Raised saturated-fat intake worsens vascular function in virgin and pregnant offspring of streptozotocin-diabetic rats

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Adult offspring of severely diabetic pregnant rats are insulin resistant and display cardiovascular dysfunction. When pregnant they develop mild hyperglycaemia. Diets high in saturated fat have been implicated in the development of cardiovascular disease and vascular dysfunction. In the present study we have determined vascular function in small mesenteric arteries from offspring of normal (OC) and diabetic (OD) rats fed standard chow and offspring of diabetic rats fed a diet high in saturated fats (OD-HF) from weaning to adulthood, and throughout their subsequent pregnancies. OD rats displayed an increased sensitivity to noradrenaline ($P < 0{\cdot}05$) and impaired sensitivity to the endothelium-dependent vasodilator, acetylcholine. The component of acetylcholine-induced relaxation attributable to endothelium-derived hyperpolarizing factor was reduced in OD-HF rats. Pregnant OD rats also demonstrated impaired maximum relaxation to acetylcholine (pregnant OD rats *v.* pregnant OC rats $P < 0{\cdot}05$). In pregnant OD-HF rats noradrenaline sensitivity was enhanced and endothelium-dependent relaxation further reduced (pregnant OD-HF rats *v.* pregnant OC rats $P < 0{\cdot}001$). The isoprostane, 8-epi-prostaglandin $F_{2\alpha}$, a marker of oxidative stress, was increased in pregnant OD rats (pregnant OD rats *v.* pregnant OC rats $P < 0{\cdot}001$) and further increased in pregnant OD-HF rats (pregnant OD-HF rats *v.* pregnant OD rats $P < 0{\cdot}05$). We conclude that a high-saturated-fat diet leads to deterioration in specific components of vascular function in OD rats. When pregnant, vascular function of OD-HF rats is further compromised. Pregnancy in the OD rats is associated with a striking increase in a marker of oxidative stress, which increases further if the saturated fat intake is raised.

Diabetes: Pregnancy: High-fat diet: Vascular function

Over recent decades the incidence of hypertension, obesity, atherosclerosis and diabetes has dramatically increased in Western populations, and has been attributed in part to environmental factors, notably diet and reduced physical activity (Rosenthal *et al.* 1983; Reaven, 1988). Epidemiological (Knowler *et al*. 1991; Taylor *et al.* 1992) and animal (Grundlegger & Thenen, 1982; Storlien *et al.* 1986; Storlien *et al.* 1991) studies suggest that the high saturated fat content of the typical Western diet is a major cause of obesity, insulin resistance, non-insulin-dependent diabetes mellitus (NIDDM) and cardiovascular diseases. Evidence has accumulated from studies of populations who once practised a traditional lifestyle, but who were abruptly exposed to the Western lifestyle, and in whom there is an unparalleled high prevalence of obesity and NIDDM (Knowler *et al.* 1991; Taylor *et al.* 1992). Susceptible subjects also risk development of NIDDM with a Western diet, as glucose tolerance deteriorates in NIDDM if consumption of dietary fats is increased (O'Dea *et al.* 1989).

Susceptibility to cardiovascular disease may also be acquired *in utero*. Barker and co-workers (Phillips *et al.* 1994) have associated thinness at birth with the development of cardiovascular disease in adulthood. Diabetes *in utero* has also been implicated in the transmission of a diabetogenic tendency to the offspring in human subjects (Pettitt *et al.* 1988; Silverman *et al.* 1995) and in animal models (Aerts & Van Assche, 1979; Gauguier *et al.* 1990; Oh *et al.* 1991). In our laboratory we have developed an

Abbreviations: ACh, acetylcholine; DEXA, dual-energy X-ray absorptiometry; L-NAME, Nω-nitro L-arginine methyl ester; NA, noradrenaline; NIDDM, non-insulin-dependent diabetes mellitus; OC, POC, virgin and pregnant offspring of control rats respectively; OD, POD, virgin and pregnant offspring of diabetic rats fed standard chow respectively; OD-HF, POD-HF, virgin and pregnant offspring of diabetic rats fed a high-saturated-fat diet respectively; ODQ, oxadiazole quinoxaline; PG, prostaglandin; PSS, physiological salt solution; STZ, streptozotocin.

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animal model which demonstrates transmission of the diabetogenic tendency. We have found that female adult offspring of streptozotocin (STZ)-diabetic pregnant rats show overt insulin resistance (Holemans *et al.* 1991*a*) and endothelial dysfunction (Holemans *et al.* 1999). Moreover, when pregnant these offspring develop gestational diabetes (Holemans *et al.* 1991*b*).

Recently, we have shown that a diet high in saturated fat induces profound vascular dysfunction in control rats, i.e. the offspring of normal animals (Gerber *et al.* 1999*b*). In the present study we have explored the possibility that a combination of the susceptibility to NIDDM acquired *in utero* together with the added insult of a diet high in saturated fat may predispose the offspring of the STZ-diabetic rat to overt vascular dysfunction. Female offspring of STZ-diabetic rats were weaned on a high-saturated-fat diet, and the diet was continued in a subgroup which were mated with a control male. Vascular function was assessed in small mesenteric arteries of the virgin offspring and the pregnant offspring, and compared with values obtained from the offspring of diabetic rats fed standard chow. Insulin resistance was investigated by analysis of plasma glucose, insulin and lipids. Since vascular dysfunction brought about by a high-fat diet or diabetes has been associated with oxidative stress, we have also evaluated plasma concentrations of the isoprostane 8-epi-prostaglandin (PG)$F_{2\alpha}$, a stable peroxide of arachidonic acid.

Material and methods

The entire protocol was reviewed and approved by the local Ethical Committee for Animal Procedures (Katholieke Universiteit Leuven, Belgium).

Animals and dietary protocol

The animals were Wistar rats (outbred, pfd Leuven) obtained from the KULeuven Breeding Center (Leuven, Belgium). Female offspring of control (OC) and of severely diabetic (OD) pregnant Wistar rats were studied. Diabetes in the dams was induced with a single intravenous injection of 35 mg streptozotocin (STZ)/kg body weight on day 1 of pregnancy (i.e. the day of the copulation plug). This dose of STZ induces severe diabetes; only rats with a plasma glucose concentration higher than 20 mmol/l on day 20 of pregnancy entered the study. After delivery all pups were suckled by their mothers, and at weaning at 20 d of age only female offspring were kept. All dams received a standard non-purified rat diet (Trouw, Ghent, Belgium) during pregnancy and lactation. The female offspring were fed either a standard non-purified rat diet (OC and OD) or a semi-synthetic diet (Special Diet Services Ltd, Witham, Essex, UK) containing 200 g saturated fat/kg from weaning to adulthood (100–120 d of age; OD-HF). The high-fat diet consisted of (g/kg): 480 carbohydrate, 210 protein, 160 lard (lard fatty acid (g/100 g total fatty acids): 2·7 palmitoleic acid, 32·8 oleic acid, 8·1 linoleic acid, 0·4 linolenic acid, 1·55 myristic acid, 21·2 palmitic acid, 9·6 stearic acid) and 40 maize oil (maize-oil fatty acids (g/100 g total fatty acids): 23·4 oleic acid, 42·9 linoleic acid, 0·8 linolenic acid, 9·8 palmitic acid, 2 stearic acid) supplemented with essential micronutrients and vitamins to ensure the same final content (w/w) as the standard non-purified rat diet. The standard non-purified rat diet consisted of (g/kg) 510 carbohydrate, 210 protein, 40 fat (maize oil). Rats were weighed weekly from 7 d of age to 98 d of age.

At 90–100 d of age a subgroup of OC, OD and OD-HF rats were mated and they remained on the same diet (standard non-purified rat diet (POC and POD) and 200 g saturated fat/kg diet (POD-HF) respectively) until assessment of vascular function at 19–22 d gestation.

Plasma measurement of glucose, insulin, cholesterol, triacylglycerols and non-esterified fatty acids

After an overnight fast blood was taken from virgin rats (98 d of age) from an incision made at the tip of the tail for determination of plasma glucose, insulin, cholesterol, triacylglycerol and free fatty acids. In some cases the sample obtained was insufficient for complete analysis. Plasma glucose was determined by the glucose oxidase method using a glucose analyser (glucose analyzer 2300STAT; Yellow Spring Instruments, Yellow Springs, OH, USA). Plasma insulin was assessed by radio-immunoassay using rat insulin (Novo Industri, Bagsvaerd, Denmark) as a standard. The antibody, raised in guinea-pigs, was donated by Dr A Kervran (Collège de France, Paris, France). Plasma triacylglycerols (Triglycerides GPO-PAP; Boehringer Mannheim GmbH, Mannheim, Germany), cholesterol (Cholesterol CHOL-PAP; Boehringer Mannheim) and free fatty acids (free fatty acids half-micro test; Boehringer Mannheim) were evaluated using commercially-available kits. In pregnant rats no fasting plasma samples were taken for practical reasons; plasma glucose and insulin vary significantly from day to day in late pregnancy and as an overnight fast might have influenced vascular function, it was considered inappropriate to take fasting blood samples on the same day as vascular function was assessed.

Assessment of vascular function

Virgin rats were killed by CO_2 inhalation at 100–120 d of age, and pregnant rats between 19 and 22 d of gestation. Small mesenteric resistance arteries were mounted on a small-vessel wire myograph as previously described (Mulvany & Halpern, 1977). Briefly, third-order branches of the mesenteric tree were dissected free of connective tissue and mounted on fine W wires in pairs as ring preparations for the measurement of isometric tension and bathed in physiological salt solution (PSS; mmol/l; NaCl 119, KCl 4·7, $CaCl_2$ 2·5, $MgSO_4$ 1·17, $NaHCO_3$ 25, KH_2PO_4 1·16, EDTA 0·026, glucose 6·0), pH 7·4 at 37°C and gassed with 50 litres CO_2/l in O_2. The passive tension–internal circumference characteristics of the arteries were determined by stretching to achieve an internal circumference equivalent to 90 % of that which would be attained when relaxed *in situ* under a transmural pressure of 100 mmHg. To confirm viability of the arteries four contractions (4 min duration) were performed to 5×10^{-6} mol noradrenaline (NA)/l, KPSS (PSS containing 125 mmol KCl/l) or a combination of both. Arteries failing to produce

active tension equivalent to 100 mmHg were rejected. Concentration–response curves, at increments of 2 min duration, were then constructed for NA (10^{-9}–10^{-5} mol/l), and following repeated washing and recovery endothelium-dependent relaxation to acetylcholine (ACh; 1 nmol–10 μmol/l) and sodium nitroprusside (1 nmol–10 μmol/l) were assessed in arteries submaximally preconstricted with 5 μmol NA/l. To deduce the relative contributions of PG and/or NO to the ACh-induced relaxation two further concentration responses to ACh were repeated first after 20 min incubation with and in the presence of 10 μmol indomethacin/l and second with indomethacin (10 μmol/l), Nω-nitro L-arginine methyl ester (L-NAME; 100 μmol/l) and the soluble guanylate cyclase inhibitor, oxadiazole quinoxaline (ODQ; 1 μmol/l). Finally, to investigate a potential role of a hyperpolarizing factor, ACh responses were studied in the presence of the PG and NO inhibitors in partially-depolarizing PSS (25 mmol KCl/l). Arteries were preconstricted with 2–4 μmol NA/l, the concentration being adjusted in order to evoke similar preconstrictor tone to that observed in normal 5 mmol KCl/l without inhibitors.

Determination of F_2-isoprostanes

Blood samples were taken for F_2-isoprostane (8-epi-$PGF_{2\alpha}$) analysis by cardiac puncture in all groups at 100–120 d of age and in pregnant animals between 19 and 22 d gestation (when vascular function was assessed). Samples were collected into (final concentration) 38 g trisodium citrate/l (blood : anticoagulant 9 : 1), 15 μmol indomethacin/l (in phosphate buffer adjusted to pH 7·4) and 20 μmol butylated hydroxytoluene/l (in ethanol). Total (sum of free and esterified) F_2-isoprostanes were assessed in the plasma of all groups as previously described (Nourooz-Zadeh *et al.* 1996; Palmer *et al.* 1998). Briefly, this procedure involved hydrolysation of the plasma sample (1 ml) and derivatization of the dry residue. The derivatized sample was reconstituted in iso-octane (25 μl) and analysed subsequently by GC–MS. This analysis was carried out using a Hewlett Packard 5890 gas chromatograph (Hewlett Packard, Bracknell, Berks., UK) linked to a VG70SEQ mass spectrometer (Fisons Instruments, Manchester, UK), with NH_3 as a reagent gas.

Determination of body composition

Body composition was determined by dual-energy X-ray absorptiometry (DEXA) using a Hologic QDR-1000/W absorptiometer (line spacing 1·511 mm, point resolution 0·76 mm). The total lean and fat tissue mass and the bone mineral content were recorded for each rat; the total mass was calculated as the sum of these three values. The intra-assay CV were 1·7, 2·1, and 0·03 for lean tissue mass, fat mass and total mass respectively (n 5). Total mass, as measured by DEXA, differed by between 0 and 2·2 % from the body weight determined by weighing the animal.

Chemicals

NA was obtained from Winthrop, Guilford, Surrey, UK. ACh, indomethacin, sodium nitroprusside and L-NAME were obtained from Sigma, Poole, Dorset, UK and ODQ from Alexis Corporation, Nottingham, UK. $PGF_{2\alpha}$-d_4 and 8-epi-$PGF_{2\alpha}$ were purchased from Cayman Chemicals, Ann Arbor, MI, USA.

Data analysis

Data are given as means with their standard errors. For vascular protocols tension was calculated as mN/mm artery length. To account for variation in artery diameter, concentration responses to NA were expressed as a percentage of the contractile response to a depolarizing K^+ buffer (124 mmol K/l substituted physiological saline (9 g NACl/l)). Relaxation to ACh was expressed as a percentage of the initial precontration to NA. Constrictor responses to NA and relaxation responses to ACh were assessed as concentration which produces 50 % maximum response (EC_{50}; $pEC_{50} = -\log EC_{50}$) and maximum relaxation (% NA-induced tone) using the curve-fitting program Graphpad (Graphpad Software, San Diego, CA, USA). Two arteries were used from each animal and means were calculated. When it was not possible to fit accurate sigmoidal curves, comparisons were made between maximal

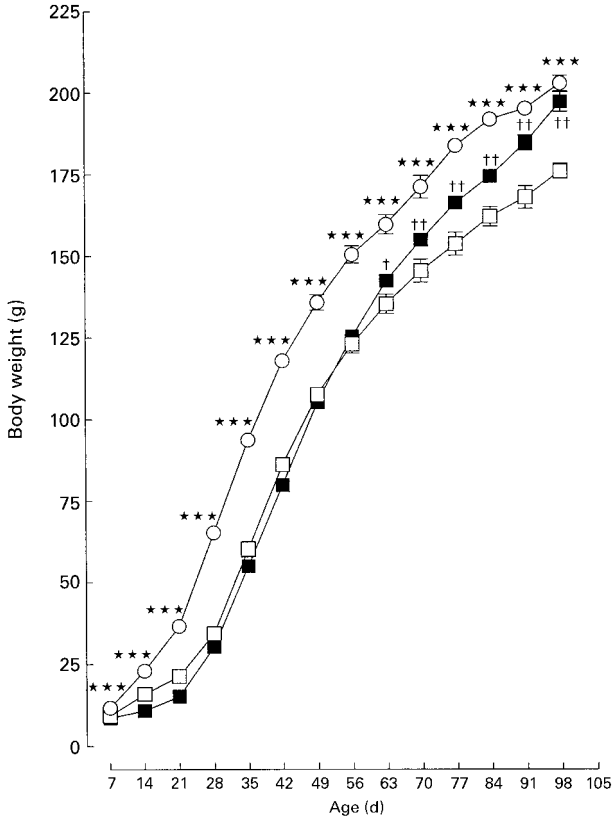

Fig. 1. Growth curves for virgin female offspring of control rats (OC; ○) and of severely diabetic rats fed standard chow (OD; □) and for virgin offspring of severely diabetic rats fed a high-saturated-fat diet (OD-HF; ■) from 7 until 98 d of age. For details of diets and procedures see pp. 286–288. Values are means with their standard errors represented by vertical bars. Mean values for OC rats were significantly different from those for OD rats: ***$P < 0{\cdot}001$. Mean values for OD-HF rats were significantly different from those for OD rats: †$P < 0{\cdot}05$, ††$P < 0{\cdot}01$.

Table 1. Food intake in adult (90 d of age) female offspring of control rats (OC) and offspring of severely diabetic rats (OD) on a normal chow and of offspring of severely diabetic rats on a high-saturated-fat diet (OD-HF)‡

(Values are means with their standard errors for ten animals in each group)

	OC		OD		OD-HF	
Food intake	Mean	SE	Mean	SE	Mean	SE
g/d	15·2	0·4	13·3**	0·4	12·7***	0·4
g/kg per d	78	2	81	3	69*††	2
kJ/d	0·228	0·006	0·201*	0·006	0·245†††	0·008
kJ/kg per d	1·071	0·029	1·215	0·038	1·331**†	0·043

Mean values were significantly different from those of OC rats. *$P<0·05$, **$P<0·01$, ***$P<0·001$.
Mean values were significantly different from those of OD rats: †$P<0·05$, ††$P<0·01$, †††$P<0·001$.
‡For details of diets and procedures, see pp. 286–287.

Table 2. Fasting plasma glucose, insulin, triacylglycerols, cholesterol and non-esterified fatty acids (NEFA) in adult offspring of control rats (OC) and offspring of severely diabetic rats (OD) on a standard chow and of offspring of severely diabetic rats on a high-saturated-fat diet (OD-HF) at 98 d of age‡

(Values are means with their standard errors)

n	OC 10		OD 6		OD-HF 10	
	Mean	SE	Mean	SE	Mean	SE
Glucose (mmol/l)	4·96	0·10	5·63*	0·34	5·88*	0·33
Insulin (pmol/l)	41	3	80*	27	96*	22
Triacylglycerols (mmol/l)	0·61	0·05	0·74	0·06	1·14	0·56
Cholesterol (mmol/l)	1·94	0·07	2·04	0·11	1·83†	0·04
NEFA (mmol/l)	0·53	0·07	0·83	0·11	0·61	0·07

Mean values were significantly different from those for OC rats: *$P<0·05$.
Mean value was significantly different from that for OD rats: †$P<0·05$.
‡For details of diets and procedures, see pp. 286–287.

responses. Total 8-epi-$PGF_{2\alpha}$ was calculated by equating the ratio of the peak areas of 8-epi-$PGF_{2\alpha}$ (R and S enantiomers): to the internal standard $PGF_{2\alpha}$-d_4, calculated from MS traces for each individual sample. From this ratio, 8-epi-$PGF_{2\alpha}$ could be quantified using calibration curves previously plotted for purchased standards extracted in PSS, as described earlier (but to known concentrations). Comparisons between groups were made using ANOVA with the Bonferroni correction for multiple comparisons (InStat; Graphpad Software). Significance was assumed if $P<0·05$.

Results

Effect of a diet high in saturated fats in virgin offspring of severely diabetic rats

Growth curves. Growth-curve profiles in OD rats and OD-HF rats were similar from weaning (21 d old) until 56 d of age (Fig. 1). From 56 d of age onward saturated fat intake led to a significantly greater increase in body weight in OD-HF rats compared with OD rats (g; day 63: OD-HF 142 (SE 2) *n* 30 *v.* OD 135 (SE 3) *n* 10, $P<0·05$; day 98: OD-HF 197 (SE 3) *n* 20 *v.* OD 176 (SE 2) *n* 10, $P<0·01$).

Food intake. Daily food intake (g/d) was lower in both OD and OD-HF rats at 90 d of age compared with OC rats (Table 1; $P<0·05$ and $P<0·001$ respectively). When expressed relative to body weight food intake was similar in OC and OD rats, but remained lower in OD-HF rats ($P<0·01$). Absolute daily energy intake was lower in OD rats than in OC rats ($P<0·05$) or in OD-HF rats ($P<0·001$). However, when daily energy intake was expressed relative to body weight OC and OD rats did not differ, but relative daily energy intake was significantly higher in OD-HF rats compared with OC ($P<0·01$) and OD rats ($P<0·05$).

Plasma glucose, insulin, triacylglycerols, free fatty acids and cholesterol. OD and OD-HF rats displayed raised fasting glucose and insulin concentrations compared with OC rats (Table 2; $P<0·001$). These concentrations were not significantly different between OD and OD-HF rats.

Table 3. Body composition measured by dual-energy x-ray absorptiometry in the offspring of control rats (OC) and offspring of severely diabetic rats (OD) on standard chow and the offspring of diabetic rats on a high-saturated-fat diet (OD-HF) at 100–120 d of age‡

(Values are means with their standard errors)

n . . .	OC 9		OD 10		OD-HF 10	
	Mean	SE	Mean	SE	Mean	SE
BMC (g)	7·24	0·15	6·47**	0·19	7·06†	0·10
BMD (g/cm^2)§	0·175	0·012	0·179	0·01	0·161*††	0·007
Lean tissue mass (g)	186·14	4·31	169·61**	3·38	169·12**	2·58
Fat tissue mass (g)	15·68	1·23	9·49	0·35	25·96**†††	3·28
Fat tissue (% total mass)	7·59	0·69	5·12	0·11	12·65**†††	1·39
Total mass‖ (g)	209	3·68	185·53***	3·82	202·14†	4·08

BMC, bone mineral content; BMD, bone mineral density.
Mean values were significantly different from those of OC rats: *$P<0·05$, **$P<0·01$, ***$P<0·001$.
Mean values were significantly different from those of OD rats: †$P<0·05$, ††$P<0·01$, †††$P<0·001$.
‡For details of diets and procedures, see pp. 286–287.
§Calculated as BMC per area (surface of the animal, determined by dual-energy X-ray absorptiometry).
‖Represents the sum of lean tissue mass, fat mass and BMC.

Table 4. Responses to constrictor and dilator agonists in rat small mesenteric arteries from virgin offspring of control rats (OC) and offspring of severely diabetic rats (OD) on a standard chow and of offspring of severely diabetic rats on a high-saturated-fat diet (OD-HF) at 100–120 d of age‡

(Mean values with their standard errors)

n	OC 10		OD 9		OD-HF 9	
	Mean	SE	Mean	SE	Mean	SE
NA						
pEC_{50} (μmol/l)	5·78	0·06	5·94*	0·06	5·98*	0·08
Maximum constriction (% K^+-induced tension)	107·32	3·20	111·71	1·93	109·95	2·55
ACh						
pEC_{50} (μmol/l)	7·22	0·09	6·47***	0·07	6·65***	0·09
Maximum relaxation§	89·49	3·15	91·19	2·19	80·87	3·96
ACh with INDO						
pEC_{50} (μmol/l)	6·89	0·08	6·55*	0·14	6·39	0·10
Maximum relaxation§	89·52	3·63	91·56	2·58	78·21*†	5·16
ACh with INDO, L-NAME and ODQ						
Maximum relaxation	70·12	6·39	58·47	3·89	16·24††	9·6
ACh with INDO, L-NAME and ODQ in 25 mM-KCl						
Maximum relaxation§	3·64	2·21	2·60	1·06	1·18	2·99
SNP						
pEC_{50} (μmol/l)	7·01	0·17	6·72	0·12	6·79	0·14
Maximum relaxation§	68·68	3·03	75·02	4·44	70·16	3·57

NA, noradrenaline; ACh, acetylcholine; INDO, indomethacin; L-NAME, Nω-nitro L-arginine methyl ester; ODQ, oxadiazole quinoxaline; SNP, sodium nitroprusside; pEC_{50}, –log EC_{50}, where EC_{50} is the concentration which produces 50 % maximum response.
Mean values were significantly different from those for OC rats: * $P < 0·05$, *** $P < 0·001$.
Mean values were significantly different from those for OD rats: † $P < 0·05$, †† $P < 0·01$.
‡ For details of diets and procedures, see pp. 286–287.
§ % NA-induced tone, i.e. % initial precontraction to NA.

Plasma cholesterol concentrations were lower ($P < 0·05$), and triacylglycerols had a tendency to be higher in OD-HF rats when compared with OD rats. Plasma fasting free fatty acids were comparable in all three groups.

Body composition. Total mass was higher in OD-HF rats than in OD rats ($P < 0·01$), which was apparently due to an increase in fat mass in OD-HF rats, as lean tissue mass was comparable in both groups (Table 3). The fat mass, whether expressed in absolute values or as a percentage of total mass was significantly higher in OD-HF rats than in OD rats ($P < 0·0001$). Bone mineral content was lower in OD rats compared with OC and OD-HF rats. However, when expressed per unit area, bone mineral density was comparable in OC and OD rats, but lower in OD-HF rats than in OC ($P < 0·05$) and OD rats ($P < 0·01$).

Vascular function. Mean internal diameter of the arteries from OC, OD and OD-HF rats were comparable (296·63 (SE 11·34) μm, *n* 17; 300·33 (SE 8·10) μm, *n* 18; 299·48 (SE 11·46) μm, *n* 18 respectively).

In respect of the constrictor response to NA, as described previously (Holemans *et al.* 1999), the small mesenteric arteries of OD rats demonstrated enhanced sensitivity to NA (OD *v.* OC $P < 0·05$). When fed the high-saturated-fat diet there was no further abnormality in NA responses, sensitivity being similar in OD and OD-HF rats (Table 4 and Fig. 2(a)).

In respect of endothelium-dependent relaxation, before evaluation of ACh responses, preconstriction to NA was no different between arteries from OC, OD and OD-HF rats, or in the presence of any inhibitor. The sensitivity to the endothelium-dependent vasodilator ACh was impaired in OD rats compared with OC rats. In the OD-HF rats sensitivity to ACh was no different from that of OD rats, but maximum relaxation was marginally but not significantly reduced (Table 4 and Fig. 3(a)).

In the presence of indomethacin the blunted sensitivity to ACh persisted in OD *v.* OC. In addition maximum relaxation in OD-HF rats was significantly reduced ($P < 0·05$) in the presence of this cyclooxygenase inhibitor when compared with OC and OD (Table 4 and Fig. 3(b)). In the presence of both NO (L-NAME and ODQ) and cyclooxygenase blockade, the difference in ACh-induced relaxation between OC and OD was no longer evident, but the difference in maximal relaxation to ACh between OD and OD-HF was exaggerated ($P < 0·01$; Table 4 and Fig. 3(c)). Relaxation to ACh was completely inhibited in all groups in the presence of indomethacin, L-NAME, ODQ and partially-depolarizing PSS (25 mmol KCl/l; Table 4 and Fig. 3(d)). Relaxation to the endothelium-independent vasodilator sodium nitroprusside was no different between OC, OD and OD-HF rats (Table 4).

8-Epi-prostaglandin $F_{2\alpha}$. Total (free and esterified) plasma levels of the F_2-isoprostane 8-epi-$PGF_{2\alpha}$ were similar in all groups (OC 322·93 (SE 32·01) pg/ml, *n* 18; OD 290·39 (SE 66·17) pg/ml, *n* 10; OD-HF 345·70 (SE 39·04) pg/ml, *n* 20; not significant; Fig. 4).

Effect of a diet high in saturated fats in pregnant offspring of severely diabetic rats

Vascular function. Mean internal diameter of the arteries from POC, POD and POD-HF rats were similar (319·86

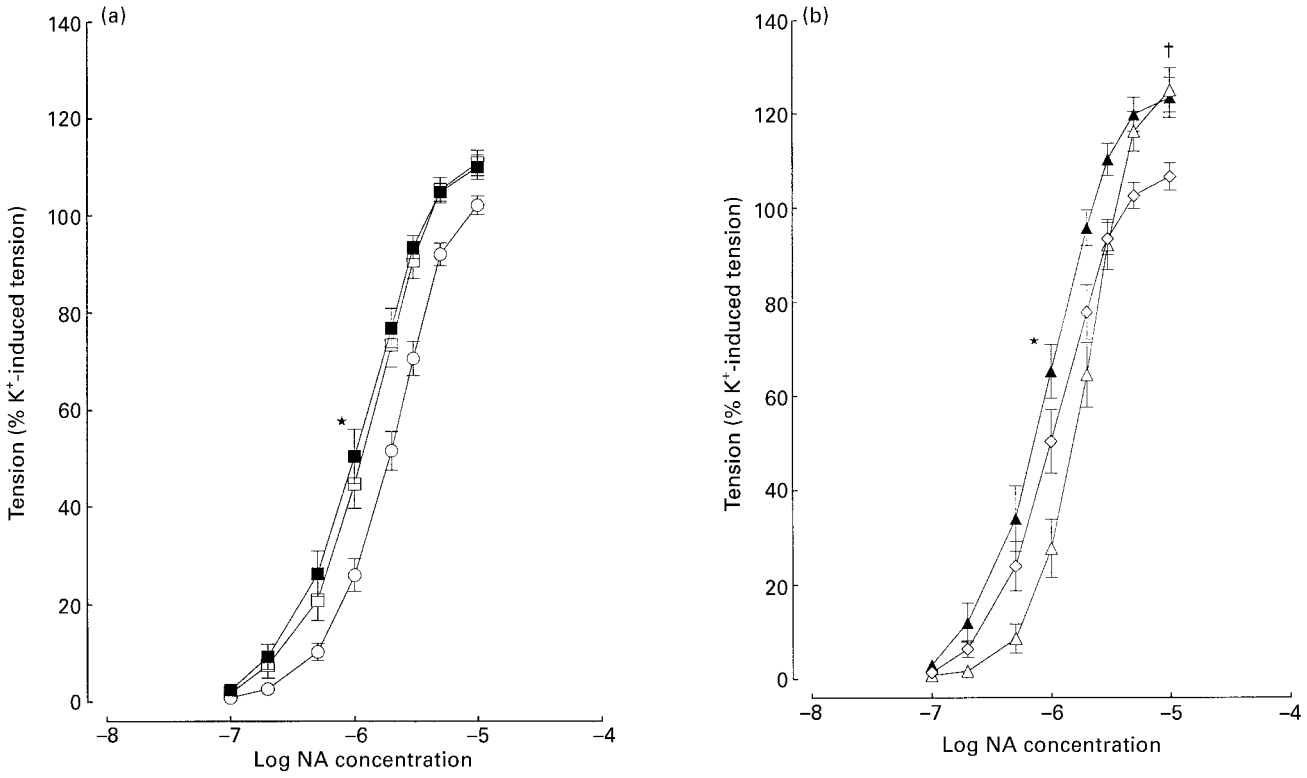

Fig. 2. Concentration–response curves to noradrenaline (NA) in mesenteric small arteries from (a) offspring of control rats (OC; ○; *n* 10) and offspring of severely diabetic rats fed standard chow (OD; □; *n* 9), and virgin offspring of severely diabetic rats fed a high-saturated-fat diet (OD-HF; ■; *n* 9); (b) Pregnant offspring of OC rats (POC; ◇; *n* 9), pregnant offspring of OD rats (POD; △; *n* 10) and pregnant offspring of OD-HF rats (POD-HF; ▲; *n* 8). For details of diets and procedures, see pp. 286–287. Values are means with their standard errors represented by vertical bars. (a) For pEC_{50} (–log EC_{50}, where EC_{50} is the concentration which produces 50 % maximum response), mean value for OD rats was significantly different from that for OC rats: * $P < 0{\cdot}05$. (b) For pEC_{50}, mean value for POD-HF rats was significantly different from that for POD rats: * $P < 0{\cdot}05$. For maximum contraction, mean value for POD rats was significantly different from that for POC rats: † $P < 0{\cdot}05$.

(SE 12·08) μm, *n* 17; 315·82 (SE 9·93) μm, *n* 17; 318·99 (SE 8·62) μm, *n* 16 respectively).

In respect of constrictor response to NA, POD rats demonstrated a higher maximal response to NA than POC rats. The POD-HF rats developed a further defect of enhanced sensitivity to NA (Table 5 and Fig. 2(b)).

In respect of endothelium-dependent relaxation, before evaluation of ACh responses preconstriction to NA was no different between arteries from POC, POD and POD-HF rats, or in the presence of any inhibitor. Although sensitivity to ACh was similar between POC and POD rats, maximum relaxation was slightly but significantly impaired in POD ($P < 0{\cdot}05$). Similar relaxation to ACh was observed in POD and POD-HF rats (Table 5 and Fig. 5(a)).

The addition of indomethacin had a similar effect on ACh-induced relaxation in POC and POD rats, but revealed a marked blunting of sensitivity to ACh in POD-HF rats when compared with POD rats (Table 5 and Fig. 5(b)). In the presence of both NO and cyclooxygenase inhibition the difference in ACh-induced relaxation between POD and POD-HF rats was no longer evident (Table 5 and Fig. 5(c)), and became similar to that of POC rats in the presence of these inhibitors. Relaxation to ACh in all groups was completely inhibited in the presence of INDO, L-NAME, ODQ and partially-depolarizing PSS (25 mmol KCl/l; Table 5 and Fig. 5(d)). Relaxation to the endothelium-independent vasodilator sodium nitroprusside was not different between the three groups (Table 5).

8-Epi-prostaglandin $F_{2\alpha}$. The plasma concentrations of total (free and esterified) 8-epi-$PGF_{2\alpha}$ were significantly higher in POD than POC rats (POC 297·16 (SE 14·64) pg/ml, *n* 10; POD 468·86 (SE 40·41) pg/ml, *n* 10; $P < 0{\cdot}001$) and further increased in POD-HF rats (688·77 (SE 77·23) pg/ml, *n* 10; POD-HF *v.* POD $P < 0{\cdot}05$; Fig. 4).

Discussion

The present study has suggested that during development of the rat dietary factors can exacerbate susceptibility to cardiovascular disease induced *in utero*. The diet high in saturated fat led to further slight deterioration of vascular function in rats in which insulin resistance was induced *in utero* by maternal diabetes. Moreover, vascular function worsened when these fat-fed offspring became pregnant. Importantly, the pregnancy-related vascular disorder was associated with an increase in a stable plasma marker of oxidative stress, the isoprostane 8-epi-$PGF_{2\alpha}$. To our knowledge, the present study is the first to have investigated the effect of feeding saturated fat to animals in which insulin resistance has been acquired *in utero*.

As shown previously, the offspring of diabetic rats developed mild hyperinsulinaemia (Holemans *et al.* 1991*b*,

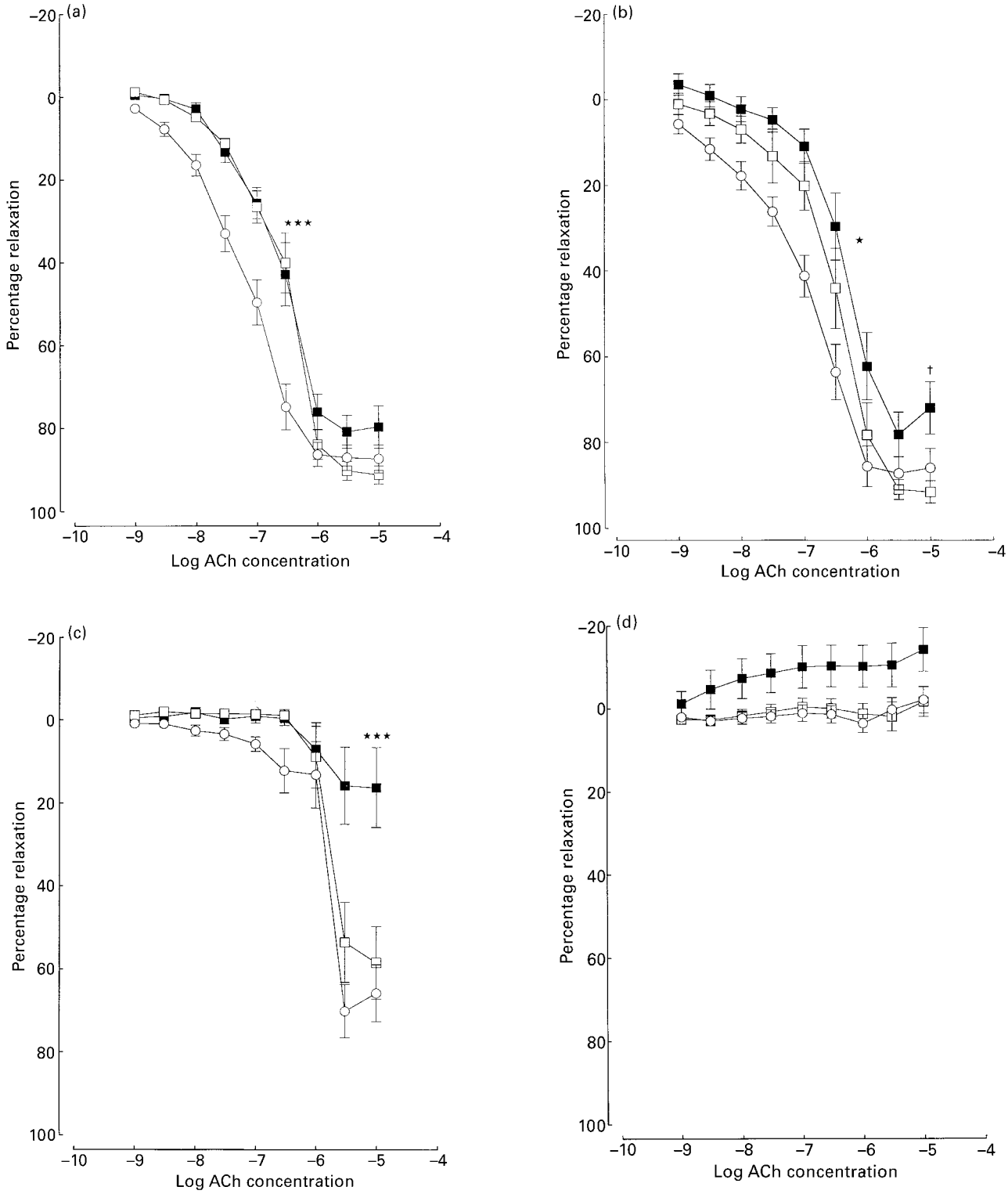

Fig. 3. Concentration–response curves to acetylcholine (ACh) in mesenteric small arteries from virgin offspring of control rats fed standard chow (OC; ○; *n* 10) and offspring of severely diabetic rats fed standard chow (OD; □; *n* 9) or a high-saturated-fat diet (OD-HF; ■; *n* 9). For details of diets and procedures, see pp. 286–287. Values are means with their standard errors represented by vertical bars. (a) Without inhibitors, for pEC_{50} (–log EC_{50}, where EC_{50} is the concentration which produces 50 % maximum response) mean value for OD rats was significantly different from that for OC rats: *** $P < 0{\cdot}001$. (b) In the presence of indomethacin, for pEC_{50} mean value for OD rats was significantly different from that for OC rats: * $P < 0{\cdot}05$; for maximum relaxation (% noradrenaline-induced tone) mean value for OD-HF rats was significantly different from those for OC rats and OD rats: † $P < 0{\cdot}05$. (c) In the presence of indomethacin, Nω-nitro L-arginine methyl ester and oxadiazole quinoxaline, for maximum relaxation mean value for OD-HF rats was significantly different from that for OD rats: *** $P < 0{\cdot}001$. (d) In the presence of indomethacin, Nω-nitro L-arginine methyl ester and oxadiazole quinoxaline in 25 mmol KCl/l.

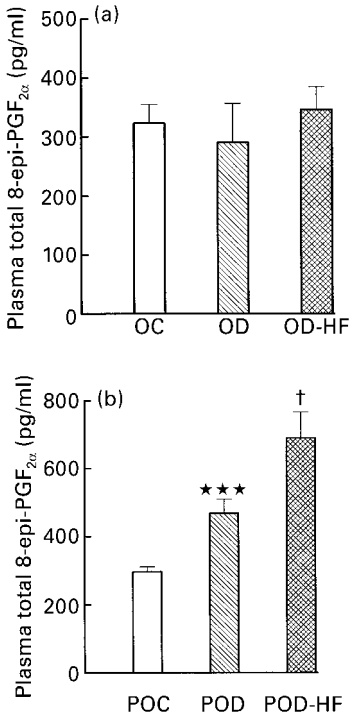

Fig. 4. Plasma concentrations (pg/ml) of 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$) for (a) virgin offspring of control rats (OC; □; *n* 18), of severely diabetic rats (OD; ▨; *n* 10) fed a standard chow and of severely diabetic rats fed a high-saturated-fat diet (OD-HF; ▩; *n* 20). (b) Pregnant offspring of OC rats (POC; □; *n* 10), of OD rats (POD; ▨; *n* 10) and of OD-HF rats (POD-HF; ▩; *n* 10). Values are means with their standard errors represented by vertical bars. Mean value for POD rats was significantly different from that for POC rats: *** $P < 0{\cdot}001$. Mean value for POD-HF rats was significantly different from that for POD rats: † $P < 0{\cdot}05$.

1993, 1997), due to insulin resistance (Holemans *et al.* 1991*a*, 1993; Ryan *et al.* 1995). We have shown recently that significant vascular dysfunction in these offspring is evident in mesenteric small arteries (Holemans *et al.* 1999). As confirmed in the present study, these animals have enhanced sensitivity to NA and a highly significant impairment of endothelial function. The normal sensitivity to sodium nitroprusside also suggests that the defect does not arise from reduced sensitivity of the smooth muscle to NO, but from reduced NO synthesis. This abnormality is similar to that observed in mesenteric arteries of the STZ-diabetic rat, although less pronounced (Taylor *et al.* 1995). The findings also suggest an association between insulin resistance and vascular endothelial function, as previously described (O'Brian *et al.* 1998). Of the several pathways proposed to underlie this association (Chowienczyck & Watts, 1997), we have recently suggested that an elevation in triacylglycerols may play an important role (Holemans *et al.* 1999).

When the virgin offspring were fed the high-saturated-fat diet there was no further increase in markers of insulin resistance (insulin and glucose), no further increase in NA-induced vasoconstriction and no significant deterioration in ACh response. However, investigation of the components of ACh-induced relaxation indicated a modest reduction in synthesis of the endothelium-derived hyperpolarizing factor, as ACh-induced relaxation was impaired in the fat-fed group in the presence of NO synthase and cyclooxygenase inhibition. The role of endothelium-derived hyperpolarizing factor was confirmed by the demonstration that K^+-induced depolarization completely negated the difference between the groups. There is some evidence that inhibition of NO induces stimulation of the synthesis of endothelium-derived hyperpolarizing factor (Gerber *et al.* 1998), and this process could provide an explanation for the lack of difference in ACh-induced relaxation before selective unmasking of the contributory components by the pharmacological inhibitors (Bauersachs *et al.* 1996).

These observations of modest defects induced by the saturated-fat diet in the virgin 'insulin-resistant' offspring of diabetic rats are in contrast to those of our recent study (Gerber *et al.* 1999*b*) in which feeding the same high-fat diet to control offspring led to highly significant vascular disorders. These disorders included an increase in NA sensitivity and reduced sensitivity to ACh, attributable to reduced NO synthesis. The present study and our previous investigation (Gerber *et al.* 1999*b*) have thus shown that a high-saturated-fat diet and maternal diabetes both lead to reduced NO synthesis in virgin offspring, but that the effects of these interventions are unlikely to be additive. As a high-saturated-fat diet in rats (Storlien *et al.* 1986, 1991; Pascoe & Storlien, 1990) leads to insulin resistance, and as insulin resistance is also a characteristic of the offspring of diabetic rats (Holemans *et al.* 1991*a*, 1993; Ryan *et al.* 1995), it could be argued that the vascular defect induced by each intervention is similar and maximal.

In normal rats, and in contrast to human subjects, a diet high in saturated fat is usually associated with a significant fall in plasma cholesterol (Gerber *et al.* 1999*b*; Salter *et al.* 1991). In this study also we found that the saturated-fat diet led to lowering of plasma cholesterol in the offspring of the diabetic rats. The minor alterations in vascular function observed here were therefore unrelated to an elevation of plasma cholesterol.

There was no evidence for an increase in oxidative stress, as assessed by measurement of the isoprostane, 8-epi-$PGF_{2\alpha}$ in OD rats, or any indication that lipid peroxidation was induced by the saturated-fat diet, despite the suggestion of insulin resistance. This finding contrasts with the increased circulating levels of 8-epi-$PGF_{2\alpha}$ observed in insulin-resistant subjects with NIDDM (Chowienczyck *et al.* 1998), but as these rats were young adults it would be of interest in future studies to determine whether oxidative stress develops in older animals. The absence of an increase in 8-epi-$PGF_{2\alpha}$ when the animals were fed the saturated-fat diet in the present and our previous investigation in normal animals (Gerber *et al.* 1999*b*) apparently contrasts with reports of oxidative stress induced by raised dietary saturated fat in mice (Ibrahim *et al.* 1997) and in human subjects (Erhardt *et al.* 1997). However, despite the recognition that the plasma concentration of 8-epi-$PGF_{2\alpha}$ is a reliable and stable indicator of oxidative stress (Morrow & Roberts, 1996), measurements of other indicators, e.g. plasma and intracellular antioxidants, would be required to justify the conclusion that the diet has no effect at all on free radical production.

Table 5. Responses to constrictor and dilator agonists in rat small mesenteric arteries from pregnant offspring of control rats (POC) and pregnant offspring of severely diabetic rats on a standard chow (POD) and of pregnant offspring of severely diabetic rats on a high-saturated-fat-diet (POD-HF) at 100–120 d of age‡

(Mean values with their standard errors)

n	POC 9		POD 10		POD-HF 8	
	Mean	SE	Mean	SE	Mean	SE
NA						
pEC_{50} (μmol/l)	6·01	0·08	5·76	0·07	6·08†	0·11
Maximum constriction (% K^+-induced tension)	106·51	2·91	122·53*	6·07	123·32*	5·27
ACh						
pEC_{50} (μmol/l)	7·18	0·11	6·99	0·09	6·84	0·11
Maximum relaxation§	98·94	0·62	94·94*	1·41	90·94*	2·55
ACh with INDO						
pEC_{50} (μmol/l)	6·62	0·25	7·07	0·29	6·37†	0·13
Maximum relaxation§	93·58	2·37	94·87	1·40	86·50	4·71
ACh with INDO, L-NAME and ODQ						
Maximum relaxation§	73·72	9·15	52·15	10·55	32·18	9·57
ACh with INDO, L-NAME and ODQ in 25 mM-KCl						
Maximum relaxation§	1·62	3·69	0·39	2·02	0·80	1·30
SNP						
pEC_{50} (μmol/l)	6·91	0·17	7·43	0·24	6·80	0·15
Maximum relaxation§	66·19	7·19	64·17	2·30	71·58	5·02

NA, noradrenaline; ACh, acetylcholine; INDO, indomethacin; L-NAME, Nω-nitro L-arginine methyl ester; ODQ, oxadiazole quinoxaline; SNP, sodium nitroprusside; pEC_{50}, –log EC_{50}, where EC_{50} is the concentration which produces 50 % maximum response.
Mean values were significantly different from those for POC rats: * $P < 0·05$.
Mean values were significantly different from those for POD rats: † $P < 0·05$.
‡ For details of diets and procedures, see pp. 286–287.
§ % NA-induced tone, i.e. % initial precontraction to NA.

In the offspring of diabetic rats perinatal growth is stunted, probably as a result of decreased utero-placental blood flow during pregnancy (Rosso & Kava, 1980) and reduced availability of milk during lactation (Ramussen & Warman, 1983). As reported previously (Holemans *et al.* 1997), there was no postnatal catch-up growth in offspring of diabetic rats, but if the offspring were fed the saturated-fat diet, body weight increased significantly. This finding was entirely the result of an increase in fat mass, as determined by the DEXA method, and concurs with findings of our previous study in normal rats (Gerber *et al.* 1999*b*) and reports by other researchers (Storlien *et al.* 1986; Pascoe & Storlien, 1990) demonstrating obesity in rats fed a high-fat diet. The increase in body weight in OD-HF rats was apparently not due to an increased food intake, but to an increased energy intake relative to body weight. The DEXA method also showed a reduction in bone mineral density in the offspring of the STZ-diabetic rats fed the high-fat diet. This finding may have implications for bone health, and is an interesting observation which warrants further investigation.

Whereas the effects of a saturated-fat diet in the virgin offspring of the diabetic animals (OD-HF rats) were modest, vascular function in the pregnant offspring (POD-HF rats) was considerably affected by the diet. Sensitivity to NA was enhanced and dilator responses to ACh were impaired in comparison with the pregnant animals on the normal diet (POD rats). These rats also displayed significantly greater maximal constriction to NA, but similar relaxation to ACh to that of pregnant controls fed saturated fat (Gerber *et al.* 1999*b*). These defects may counteract the normal fall in peripheral vascular resistance in pregnancy, and could potentially influence pregnancy outcome (Poston, 1996). In contrast to the virgin animals (OD and OD-HF rats), the defect in ACh-induced relaxation in saturated-fat-fed pregnant animals (POD-HF rats) was NO dependent and associated with an increase in 8-epi-$PGF_{2\alpha}$. This finding is consistent with our previous study (Gerber *et al.* 1999*b*) in which oxidative stress, as assessed by this method, was also increased in pregnant saturated-fat-fed controls when compared with virgin control rats fed the saturated-fat diet. The rise in 8-epi-$PGF_{2\alpha}$ was, however, similar in both pregnant control rats fed the saturated-fat diet (Gerber *et al.* 1999*b*) and POD-HF rats (present study). It would appear therefore that the offspring of the diabetic animal is no more susceptible to oxidative stress due to saturated fat consumption than are the offspring of the normal animal. Free radicals may cause DNA and membrane damage (Halliwell, 1996), and through synthesis of lipid peroxides, reduce endothelial cell NO synthesis, thus providing a potential mechanism for both reduced ACh-induced relaxation, and also increased NA constriction (Cooke & Dzau, 1997). As pregnancy itself is recognized to provoke oxidative stress (Wisdom *et al.* 1991), the added stimulus to free radical synthesis by saturated fat may be sufficient to 'tip the balance' in favour of lipid peroxidation.

Interestingly, the plasma concentration of 8-epi-$PGF_{2\alpha}$ in POD rats was also raised above that of the POC rats (Gerber *et al.* 1999*a*). Previously we have shown that these pregnant animals develop mild hyperglycaemia (Holemans *et al.* 1991*b*, 1993, 1997), which could contribute directly to enhanced free radical synthesis and lipid peroxidation (Hunt

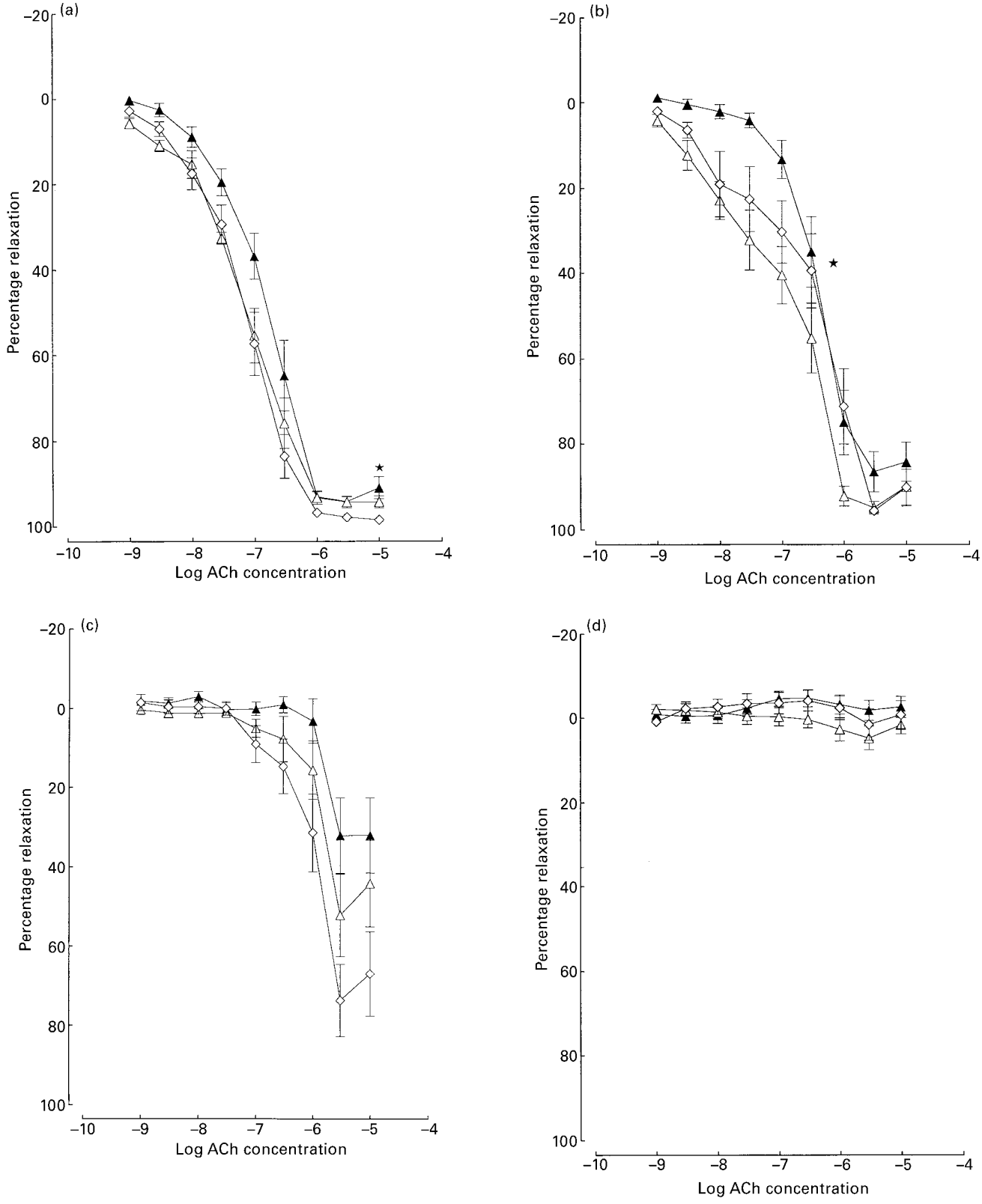

Fig. 5. Concentration–response curves to acetylcholine in mesenteric small arteries from pregnant offspring of control rats (POC; ◇; *n* 9) and from pregnant offspring of severely diabetic rats fed standard chow (POD; △; *n* 10) or a high-saturated-fat diet (OD-HF; ▲; *n* 8). For details of diets and procedures, see pp. 286–287. Values are means with their standard errors represented by vertical bars. (a) Without inhibitors, for maximum relaxation (% noradrenaline-induced tone) mean value for POD rats was significantly different from that for POC rats: $^{*}P < 0{\cdot}05$. (b) In the presence of indomethacin, for pEC_{50} (–log EC_{50}, where EC_{50} is the concentration which produces 50 % maximum response) mean value for POD-HF rats was significantly different from that for POD rats: $^{*}P < 0{\cdot}05$. (c) In the presence of indomethacin, Nω-nitro L-arginine methyl ester and oxadiazole quinoxaline. (d) In the presence of indomethacin, Nω-nitro L-arginine methyl ester and oxadiazole quinoxaline in 25 mmol KCl/l.

et al. 1988). Again, as in normal pregnant rats (Gerber *et al.* 1999*b*), pregnancy seems to confer additional 'stress', and so unmasks an already compromised balance between free radical synthesis and antioxidant status. Oxidative stress in the diabetic pregnant rat has been implicated in embryopathy (Siman & Eriksson, 1997) and, we suggest, could potentially play a role in fetal 'programming' through permanent alteration of DNA and tissue damage in the developing fetus. We have reported oxidative stress in fetuses of STZ-diabetic pregnant dams (Gerber *et al.* 1999*a*), and it could be hypothesized that this original insult underlies the vascular and metabolic sequelae we observed in these offspring.

In conclusion, dietary saturated fat may further compound defects of the cardiovascular system acquired by female rats in a diabetic environment *in utero*. Whilst relatively minor in the virgin animals, offspring which become pregnant are particularly compromised. However, the defects induced by feeding saturated fat to the pregnant offspring of diabetic rats were in general of similar severity to those we observed in normal pregnant offspring on the same diet (Gerber *et al.* 1999*b*). Thus, it can be concluded that fat feeding *per se* is injurious to pregnant animals and their offspring independent of a maternal diabetes.

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