Dietary obesity in the rat induces endothelial dysfunction without causing insulin resistance: a possible role for triacylglycerols

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ABSTRACT

Impaired arterial vasorelaxation, due primarily to endothelial dysfunction, is associated with obesity. To clarify the relationship with insulin resistance and other metabolic disturbances, we studied endothelial-dependent and -independent vascular responses in rats with dietary-induced obesity. Dietary-obese rats had significantly higher body weights (10–32%; P < 0.001) and fat-pad masses (220–280%; P < 0.001) than lean controls, together with raised plasma levels of triacylglycerols (15–80%; P < 0.001), non-esterified fatty acids (13–38%; P < 0.05) and leptin (85–180%; P < 0.001). However, measures of insulin sensitivity (including the hyperinsulinaemic–euglycaemic clamp in a parallel experiment) were comparable with those in controls. Contractions induced in mesenteric arteries by noradrenaline (0.5–8 μmol/l) were comparable in lean and obese groups, but vasorelaxation in noradrenaline-preconstricted arteries was markedly reduced in dietary-obese rats of both sexes. Concentration–response curves to endothelium-dependent vasorelaxants (acetylcholine, A23187 and insulin) showed significant reductions in maximal relaxation (20–95% less than in leans; P < 0.001) and significant rightward shifts in EC40 (concentration giving 40% of maximal response) (P < 0.01). Relaxation in response to the direct NO donor, sodium nitroprusside, showed a lesser impairment (12%; P < 0.01) in dietary-obese rats. Maximal relaxation to acetylcholine was correlated inversely in both sexes with fat-pad mass (r² = 0.37, P < 0.05) and plasma triacylglycerols (r² = 0.51, P < 0.01), and with leptin in males only (r² = 0.35, P < 0.05). Independent determinants of acetylcholine-induced relaxation were fat mass and plasma triacylglycerols; plasma insulin and insulin sensitivity had no effect. Dietary-induced obesity severely impaired arterial relaxation in both sexes, particularly at the endothelial level. This is not attributable to insulin resistance, but may be related to moderate hypertriglyceridaemia.

INTRODUCTION

Obesity is an important risk factor for atheroma, and predisposes to hypertension and dyslipidaemia, in which hypertriglyceridaemia is now thought to be important [1,2]. Obesity is also accompanied by specific abnormalities of endothelial function that also occur in other pro-atherogenic conditions [3,4]. The endothelium is...
crucial to key arterial functions, including the regulation of vascular tone [5]. Endothelial cells produce various vasoactive mediators, notably the vasodilator nitric oxide (NO), which acts on the underlying vascular smooth muscle [6]. NO production is regulated by the endothelial form of NO synthase, which is stimulated by mediators and hormones that include acetylcholine (ACh), bradykinin and insulin [6,7].

Impaired NO-mediated vasorelaxation has been demonstrated in obesity, Type II diabetes and hypertension [8–10], with the most striking abnormality being blunted vasodilatation in response to ACh. Impaired endothelium-mediated vasorelaxation could contribute to hypertension [11], and its presence in subjects at high risk of atherogenesis suggests that it may predict atheroma formation, and ultimately coronary and peripheral arterial disease [11,12].

The causes of endothelial dysfunction and its pathophysiological relationship with other proatherogenic conditions are unknown. Obesity (especially abdominal), hypertension, Type II diabetes and dyslipidaemia are all commonly associated with reduced sensitivity to the metabolic actions of insulin [3,13]. This has led to the hypothesis that their co-segregation represents a specific ‘metabolic syndrome’ (syndrome X), whose fundamental cause is insulin resistance [3,13–15]. It has also been suggested that insulin resistance is the cause of vascular dysfunction in these conditions [13–17]. This hypothesis remains unproven.

Much of the work relating defects in endothelial function and vascular reactivity to insulin resistance has been done in genetically obese rats [fa/fa Zucker, JCR-LA cp/cp and ZDF (Zucker diabetic fatty)], which have massive insulin resistance that is due ultimately to mutations affecting the leptin receptors [18,19]. However, these mutants are imperfect models of human obesity, which is not due to a primary loss of leptin signalling and in which insulin resistance is less marked. We have therefore studied a rat model more relevant to obesity, Type II diabetes and hyperlipidaemia, characterized by a selective increase in body fat mass (> 100% above lean values), with proportionate rises in plasma leptin, and by variable but relatively mild insensitivity to insulin and leptin [20].

Here, we have characterized in detail the vascular responses to endothelium-dependent vasodilatation [ACh, carbamylcholine (CCh), A23187 and insulin] and to the direct NO donor, sodium nitroprusside (SNP). Endothelial function has not been reported previously in dietary-induced obesity. To try to clarify the causes of endothelial dysfunction in obesity, we also sought to identify the metabolic determinants of impaired relaxation in response to ACh. In particular, we assessed changes in insulin sensitivity from plasma insulin and glucose levels, and in a parallel study using the hyperinsulinaemic–euglycaemic clamp.

**MATERIALS AND METHODS**

**Animals and experimental protocol**

In experiment 1, adult male Wistar rats (n = 28) were randomly assigned to a control group (n = 10) or a dietary-obese group (n = 18). In experiment 2, adult female Wistar rats (n = 18) were randomly assigned to a control (n = 5) or a dietary-obese (n = 13) group. All 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). Controls were fed on standard pelleted laboratory chow (CRM Biosure, Cambridge, U.K.), while dietary-obese groups had free access to a highly palatable, high-energy diet consisting of 33% (by weight) ground chow, 33% Nestlé condensed milk, 7% sucrose and 27% water. Male rats were studied for 16 weeks and females for 12 weeks.

The rats were killed by CO₂ inhalation, and the gonadal and perirenal fat masses and the gastrocnemius muscle were dissected and weighed. Blood was collected for measurement of glucose, insulin, leptin, non-esterified (‘free’) fatty acids (NEFA) and triacylglycerols (TG; triglycerides). The plasma glucose concentration was determined by the glucose oxidase method, and NEFA and TG concentrations were measured with commercial diagnostic kits from Boehringer Mannheim and Sigma Diagnostics respectively. Insulin and leptin concentrations were measured by RIA kits (Pharmacia/Upjohn Diagnostics and Linco Research respectively).

In experiment 3, 20 adult male Wistar rats were randomly assigned to a control (n = 10) or a dietary-obese (n = 10) group and treated as described above for 12 weeks. They underwent a hyperinsulinaemic–euglycaemic clamp as a terminal procedure [21]. Rats were anaesthetized with an intraperitoneal injection of a Diazemuls (Dumex Ltd)/Hypnorm (Janssen Pharmaceutica Ltd)/water mixture (1:1:2, by vol.). Human soluble insulin (Humulin-S; Eli Lilly, Basingstoke, Hants., U.K.), in isotonic saline containing 1% (w/v) BSA (Sigma), was infused at a constant rate of 14 m-units/min into the right jugular vein to produce hyperinsulinaemia. Glucose solution (20% in water) was co-infused with the insulin, and the rate was adjusted to maintain blood glucose at 5 mmol/l; tail-vein blood glucose concentrations were measured every 5 min using an Exactech® electrochemical meter (Medisense, Oxford, U.K.). The M-value was derived from the mean steady-state glucose infusion rate during the last 30 min of the 180 min clamp divided by the terminal plasma insulin concentration [21]. At the end of the clamp, rats were
killed by exsanguination, and plasma glucose and insulin concentrations were measured as above.

Assessment of vascular function
In experiments 1 and 2, eight third-order mesenteric arteries were carefully dissected from each animal and mounted on two 40 μm-diameter stainless-steel wires in an automated myograph (Cambustion, Cambridge, U.K.). Pairs of arteries were incubated in a 5 ml organ bath containing physiological salt solution [composition (in mmol/l): NaCl 119, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.17, NaHCO₃ 25, KH₂PO₄ 1.18, EDTA 0.026 and glucose 5.5] gassed with 95% O₂/5% CO₂ at 37 °C.

After a 30 min equilibration period, the length–tension characteristics for each vessel were determined [22]. Arteries were allowed a further 30 min to equilibrate before being depolarized twice with high-potassium physiological salt solution, in which NaCl in normal physiological salt solution was replaced by an equimolar concentration of KCl (125 mmol/l). Cumulative concentration–response curves to either KCl (10–125 mmol/l) or noradrenaline (NA; 0.5–8 μmol/l) were then carried out. Any vessel failing to reach its predetermined target tension in response to KCl (125 mmol/l) was discarded.

Assessment of endothelium-dependent and -independent vascular relaxation
To characterize endothelial defects, we measured the effects of selected endothelium-dependent vasodilators (ACh, CCh, the calcium ionophore A23187 and insulin) in arteries preconstricted with 8 μmol/l NA. We similarly investigated direct effects on vascular smooth muscle using the endothelium-independent vasodilator SNP.

Reagents
NA, ACh, CCh, A23187 and SNP were all obtained from Sigma. All solutions except A23187 were freshly made up before use in distilled water. A23187 was prepared in ethanol; working concentrations of ethanol in the bath (< 0.01%, v/v) do not affect vascular contractility [22,23].

Data interpretation and statistical analyses
Vasoconstriction in response to NA and KCl is expressed as absolute force generated. Relaxation in response to ACh, CCh, A23187, SNP or insulin was calculated as the percentage reduction from the maximal tension generated in response to a supramaximal concentration of NA (8 μmol/l).

Data are means ± S.E.M. Statistical significance was tested using repeated-measures ANOVA or the Mann–Whitney test, as appropriate. Univariate correlations between maximal ACh-induced vasorelaxation and various metabolic parameters were determined by linear regression followed by two-tailed analyses, using Arcus Pro-stat (Version 3.23; Iain Buchan, University of Liverpool, U.K.). Independent determinants of ACh-induced vasorelaxation were identified from partial correlation coefficients, derived from stepwise analysis using the SPSS statistical package (Version 8; SPSS Inc.). Differences were considered statistically significant at the P < 0.05 level.

RESULTS

Body weight and metabolic data
Rats of either sex given the highly palatable diet gained progressively more weight than chow-fed controls, being significantly heavier (P < 0.01) after 4 weeks. Diet-induced weight gain was significantly greater in males (experiment 1) than in females (experiment 2), with the final body weights of diet-fed rats being 32% (males) and 9% (females) greater than those of their respective chow-fed controls (Figure 1, Table 1). The weight gain of the dietary-obese male rats in experiment 3 (18% over control) fell mid-way between those in experiments 1 and 2 (Table 2). The epididymal and perirenal fat masses weighed significantly more (> 200%; P < 0.001) in all dietary-obese groups than in controls, while gastrocnemius muscle weight was comparable (Table 1).

In dietary-obese rats of either sex, terminal plasma TG levels were significantly higher (by 15–80%; P < 0.001) than in lean controls, as were NEFA levels (by 13–38%; P < 0.05). Leptin levels were also significantly raised above those in controls, by 180% in males and 85% in females (Table 1).

There were no significant differences in either plasma insulin or glucose levels between dietary-obese rats and controls in either experiment 1 or 2 (Table 1), suggesting that the moderate obesity in these rats was not accompanied by insulin resistance. This was confirmed by

![Figure 1: Effects of a highly palatable diet on weight gain in male and female Wistar rats](https://example.com/figure1.png)

Lean controls were fed standard laboratory pelleted diet, while dietary-obese rats were given a highly palatable diet throughout the experimental period. Data represent means ± S.E.M.
Table 1  Physiological and metabolic characteristics of chow-fed and dietary-obese male and female rats
Data are means ± S.E.M. NS, not significant.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Males</th>
<th>Females</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chow-fed (n = 10)</td>
<td>Dietary-obese (n = 18)</td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>200 ± 5</td>
<td>211 ± 3</td>
<td>NS</td>
</tr>
<tr>
<td>Final</td>
<td>562.8 ± 17.7</td>
<td>742.0 ± 14.4</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Gain</td>
<td>363.0 ± 16.3</td>
<td>530.1 ± 14.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Epididymal fat pad mass (g)</td>
<td>8.38 ± 1.0</td>
<td>18.6 ± 0.8</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Gonadal fat mass (g)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Penitrenal fat mass (g)</td>
<td>11.5 ± 1.8</td>
<td>25.7 ± 1.8</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Gastrocnemius muscle mass (g)</td>
<td>3.4 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>11.2 ± 0.8</td>
<td>12.2 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma insulin (m-units/l)</td>
<td>2.00 ± 0.42</td>
<td>2.21 ± 0.38</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma leptin (ng/ml)</td>
<td>25.30 ± 5.15</td>
<td>75.63 ± 7.0</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Plasma TG (mmol/l)</td>
<td>0.44 ± 0.03</td>
<td>0.79 ± 0.07</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Plasma NEFA (mmol/l)</td>
<td>0.34 ± 0.04</td>
<td>0.47 ± 0.04</td>
<td>&lt; 0.05</td>
</tr>
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</table>

Table 2  Physiological and metabolic characteristics of chow-fed and dietary-obese male rats used for glucose clamping
Data are means ± S.E.M. NS, not significant.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chow-fed (n = 10)</th>
<th>Dietary-obese (n = 10)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>244.6 ± 5.1</td>
<td>252.0 ± 2.3</td>
<td>NS</td>
</tr>
<tr>
<td>Final</td>
<td>465.6 ± 8.6</td>
<td>523.5 ± 11.1</td>
<td>&lt; 0.001</td>
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<tr>
<td>Gain</td>
<td>221.5 ± 10.3</td>
<td>271 ± 14.9</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>5.48 ± 0.38</td>
<td>6.07 ± 0.44</td>
<td>NS</td>
</tr>
<tr>
<td>Post-clamp plasma insulin (µ-units/ml)</td>
<td>175 ± 21</td>
<td>205 ± 42</td>
<td>NS</td>
</tr>
<tr>
<td>M-value</td>
<td>0.50 ± 0.07</td>
<td>0.52 ± 0.06</td>
<td>NS</td>
</tr>
</tbody>
</table>

Figure 2  Effects of NA on arteries from dietary-obese and lean control rats
Data represent means ± S.E.M. The concentration–response curves for the two groups did not differ significantly for either sex.

the clamp study in experiment 3. Steady-state glucose and insulin concentrations during the clamp were closely similar in lean and obese rats (Table 2), and the M values were almost identical (lean, 0.50 ± 0.07; obese, 0.52 ± 0.06; P > 0.1).

Vascular contractile responses
There were no significant differences in arterial diameter between the obese and lean groups [males: obese, 235 ± 15 µm; lean, 230 ± 11 µm (P > 0.5); females: obese, 230 ± 15 µm; lean, 231 ± 10 µm (P > 0.5)].

The contractile responses of arteries to increasing concentrations of NA (0.5–8 µmol/l) were measured in all the rats, and displayed the characteristic sigmoid relationship. Maximal tension generated was consistently greater in males, but there were no significant differences between any lean and obese groups (Figure 2). KCl concentration–response curves (tested in arteries from female rats) similarly showed no significant differences between lean and obese groups (results not shown).

Endothelium-dependent relaxation
In both sexes, arteries from lean rats that were precontracted with NA (8 µmol/l) demonstrated pro-
Progressive relaxation to cumulative additions of ACh (10 nmol/l–100 μmol/l). Maximal relaxation at 100 μmol/l ACh was 89 ± 3 % and 83 ± 3 % for arteries from male and female rats respectively (Figure 3a). Arteries from dietary-obese rats that were similarly exposed to ACh displayed marked impairment of the relaxation responses (Figure 3a), with maximum relaxation of only 72 ± 4 % in males and 42 ± 1 % in female rats (both P < 0.001 compared with lean controls). The dose–response curves for arteries from obese rats also displayed significant rightward shifts as compared with those from lean controls. EC₅₀ values (concentration giving 50 % of maximal response) were as follows: obese males, 0.27 ± 0.1 μmol/l; lean males, 0.09 ± 0.01 μmol/l (P < 0.001); obese females, 97.53 ± 0.48 μmol/l; lean females, 0.36 ± 0.02 μmol/l (P < 0.001).

The effects of CCh, tested in arteries from males, were similar to those of ACh. Maximum relaxation in response to 100 μmol/l CCh was 94 ± 1 % in arteries from lean rats, but only 75 ± 4 % (P < 0.001) in arteries from obese rats, and there was a marked rightward shift of the dose–response curve (EC₅₀ values: obese, 1.2 ± 0.10 μmol/l; lean, 0.22 ± 0.02 μmol/l; P < 0.001).

A23187, tested in arteries from males, induced the concentration-dependent relaxation of NA-preconstricted arteries. This effect was significantly attenuated in arteries from obese rats at A23187 concentrations of between 3.16 nmol/l and 31.6 μmol/l, but the responses were comparable at the highest concentration (100 μmol/l) (Figure 3b). The EC₅₀ was significantly higher in the dietary-obese group than in the controls (4.64 ± 0.08 and 1.37 ± 0.02 μmol/l respectively; P < 0.01).

Insulin induced concentration-dependent arterial relaxation in arteries from lean rats of both sexes. Arteries from dietary-obese rats consistently showed a marked loss of insulin-induced relaxation, with reductions of > 90% (P < 0.001) at a concentration of 250 m-units/l compared with those from their lean counterparts (Figure 4).

Endothelium-independent relaxation
The direct NO donor SNP, tested in females, also induced vasorelaxation that was attenuated in dietary obesity. The maximum relaxation at 100 μmol/l SNP of arteries from chow-fed rats was 81 ± 3 %, but only 71 ± 2 % in those from obese rats (P < 0.01). In addition, the concentration–response curve showed a significant rightward shift (EC₅₀ values: obese, 2.05 ± 0.02 μmol/l; lean, 0.21 ± 0.03 μmol/l; P < 0.001) (Figure 5).

Metabolic determinants of vascular dysfunction
We examined the relationships between endothelial-mediated vasorelaxation (measured as the percentage reduction of maximal NA-induced vasorelaxation induced by 100 μmol/l ACh) and major metabolic changes of obesity, namely body weight, fat-pad mass (sum of
perirenal and gonadal fat pads) and plasma levels of TG, NEFA, leptin and insulin (Table 3).

There was an inverse correlation between body weight and maximal ACh-induced vasorelaxation in male but not female rats, whereas both sexes showed highly significant inverse correlations between ACh-induced vasorelaxation and fat-pad mass. In both sexes, plasma TG levels were also significantly inversely correlated with maximal ACh-induced vasorelaxation (Table 3). Stepwise multivariate analysis on data from both sexes demonstrated that the independent determinants for endothelial dysfunction in dietary obesity were total fat-pad mass and plasma TG levels. Univariate analysis showed that body weight and plasma leptin levels were inversely related to maximal ACh-induced vasorelaxation in male rats, but not in females. Moreover, stepwise analysis also indicated a significant independent contribution of leptin to vascular dysfunction in male rats only (Table 3).

These analyses also confirmed that endothelial dysfunction was not related to metabolic insulin resistance. There was no significant correlation between attenuation of maximal ACh-induced vasorelaxation and plasma levels of either glucose or insulin, and stepwise multiple variant analyses similarly showed no significant effect attributable to these parameters. Neither was there any influence of NEFA (Table 3).

### DISCUSSION

This study has yielded two novel and important findings. Firstly, rats with dietary-induced obesity – like the widely studied genetic models – show markedly impaired arterial relaxation, mainly due to endothelial dysfunction. Secondly, these defects occurred in the absence of significant insulin resistance, as shown by unchanged plasma glucose and insulin concentrations and by normal M-values in the parallel hyperinsulinaemic–glucose clamp study. Our findings therefore throw new light on the causes of arterial disease in obesity, and also directly challenge the notion that vascular dysfunction in obesity is caused primarily by insulin resistance [3,13].

The vascular dysfunction induced by dietary obesity was severe and multifactorial. Impaired relaxation in response to ACh was comparable in magnitude to that in the widely studied genetic models – show markedly impaired arterial relaxation, mainly due to endothelial dysfunction. Furthermore, despite a larger increase in total body weight of obese male rats, attenuation of ACh-induced vasodilatation was more pronounced in obese female rats than in obese male rats, indicating a gender-dependent variation in vascular reactivity. Similar observations have also been reported previously implying an effect of gender on vascular contractility [25–27].

Dietary obesity was associated with impairment of relaxation induced by four different endothelium-dependent stimuli: ACh, CCh, A23187 and insulin. All

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**Table 3 Correlations between vasorelaxation and physiological and metabolic characteristics in dietary-obese male and female rats**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Males (n = 18)</th>
<th>Females (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate correlation coefficient</td>
<td>Partial correlation coefficient</td>
</tr>
<tr>
<td></td>
<td>( r^2 )</td>
<td>( P ) value</td>
</tr>
<tr>
<td>Body weight</td>
<td>0.504</td>
<td>0.002</td>
</tr>
<tr>
<td>Gonadal + perineal fat-pad mass</td>
<td>0.371</td>
<td>0.012</td>
</tr>
<tr>
<td>Plasma TG</td>
<td>0.513</td>
<td>0.001</td>
</tr>
<tr>
<td>Plasma NEFA</td>
<td>0.167</td>
<td>0.116</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.017</td>
<td>0.641</td>
</tr>
<tr>
<td>Leptin</td>
<td>0.347</td>
<td>0.021</td>
</tr>
</tbody>
</table>
induce vasorelaxation by stimulating endothelial NO production through endothelial NO synthase [5,6]; blunted vasodilatation in response to all these agents points to a defect in the NO synthase or in NO production, rather than to specific abnormalities, such as in the muscarinic cholinergic receptor or in the action of insulin (as has been suggested in the fa/fa Zucker rat [23]). Comparable responses to the stable ACh analogue, CCh, argue against the attenuated ACh-induced vasorelaxation being due to excessive degradation of ACh by cholinesterase.

Endothelium-independent mechanisms are also involved, as SNP-induced vasorelaxation was also attenuated, but to a lesser extent than the upstream endothelial-mediated mechanisms. This additional defect could reflect reduced responsiveness of vascular smooth muscle and/or enhanced clearance of NO, and indicates that dietary obesity induces distinct abnormalities in both endothelium and vascular smooth muscle.

Analogous vascular dysfunction has been reported in states that show the common feature of insulin resistance. Endothelium-dependent vasorelaxation is impaired in morbidly obese and massively insulin-resistant Zucker and JCR:LA-cp rats [23]. Comparable responses to the stable ACh analogue, CCh, argue against the attenuated ACh-induced vasorelaxation being due to excessive degradation of ACh by cholinesterase.

In conclusion, we have demonstrated that moderate dietary-induced obesity markedly impairs vasorelaxation, primarily at the endothelial level, but also in smooth muscle. This vascular dysfunction is comparable in magnitude with that reported in insulin-resistant states, but occurred in the absence of insulin resistance. These defects may contribute to the hypertension and atheroma formation that accompany obesity, independently of any influence of insulin resistance. Hypertriglyceridaemia may provide a link between expanded fat-pad mass and endothelial dysfunction.

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