Diet-induced endothelial dysfunction in the rat is independent of the degree of increase in total body weight

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ABSTRACT
A growing number of studies indicate an association between obesity, insulin resistance, dyslipidaemia and cardiovascular disorders, collectively known as Syndrome X. In this study we have aimed to produce a model of Syndrome X by voluntary feeding of Wistar rats with a highly palatable cafeteria diet, and examined its effects on metabolic changes and vascular reactivity of Wistar rats. At the end of the experiment, the cafeteria-diet fed group was divided into two groups of low weight gain (LWG) and high weight gain (HWG). Both LWG and HWG groups had significantly ($P < 0.01$) higher fat-pad mass than their chow-fed counterparts, while gastrocnemius muscle mass were comparable. All cafeteria-diet fed rats had significantly ($P < 0.01$) raised plasma triacylglycerol (TG) levels whereas plasma non-esterified fatty acids, glucose and insulin levels were similar between chow-fed and cafeteria-diet fed rats. Vasorelaxation responses to acetylcholine, insulin and sodium nitroprusside were significantly ($P < 0.01$) attenuated in cafeteria-diet fed animals; however, there were no differences in contractile responses of the mesenteric arteries to noradrenaline or KCl between the groups. Multiple regression analysis showed a significant ($P < 0.05$) negative association between plasma TG levels and reduction in acetylcholine-induced vasorelaxation. Acetylcholine-induced vasorelaxation was also significantly ($P < 0.05$) associated with the amount of fat-pad mass. These data suggest that diet-induced vascular dysfunction can occur in the absence of insulin resistance, and that plasma TGs may have a detrimental effect on vascular reactivity.

INTRODUCTION
Obesity is now thought to be a major factor in the development of hypertension. An association has been described between obesity, arterial hypertension, insulin resistance and dyslipidaemia [1–3], which comprises core features of the ‘metabolic syndrome’ or Syndrome X. The precise mechanisms linking obesity and insulin resistance to the development of hypertension are incompletely defined, but have been suggested to include increased sympathetic nervous system activity and resistance to the normal vasodilator effects of insulin [4–6]. Abnormalities of arterial function have been described in obese humans and in certain animal models that reflect some aspects of human obesity. In humans, these include impairment of both endothelium-dependent and -independent vasodilatation [7–10]. In animals, the relationship between vascular reactivity abnormalities and...
Obesity has been studied using fatty (fa/fa) Zucker rats [11,12] and JCR:LA-cp rats [13,14], showing a marked attenuation in endothelium-dependent vasorelaxation [11–14]. Although these well-documented models resemble human obesity and Syndrome X in some respects [15], the fa mutation [16], which results in a non-functional leptin receptor, is a very rare cause of obesity in humans. Furthermore, such obesity is phenotypically more severe than more common polygenic/environmental types. Therefore these animals may not provide an accurate picture of the effects of obesity on the vascular reactivity seen in humans.

Human studies have shown contrasting outcomes as to the role of obesity in vascular defects. One report [9] argues that obesity, independent of other risk factors, is associated with endothelial dysfunction, whereas a more recent study [17] reported that endothelial dysfunction in Type II diabetes is independent of obesity. Moreover, studies of the dietary-induced obesity model in the rat, which is more closely analogous to human obesity [18,19], has been shown to lead to hyperphagia [18] and endothelial dysfunction [19]. We therefore aimed to further elucidate whether highly palatable-diet-induced endothelial dysfunction is proportional to an increase in total body weight.

**MATERIALS AND METHODS**

**Animals**

Adult female Wistar rats (n = 18) were randomly divided into a control group (n = 5, 175.7 ± 2.8 g) and a test group (n = 13, 171.2 ± 3.5 g). At 12 weeks, animals in the test group were divided into two groups: high weight gain (HWG; n = 6) and low weight gain (LWG; n = 7) groups, according to whether or not weight gain of all animals exceeded that in lean controls (Table 1). All animals had free access to water and were housed in groups of two or three under controlled environmental conditions (19–22 °C, 30–40% humidity) and a 12 h light–dark cycle (lights on at 07:00 hours). Lean controls were fed a standard laboratory pelleted diet (CRM Biosure, Cambridge, U.K.), whereas test groups had free access to a highly palatable, high-energy diet consisting of 33% (by weight) ground pellet diet, 33% Nestle condensed milk, 7% sucrose and 27% water, as previously described [18]. Each pair of groups was initially matched for weights (Table 1). All animals in the study were maintained on their respective diet for 12 weeks before being killed.

The rats were killed by CO₂ inhalation, and the gonadal and perirenal fat masses and the gastrocnemius muscle dissected and weighed. Blood was removed by cardiac puncture into cold heparinized tubes. The plasma was immediately separated by centrifugation before being frozen for later measurements of blood analytes ([glucose, insulin, leptin, free non-esterified fatty acid (NEFA) and triacylglycerols (TGs)]). Plasma glucose concentration was determined using a glucose oxidase method, and NEFA and TG concentrations were measured using commercial diagnostic kits (Boehringer Mannheim and Sigma). Insulin and leptin concentrations were measured by RIA kits (Pharmacia/Upjohn Diagnostics, Lewes, Sussex and Linco Research Biogenesis, Poole, Dorset, U.K. respectively).

**Table 1** Physiological and metabolic characteristics of chow-fed and highly palatable diet-fed rats

<table>
<thead>
<tr>
<th>Rats . . .</th>
<th>Chow-fed</th>
<th>DiO HWG</th>
<th>DiO LWG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>175.7 ± 2.8</td>
<td>172.5 ± 2.5</td>
<td>168.7 ± 0.8</td>
</tr>
<tr>
<td>Final</td>
<td>250.1 ± 5.5</td>
<td>246.6 ± 4.9</td>
<td>293.5 ± 20.7*</td>
</tr>
<tr>
<td>Gained</td>
<td>74.4 ± 3.6</td>
<td>74.1 ± 2.8</td>
<td>124.9 ± 21.3*</td>
</tr>
<tr>
<td>Gonadal fat-pad mass (g)</td>
<td>0.76 ± 0.05</td>
<td>2.00 ± 0.17*</td>
<td>1.72 ± 0.32*</td>
</tr>
<tr>
<td>Perirenal fat mass (g)</td>
<td>2.20 ± 0.08</td>
<td>4.49 ± 0.50*</td>
<td>4.50 ± 0.75*</td>
</tr>
<tr>
<td>Gastrocnemius muscle mass (g)</td>
<td>1.60 ± 0.06</td>
<td>1.56 ± 0.08</td>
<td>1.77 ± 0.07</td>
</tr>
<tr>
<td>Fat/lean ratio</td>
<td>1.85 ± 0.07</td>
<td>4.18 ± 0.39*</td>
<td>3.47 ± 0.47*</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>0.97 ± 0.08</td>
<td>1.02 ± 0.07</td>
<td>1.01 ± 0.03</td>
</tr>
<tr>
<td>Plasma glucose (mM)</td>
<td>7.96 ± 0.35</td>
<td>8.88 ± 0.20</td>
<td>7.72 ± 0.80</td>
</tr>
<tr>
<td>Plasma insulin (μ-units/ml)</td>
<td>12.69 ± 2.39</td>
<td>11.80 ± 1.06</td>
<td>13.92 ± 1.73</td>
</tr>
<tr>
<td>HOMA index</td>
<td>4.42 ± 0.65</td>
<td>4.67 ± 0.46</td>
<td>4.87 ± 1.03</td>
</tr>
<tr>
<td>Plasma leptin (ng/ml)</td>
<td>5.97 ± 0.60</td>
<td>6.36 ± 0.59</td>
<td>10.98 ± 0.30*</td>
</tr>
<tr>
<td>Plasma TGs (mM)</td>
<td>0.23 ± 0.06</td>
<td>0.63 ± 0.14*</td>
<td>0.55 ± 0.13*</td>
</tr>
<tr>
<td>Plasma non-esterified fatty acids (mM)</td>
<td>0.33 ± 0.08</td>
<td>0.36 ± 0.03</td>
<td>0.40 ± 0.04</td>
</tr>
</tbody>
</table>

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**Assessment of vascular function**

Eight third-order mesenteric arteries (180–220 μm diameter, 2 mm lengths) were carefully dissected from each animal. Each artery was freed of fat and connective tissue, and mounted on two 40 μm diameter stainless steel wires in an automated myograph (Cambustion, Cambridge, U.K.), based on the principle of the Mulvany myograph. The vessels (in duplicate) were incubated in a 5 ml organ bath containing physiological salt solution (119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.17 mM MgSO₄, 25 mM NaHCO₃, 1.18 mM KH₂PO₄, 0.026 mM EDTA and 5.5 mM glucose) gassed with 95% O₂ and 5% CO₂ at 37 °C.

After 30 min equilibration, the length–tension characteristics for each vessel were determined, as previously described [20], using the law of Laplace (\( P = T/r \); where \( P \) is the transmural pressure, \( T \) is the tension and \( r \) is the vessel radius). Each vessel was then set to its normalized diameter, i.e. the diameter it would achieve at rest in vivo under a transmural pressure of 100 mmHg. The computer also calculated the target tension that each vessel should develop in response to a maximal stimulus. Arteries were allowed a further 30 min to equilibrate before being depolarized twice with high potassium physiological salt solution (125 mM), in which NaCl in normal physiological salt solution was replaced by an equimolar concentration of KCl. Cumulative concentration–response curves to either KCl (10–125 mM) or noradrenaline (NA; 0.5–6 μM) were then carried out. Any vessel failing to reach its predetermined target tension in response to vasoconstriction with KCl (125 mM) was discarded.

**Assessment of endothelium-dependent and -independent vascular relaxation**

To characterize defects in NO and endothelial function, we measured relaxation induced in NA-preconstricted arteries following exposure to endothelium-dependent (acetylcholine; ACh) and endothelium-independent (sodium nitroprusside; SNP) vasodilators. We also studied insulin, which has been shown to induce vasorelaxation via NO generation [12,21,22], and whose vasorelaxation properties are markedly attenuated in the fa/fa Zucker rats [12].

Arteries were contracted with a supramaximal concentration of NA (8 μM). When contraction reached a plateau after 2 min, concentration–response curves were carried out for either ACh, SNP (10 nM–100 μM) or insulin (50–2500 m-units/l) in random order.

**Data interpretation and statistical analyses**

Vasoconstriction in response to KCl and NA was expressed as absolute force generated. Relaxation in response to ACh, SNP or insulin was calculated as the percentage reduction from the maximal tension generated in response to the supramaximal concentration of NA (8 μM). Data are expressed as means ± S.E.M. Statistical significance was tested using repeated-measures ANOVA or the Mann–Whitney test, as appropriate. To evaluate relationships between changes in metabolic parameters and reduction in maximal ACh-induced vasorelaxation, linear regression analysis followed by a two-tailed test was carried out using the statistical package, Arcus Pro-stat (Version 3.23; Iain Buchan, Liverpool, U.K.). We have also used partial correlation and stepwise analysis (multiple linear regression analysis) to determine the relationship between each metabolic parameter and the reduction in ACh-induced vasorelaxation (SPSS, Version 8; SPSS Inc., San Rafael, CA, U.S.A.), incorporating Bonferroni corrections where appropriate. Results were considered statistically significant when \( P < 0.05 \).

**Homoeostasis model assessment (HOMA)**

HOMA, which employs measures of fasting plasma concentrations of glucose and insulin, was used to assess insulin resistance according to the method of Matthews et al. [23].

**RESULTS**

**Body weight and metabolic data**

Not all of the rats given highly palatable diet gained more weight than the chow-fed controls (Table 1). The final weights of HWG rats fed on highly palatable diet were 19% greater than the chow-fed and LWG rats fed on highly palatable diet (Table 1).

The gonadal and perirenal fat masses were significantly \( (P < 0.01) \) (Table 1). The final weights of HWG rats fed on highly palatable diet were significantly \( (P < 0.01) \) (Table 1). The final weights of HWG rats fed on highly palatable diet were significantly \( (P < 0.01) \) (Table 1). The final weights of HWG rats fed on highly palatable diet were significantly \( (P < 0.01) \) (Table 1).
Figure 1  Effects of highly palatable cafeteria diet on vascular contractility
Mesenteric arteries were contracted by either (upper panel) KCl (10–125 mM) or (bottom panel) NA (0.5–6 μM). Data represent means ± S.E.M. There were no significant differences between chow-fed and dietary-obese groups.

Vascular responses
There were no significant differences in vessel diameter between the HWG, LWG and chow-fed animals (225 ± 15 μm, 220 ± 10 μm and 222 ± 15 μm respectively, P > 0.5).

Contractile responses
The contractile responses of vessels from highly palatable cafeteria diet-fed (both HWG and LWG) and lean rats to increasing concentrations of KCl (10–125 mM) and NA (0.5–6 μM) displayed the characteristic sigmoidal relationship, with no significant differences between the two groups either by ANOVA or at any single concentration (Figure 1).

Endothelium-dependent relaxation
Arteries from chow-fed rats that were pre-contracted with NA (8 μM) demonstrated progressive relaxation to cumulative additions of ACh (10 nM–100 μM), achieving a maximum of 81 ± 6% relaxation at an ACh concentration of 100 μM (Figure 2). Arteries from cafeteria diet-fed rats (both HWG and LWG rats) that were similarly exposed to ACh displayed a marked impairment of the relaxation responses, with significant (P < 0.001) right- and downward-shifts compared with vessels from chow-fed rats, with >50% reduction in maximum relaxation (EC50 98.3 ± 1.12 for HWG and 100.0 ± 2.10 for LWG versus 0.42 ± 0.01 μM for chow-fed rats) (Figure 2).

SNP had similar effects to those of ACh. Maximum relaxation of arteries from chow-fed rats was 81 ± 3% at 100 μM SNP (Figure 3), and the relaxation responses of...

Figure 2  Relaxation response curves for ACh on arteries from chow-fed and dietary-obese rats
Arteries were precontracted with 8 mM NA. When contraction reached a plateau after 2 min, concentration response to ACh was carried out. Data represent means ± S.E.M. There was a significant (P < 0.001) attenuation of ACh-induced responses in arteries from dietary-obese rats compared with their control chow-fed counterparts.

Figure 3  Effects of cafeteria diet on SNP-induced vasorelaxation
Arteries were pre-contracted with 8 mM NA. When contraction reached a plateau after 2 min, concentration response to SNP was carried out. Data represent means ± S.E.M. There was a significant (P < 0.01) attenuation of SNP-induced responses in arteries from dietary-obese rats compared with their control lean chow-fed counterparts.
Body weight and endothelial dysfunction

Figure 4 Effect of insulin on arteries from chow-fed and dietary-obese animals
Arteries were precontracted with 8 mM NA. When contraction reached a plateau after 2 min, concentration response to insulin was carried out. Data represent means ± S.E.M. There was a significant (P < 0.01) attenuation of insulin-induced responses in arteries from dietary-obese rats compared with their lean chow-fed counterparts.

arteries from highly palatable diet-fed rats (both HWG and LWG) again displayed a significant (P < 0.001) right- and downward-shift, with up to 25% reduction in maximum relaxation (EC₅₀ 2.34 ± 0.04 for HWG versus 2.82 ± 0.05 for LWG versus 0.56 ± 0.03 μM for chow-fed rats) (Figure 3).

Insulin responses
Insulin induced a concentration-dependent relaxation of vessels from chow-fed rats. There was a significant (57%, P < 0.001) loss of insulin-induced relaxation effect on arteries from cafeteria-fed (both HWG and LWG) rats compared with their chow-fed counterparts (Figure 4).

Relationships between metabolic changes and vascular endothelial dysfunction
We have also examined the correlation between attenuation of maximum ACh-induced vasorelaxation and metabolic changes (increases in body weight, fat pad masses, plasma TG, NEFA, leptin and insulin). There were significant inverse correlations between reduction in maximal ACh-induced vasorelaxation and total fat-pad masses, individual fat-pad mass and TG levels (Table 2). However, there was no significant correlation between plasma NEFA, leptin, insulin or HOMA index and reduction in maximal ACh-induced vasorelaxation (Table 2).

DISCUSSION
The main findings of the present study are that vascular dysfunction, as determined by attenuation of ACh-induced vasorelaxation, was induced by the highly palatable diet, regardless of whether or not there was an overt increase in total body weight compared with chow-fed animals. However, even in those animals that did not gain excessive weight, the highly palatable diet significantly increased visceral fat mass and plasma TG levels compared with chow-fed counterparts. These parameters are thought to play important roles in the development of cardiovascular disease in obesity [24]. Moreover, in the present study, LWG rats had leptin levels similar to those of chow-fed rats, despite having higher TG levels and fat-pad masses, suggesting that they (LWG rats) had developed ‘central obesity’, which in humans is most strongly associated with the metabolic syndrome (Syndrome X). The variation in weight gain in the present study somehow resembles that of human subjects. There were no significant differences in the amount of food consumed between LWG and HWG rats (results not shown), suggesting that LWG animals might have had higher metabolism, thus wasting intake energy via excessive metabolic expenditure. However this hypothesis requires further investigation.

Table 2 Inverse correlation of physiological and metabolic characteristics of chow-fed and highly palatable diet-fed rats with reduction in maximal ACh-induced vasorelaxation

<table>
<thead>
<tr>
<th>Physiological or metabolic characteristics</th>
<th>Univariate correlation coefficient r²</th>
<th>P value</th>
<th>Partial correlation coefficient r²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total body weight</td>
<td>0.159</td>
<td>0.113</td>
<td>0.16</td>
<td>0.116</td>
</tr>
<tr>
<td>Total fat-pad masses</td>
<td>0.326</td>
<td>0.017</td>
<td>0.300</td>
<td>0.015</td>
</tr>
<tr>
<td>Gonadal fat</td>
<td>0.357</td>
<td>0.011</td>
<td>0.362</td>
<td>0.010</td>
</tr>
<tr>
<td>Perirenal fat</td>
<td>0.292</td>
<td>0.025</td>
<td>0.297</td>
<td>0.022</td>
</tr>
<tr>
<td>Plasma TG</td>
<td>0.302</td>
<td>0.022</td>
<td>0.298</td>
<td>0.027</td>
</tr>
<tr>
<td>Plasma non-esterified fatty acids</td>
<td>0.040</td>
<td>0.441</td>
<td>0.042</td>
<td>0.444</td>
</tr>
<tr>
<td>HOMA index</td>
<td>0.095</td>
<td>0.228</td>
<td>0.098</td>
<td>0.235</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.002</td>
<td>0.852</td>
<td>0.002</td>
<td>0.840</td>
</tr>
<tr>
<td>Leptin</td>
<td>0.179</td>
<td>0.091</td>
<td>0.172</td>
<td>0.100</td>
</tr>
</tbody>
</table>
Obesity is positively correlated with insulin resistance in humans [25,26] and in genetically defective animals, such as fatty Zucker rats [12,15]. In the present study, obesity was induced without insulin resistance. There were no significant differences in plasma glucose, insulin concentrations or HOMA index between chow-fed, LWG and HWG rats fed a palatable diet, although the HOMA index tended to be higher, particularly in the HWG groups. This indicates that in dietary-obese rats, vascular dysfunction can occur, independently of overt insulin resistance.

Contractile responses to KCl and NA were almost identical in all three groups, indicating the absence of dietary-induced obesity effect on vascular contractility. This is in agreement with human [7,27] and animal [12,28] studies reported previously. However, there were marked attenuations of the vasorelaxation response induced by both SNP and ACh. SNP-induced vasorelaxation involves direct activation of smooth muscle cGMP [29], whereas ACh-induced vasorelaxation may involve activation of either endothelium-dependent NO system and/or induction of endothelium-derived hyperpolarizing factor (EDHF). The NO, but not EDHF, component of ACh-induced vasorelaxation can be blocked by NO synthase inhibitors [30]. In the present study, the maximal ACh-induced vasorelaxation was attenuated by >50% in both HWG and LWG rats, indicating induction of endothelial dysfunction by the highly palatable diet. Reduction in endothelium-dependent NO-mediated vasorelaxation has been observed in humans with Type II diabetes and hypercholesterolaemia [8,10,21,26,32] and in animal [11,12,14] studies, where, in general, there is an increase in body fat content. Although in the present study we have not tested the precise general, there is an increase in body fat content. Although, we cannot rule out a possible role for EDHF.

SNP-induced vasorelaxation was also reduced in HWG and LWG rats fed a highly palatable diet, indicating either impairment of NO generation by vascular smooth muscle cells or increased degradation of NO in these groups. Our finding is in agreement with some reports which have shown attenuation of SNP-induced vasodilatation in human subjects with Type II diabetes [8,10,31] and hypercholesterolaemia [7].

Insulin-induced vasorelaxation in both humans and animals has been attributed to its ability to stimulate endothelium-dependent NO generation [12,21,22]. In the present study, pre-contracted arteries from chow-fed animals exhibited concentration-dependent relaxation by insulin. Insulin-induced vasorelaxation was severely blunted in all (both LWG and HWG) highly palatable diet fed rats. These data further strengthen the hypothesis that in normal Wistar rats highly palatable diet causes endothelial dysfunction, which, in turn, results in attenuation of insulin responses, regardless of degree of increase in total body weight.

Various studies have demonstrated a correlation between plasma TG levels and risk of coronary artery disease and an amplification of risk with combined elevations of TG and low-density lipoprotein. Animal and human studies have shown strong associations between plasma TGs [10,13,24,33–36], lower levels of high-density lipoprotein [10] and increased levels of low-density lipoprotein [37] and the magnitude of endothelial defect. Moreover, postprandial TG has also been shown to play a major role in the development of atherosclerosis in humans [38]. This negative effect of plasma lipid profile has, at least partly, been attributed to inhibition of NO synthesis [39]. In Type II diabetes, the severity of coronary atherosclerosis has been shown to be directly associated with the number of circulating TG-rich lipoprotein particles [40,41]. A similar effect was also seen in the present study (Table 2), where a strong negative correlation between plasma TG concentration and the level of attenuation of ACh-induced vasorelaxation was apparent.

In conclusion, we postulate that induction of obesity (measured as an increase in fat/lean ratio) by highly palatable diet is independent of total body mass increase. This induced obesity has no effect on vascular contractility, but attenuates both endothelium-dependent and -independent vasorelaxation, an effect which can occur in the absence of insulin resistance. These findings will help us to evaluate the role of diet in human obesity and related cardiovascular disorders.

REFERENCES