eNOS exhibit endothelial dysfunction,35 whereas statins have been shown to upregulate eNOS.15 However, effects of maternal protein restriction on eNOS activity or expression are not supported by the current data, which indicates no change in either hepatic eNOS activity or vascular mRNA levels between the groups, a finding that is in contrast to previous findings.4,26 Taken together, this would suggest that changes in eNOS in mice are unlikely to be responsible for the reduction in NO bioavailability, although eNOS mRNA levels need not necessarily be related to changes in eNOS protein or activity.23,27 Another potential candidate could be oxidative stress, an increasingly recognized component of cardiovascular disease,10,11 linked to the decrease of NO bioavailability through either the production of peroxynitrite or the uncoupling of eNOS.10,28,29 Indeed, one proposed pleiotropic action of statins is through an antioxidant pathway.17 Enhanced oxidative stress has been reported in the offspring of dams after either total nutrient23 or protein restriction,20 although in contrast to this we have recently reported finding no difference in protein carbonyl levels after maternal protein restriction.26 The present study used 2 markers of oxidative stress, hepatic protein carbonyls30 and local arterial SOD1 expression.31,32 The current data support our previous data that maternal protein restriction does not alter protein carbonylation35 nor levels of SOD1. At present, it is difficult to describe a clear role for oxidative stress in this model.

Another possible explanation for the endothelial dysfunction arises from a novel finding of the present study, namely, the presence of raised plasma inflammatory markers in the PR offspring. Vascular inflammation, a key mediator of which is CRP,33 is increasingly recognized to be associated with a number of cardiovascular diseases, including atherosclerosis34 and peripheral vascular disease.13 In female offspring, CRP was elevated in the PR group and restored to control levels by administration of atorvastatin, in a manner that mirrored the effects on MA endothelial function. Previous studies have shown that elevated CRP leads to uncoupling of eNOS,36 and, thus, a decrease in NO bioavailability and overexpression of CRP in mice also leads to endothelial dysfunction.36 Taken together, this might offer an explanation of the origin of the endothelial dysfunction in this model and of how statins can modify this. It is of interest that, in heart failure patients, atorvastatin not only lowered CRP but also improved endothelial-dependent flow-mediated dilatation.37 Furthermore, statins have been shown to be beneficial in a human population that has raised CRP but not hypercholesterolemia,38 a profile similar to the current model.

Similar mechanisms do not appear to operate in the male offspring. Although endothelial dysfunction was observed in both males and females and was corrected with atorvastatin in both genders, this was not so for the inflammatory markers. Unlike the female, male CRP levels were similar, although levels of β₃-microglobulin, granulocyte chemotactic protein, and lipocytin-2 were raised in the PR and PRS groups alike and have been linked to cardiovascular pathologies.13,39 In addition, whereas levels of the inflammatory markers (CRP) mirrored the endothelial function in females, this was not the case in the males. Although atorvastatin did restore endothelial function in the male offspring, it did not correct the raised levels of inflammatory markers. It, therefore, appears that increased inflammation may be linked to the endothelial dysfunction in females but not in males. Another gender dimorphism seen in the current study was that the TA responses to ACh were attenuated in male but not female PR offspring.

Responses in the aorta are distinctly different from those in the mesenteric resistance arteries. Although most work in this field has focused on mesenteric resistance arteries3,4 because of their contribution to peripheral resistance in the rat,40 we have demonstrated previously that altered responses in the MAs of female offspring of PR dams are not necessarily also present in the TA, albeit in late pregnancy.18 Gender differences are a common finding in many such studies of maternal dietary imbalance;22,41 although the mechanisms behind this remain to be elucidated. One candidate is estrogen and the estrogen receptors (ERα and ERβ), which are known to have a number of favorable cardiovascular actions, including increasing NO bioavailability and reducing oxidative stress,32–34 all of which are proposed to underpin the increase of cardiovascular disease in postmenopausal women.45 We have previously demonstrated impaired 17β-estradiol–mediated vasodilatation in MAs of PR dams,4 suggesting that alterations in estrogen may indeed have a role in this model.

Table 2. MA mRNA Levels of Various Markers From 145-Day-Old Offspring

<table>
<thead>
<tr>
<th>Gene</th>
<th>Male C, n=6</th>
<th>PR n=7</th>
<th>PRS n=5</th>
<th>Female C, n=6</th>
<th>PR n=7</th>
<th>PRS n=5</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTa4</td>
<td>1.25±0.12</td>
<td>1.34±0.14</td>
<td>1.11±0.09</td>
<td>1.50±0.24</td>
<td>1.16±0.16</td>
<td>1.33±0.11</td>
</tr>
<tr>
<td>NADPH</td>
<td>0.36±0.18</td>
<td>0.80±0.32</td>
<td>0.34±0.22</td>
<td>0.96±0.14</td>
<td>0.75±0.31</td>
<td>0.61±0.18</td>
</tr>
<tr>
<td>TNFα</td>
<td>1.71±0.33</td>
<td>2.18±0.37</td>
<td>1.48±0.40</td>
<td>1.56±0.17</td>
<td>1.40±0.34</td>
<td>1.23±0.30</td>
</tr>
</tbody>
</table>

Perspectives

The current study has demonstrated that maternal protein restriction leads to endothelial dysfunction and increased inflammation and suggests decreased NO bioavailability in the offspring. We demonstrate for the first time that atorvastatin can attenuate this early endothelial dysfunc-
tion in a manner that is independent of actions on cholesterol and possibly linked to effects on inflammation, at least in females. The ability of atorvastatin to modify this early endothelial dysfunction could have profound clinical implications, particularly because endothelial dysfunction is a pathological precursor for atherosclerosis.

Acknowledgments
We are grateful to Dr John Jackson for the plasma glucose and plasma lipid analysis.

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Disclosures
This work was in part supported by a research grant and the gift of atorvastatin from Pfizer.

References


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ATORVASTATIN RESTORES ENDOTHELIAL FUNCTION IN OFFSPRING OF PROTEIN RESTRICTED RATS IN A CHOLESTEROL-INDEPENDENT MANNER

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Abbreviated title: Atorvastatin corrects endothelial dysfunction
FULL METHODS
All animal procedures were in accordance with the regulations of the British Home Office Animals (Scientific Procedures) Act, 1986 and approved by the local ethical review committee.

Animals and dietary protein restriction
Virgin female Wistar rats (Harlan, U.K.) were mated (day of vaginal plug detection being defined as day 0 of pregnancy) and pregnant dams randomly assigned to one of two dietary groups: control (18% casein, n=6) or an isocaloric protein restricted diet (PR, 9% casein, n=14) the composition of which has been previously described\(^1\). Pregnant dams were fed on the experimental diets from confirmation of pregnancy until delivery and immediately postpartum returned to standard lab chow. Post-weaning, all pups were continued on standard chow for the remainder of the experiment. At weaning, a subgroup of PR litters (PRS, n=7) were given atorvastatin (10 mg/kg/day – Pfizer, Sandwich, UK) in the drinking water. As atorvastatin is insoluble in water it was suspended in 0.5% carboxymethylcellulose solution and replaced weekly. Atorvastatin was continued from weaning until the end of the experiment at 145 days when offspring were killed by CO\(_2\) inhalation and cervical dislocation.

Vascular protocol
At post mortem, thoracic aorta (TA) and small mesenteric arteries (MA, internal diameter ca. 300 µm) were dissected and stored in cold (4\(^\circ\) C) physiological salt solution (PSS) of the following composition; NaCl, 119; KCl, 4.7; CaCl\(_2\), 2.5; MgSO\(_4\), 1.17; NaHCO\(_3\), 25; KH\(_2\)PO\(_4\), 1.18; EDTA, 0.026; and D-glucose, 5.5 mM. Segments from both arteries were cleaned of connective tissue and mounted in a wire myograph (Danish Myo Technology A/S, Denmark). Segments were bathed in PSS, heated to 37\(^\circ\) C and continuously gassed with 95% O\(_2\) and 5% CO\(_2\).

Thoracic aorta
Segments were placed under a resting tension of 1 g and smooth muscle integrity was assessed by a single wash with 125 mM KPSS (PSS solution with an equimolar substitution of KCl for NaCl). Following equilibration, cumulative concentration response curves (CRCs) were constructed to the \(\alpha_1\)-adrenoceptor agonist phenylephrine (PE, 1 nM – 100 µM). Segments were preconstricted with a submaximal dose of PE equivalent to EC\(_{80}\) (effective concentration equal to 80% of maximal concentration) and CRCs to the endothelium-dependent vasodilators acetylcholine (ACh; 1 nM - 10 µM) and the NO donor sodium nitroprusside (SNP; 100 pM - 30 µM) were performed. To determine the role of NO in the ACh-mediated vasodilatation, responses to ACh were repeated in the presence of NO synthase inhibitor \(\text{N}^\omega\)-Nitro-\(\text{L}\)-Arginine Methyl Ester (L-NAME, 100 µM).

Mesenteric arteries
Segments of MA were normalised as previously described\(^2\). Functional integrity of the smooth muscle was assessed with four, 2 minute washes with 125 mM KPSS solution. Vessels failing to produce an active tension equivalent to 13.3 kPa were discarded from the study. Cumulative CRCs were constructed to PE (10 nM – 100 µM). Vessels were preconstricted with PE EC\(_{80}\) and CRCs to ACh (0.1 nM - 10 µM) and SNP (0.1 nM - 10 µM) were performed. For each response, preconstriction to PE did not differ between the groups. To investigate factors involved in the ACh-mediated vasodilatation, responses were repeated in the presence of L-NAME (100 µM) and the cyclooxygenase inhibitor, indomethacin (INDO; 10 µM). Inhibitors were incubated for 30 minutes prior to experiments. All drugs were obtained from Sigma (Poole, UK) unless otherwise stated.
**Determination of Plasma Markers**

Blood was collected by cardiac puncture and stored on ice in heparinised tubes. Samples were centrifuged and plasma collected and snap frozen in liquid nitrogen. Plasma levels of (non-fasting) glucose, triglycerides and cholesterol were analysed using standard reagents (Kone). Plasma levels of the non-esterified fatty acids (NEFAs) were analysed by the WAKO reagent kit. Levels of inflammatory biomarkers were analysed using Rules Based Medicine (Austin, TX, USA) rodent Multi Analyte Profiles.

**Hepatic eNOS Activity Assay**

Total hepatic NOS activity was determined in liver homogenates by measuring the conversion of L-[14C]arginine to L-[14C]citrulline using the commercially available kit and following the manufacturers instructions (NOSdetect Assay Kit, ALX-850-006-KI01, Alexis Biochemicals, UK) as previously described\(^3\). Activity is expressed as cpm.

**Determination of Hepatic Protein Carbonyl Concentration**

Hepatic protein carbonyl concentration was determined as previously described\(^4\). Briefly ~250 mg of frozen liver was homogenised in 500 µl of water and centrifuged for 2 minutes. 5 µl was extracted for a protein assay (Pierce Coomassie Plus\(^{TM}\) 1856210) and 5 µl for a protein carbonyl enzyme immunoassay (Zentech Protein Carbonyl ELISA Kit 850-312-KI01). Along with the samples, the protein and protein carbonyl concentrations of 5 standards (provided in the kit) were measured, which contained serum albumin with increasing known proportions of oxidised protein. The concentration of protein carbonyls in the liver samples is expressed per milligram of protein.

**Analysis of mRNA levels**

Expression of mRNA levels in liver, thoracic aorta and mesenteric arteries were determined using real time PCR relative to β-actin (TaqMan, Applied Biosystems, Warrington, U.K.). The primers and probes used are given in Table S1. The data from thoracic aorta is presented here in Table S2.

**Calculations and statistical analysis**

All data are expressed as mean ± (S.E.M). Constrictor responses were calculated as % maximum contraction induced by 125 mM KPSS and relaxant responses as % reversal of PE-induced contraction. Cumulative CRCs to agonists were analysed by fitting to a four-parameter logistic equation using non-linear regression to obtain the pEC\(_{50}\) (effective concentration equal to 50% of maximum) and a maximal response, which were compared by one-way analysis of variance (Prism 3.0, GraphPAD software Inc., San Diego, CA, U.S.A.). Significance was accepted if \(p<0.05\). At all points the investigator was blinded to the dietary group.
REFERENCES


Table S1. Primers and probes used for mRNA analysis

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<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
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<tr>
<td>β-actin</td>
<td>5’- CGTGAAAAGATGACCCAGATCA-3’</td>
<td>5’- CACAGCCTGGATGGCTACGT-3</td>
<td>5’-FAM- TTTGACCTCAACACCCAGCCAT -TAMRA-3’</td>
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<tr>
<td>eNOS</td>
<td>5’- GGAGCACCACCACTGAGACAG -3’</td>
<td>5’- GATTTCTAGCAGCATATTGGGACACA -3’</td>
<td>5’-FAM- TTTGCTGCTCTTGGCTGCG -TAMRA-3’</td>
</tr>
<tr>
<td>SOD1</td>
<td>5’- CCACCTGGAGACCTCATTTATAAT -3’</td>
<td>5’- CTCCAAACATGCCCTCCTTATCATC -3’</td>
<td>5’-FAM- CTCACCTAAGAAACATGGCGGTCCAGC -TAMRA-3’</td>
</tr>
<tr>
<td>GSTa4</td>
<td>5’- GACGGGATGCTACTGAGACAG -3’,</td>
<td>5’- TGGACACATTGCCTCCTCCTTCA-3’</td>
<td>5’-FAM- CAGAGCACATCTAGCTACTGAGACAG -TAMRA-3’</td>
</tr>
<tr>
<td>NADPH</td>
<td>5’- CAACCCCCTGAGTCTTGGACAG -3’,</td>
<td>5’- GCGATAAAAGGAGGACGTCTCCT-3’</td>
<td>5’-FAM- TGGATCTCCTGGCCGATTG -TAMRA-3’</td>
</tr>
<tr>
<td>TNFα</td>
<td>5’- CCCACGTCGATGCAAACACC -3’,</td>
<td>5’- CTTGGAGGAAACCTGGGAGTGA-3’</td>
<td>5’-FAM- AGCCAGCGTGCCAACGCCC -TAMRA-3’</td>
</tr>
<tr>
<td>HMG</td>
<td>5’- TGCAGAGAGGGTGCAAG -3’,</td>
<td>5’- CTCTGCTCAAGGGTTTCCA -3’</td>
<td>5’-FAM- TCAATGCTAAGCATAACCCAGCTACAAA -TAMRA-3’</td>
</tr>
</tbody>
</table>

RESULTS

Thoracic Aorta mRNA levels

Table S2. Levels of mRNA in the thoracic aorta of male and female offspring

<table>
<thead>
<tr>
<th>mRNA</th>
<th>C (n=6)</th>
<th>PR (n=7) PRS (n=5)</th>
<th>C (n=6)</th>
<th>PR (n=7) PRS (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>0.78 ± 0.12</td>
<td>0.64 ± 0.13</td>
<td>0.50 ± 0.11</td>
<td>0.64 ± 0.11</td>
</tr>
<tr>
<td>SOD1</td>
<td>0.22 ± 0.03</td>
<td>0.19 ± 0.02</td>
<td>0.20 ± 0.02</td>
<td>0.17 ± 0.02</td>
</tr>
</tbody>
</table>