Augmentation of diabetes-associated renal hyperfiltration and nitric oxide production by pregnancy in rats

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Abstract

We tested the hypothesis that pregnancy might increase diabetes-associated nitric oxide (NO) production and renal hyperfiltration. Two weeks following i.v. streptozotocin (40 mg/kg), mean arterial pressure (MAP) was not modified by diabetes; glomerular filtration rate (GFR), renal plasma flow (RPF) and filtration fraction (FF) were higher in pregnant than in virgin controls and increased by diabetes to a greater extent in pregnant than in virgin rats. Urinary volume (UV), creatinine, albumin and sodium ($U_{Na}V$) were significantly increased by diabetes. Diabetes led to an increase in renal, cardiac, aortic and uterine but not in placental NO synthase activities. Infusion of N^G -nitro-L-arginine (L-NA) caused a dose-dependent reduction in GFR, RPF, plasma NO_2^-/NO_3^- , UV and

$U_{Na}V$; in general, diabetes increased these effects to a greater extent in pregnant than in virgin rats. L-NA increased MAP in all groups of rats but did not alter FF. Diabetes did not alter responses of thoracic aorta rings to vasoconstrictor effects of phenylephrine and the vaso-relaxant effects of sodium nitroprusside but increased endothelium-dependent relaxant effects of acetylcholine. In general the effects of diabetes of 7 days duration were similar to those described above for diabetes of 14 days duration. These data suggest that diabetes-associated renal hyperfiltration and NO production are augmented by pregnancy.

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Introduction

Nephropathy is one of the serious long-term complications of diabetes and may develop in 30–40% of diabetes mellitus patients (Rudberg *et al.* 1992, Chowdhury *et al.* 1996). Although several factors are involved in the genesis of diabetic nephropathy, glomerular hyperfiltration with increased intraglomerular pressure antedates the development of nephropathy and appears to contribute to the diabetes-associated renal injury (Hostetter *et al.* 1981, Chowdhury *et al.* 1996). Increased renal plasma flow (RPF) often accompanies diabetes mellitus (Chowdhury *et al.* 1996). Vascular endothelial nitric oxide (NO) plays a role in renal haemodynamics (Shultz *et al.* 1990, Raji 1993, Danielson & Conrad 1995, Baylis & Qiu 1996) and contributes to renal hyperfiltration during streptozotocin diabetes in rats (Bank & Aynedjian 1993, Tolins *et al.* 1993, Komers *et al.* 1994).

The endothelium-dependent vasorelaxant effects of acetylcholine (ACh) (Furchgott & Zawadzki 1980) and several other substances (Furchgott 1983) seem to be caused primarily by a release of NO (Palmer *et al.* 1987, Moncada *et al.* 1991). Diabetes can lead to endothelial dysfunction (Poston & Taylor 1995, Sobrevia & Mann

1997), which can alter NO homeostasis. Clinical data suggest that the role of NO is not uniform on different blood vessels and is influenced by the presence of different diabetic complications (Hicks *et al.* 1997, Veves *et al.* 1998); a variability and heterogeneity in endothelial NO functions in different blood vessels is also observed in experimental diabetes (Kiff *et al.* 1991).

NO is synthesized from the terminal guanidino-nitrogen atom of the amino acid L-arginine; its synthesis is catalysed by NO synthase (NOS) and can be blocked by L-arginine analogues such as N^G -monomethyl-L-arginine (L-NMMA), N^G -nitro-L-arginine methyl ester (L-NAME) and N^G -nitro-L-arginine (L-NA) (Moncada *et al.* 1991). Inhibition of NOS activity by different L-arginine analogues has been extensively utilized as a tool to assess the role of NO in modulating vascular tone (Poston & Taylor 1995).

Pregnancy is associated with profound renal haemodynamic changes including a marked increase in glomerular filtration rate (GFR) both in humans (Gallery 1984) and rats (Baylis 1984), which might be contributed by NO (Danielson & Conrad 1995). Diabetic complications are aggravated by pregnancy (Kitzmiller & Combs 1996) but a contribution of NO in renal complications during

diabetic pregnancy has not, to our knowledge, been reported. Here we tested the hypothesis that pregnancy might increase the role of NO in diabetes-associated renal hyperfiltration using virgin and pregnant rats as the model, 7 and 14–15 days after the induction of streptozotocin diabetes.

Materials and Methods

Chemicals

The following agents were purchased: inulin-methoxy [methoxy- ^3H] (500 mCi/g) and *p*-aminohippuric acid (PAH) [glycyl- $1\text{-}^{14}\text{C}$] (60 Ci/mmol) (NEN, Boston, MA, USA); [^3H]L-arginine (63 Ci/mmol) (Amersham, Oakville, ON, Canada); L-NA, NADPH, nitrate reductase, calmodulin, tetrahydrobiopterin, Griess reagent, soybean trypsin inhibitor, aprotinin, L-valine, NaNO_2 , NaNO_3 , dithiothreitol, ACh, phenylephrine and sodium nitroprusside (SNP) (Sigma Chemical Co., St Louis, MO, USA); all other high purity chemicals (BDH, Montreal, QC, Canada).

Animals and induction of diabetes

Experiments were done on Sprague–Dawley rats (Charles River, St Constant, QC, Canada) according to a protocol of the McGill University Animal Care Committee. Animals were housed at 22–24 °C, 55–70% humidity and a schedule of 12 h light:12 h darkness (lights on 0700–1900 h) and freely fed rat chow and tap water. Female rats weighing 200–225 g were housed with 250–275 g males overnight; the presence of sperm in the vaginal wash the following morning was designated as day 0 of pregnancy. Diabetes was induced by a single injection of streptozotocin (40 mg/kg) into the tail vein of rats on day zero of pregnancy and in age-matched virgin rats; controls were injected with the vehicle (citrate saline buffer, pH 4.5) (Conliffe *et al.* 1992). Urine was tested for the presence of glucose by Chemstrips (Boehringer Mannheim, Montreal, QC, Canada) for 2 successive days and animals which did not exhibit glucosuria were discarded. A small number of studies was done 7 days after the induction of diabetes; effects of 14–15 days duration of diabetes were studied in greater detail. Blood was collected from all animals at the time of killing and plasma glucose was determined by a CX-7 Beckman autoanalyser (Beckman, Palo Alto, CA, USA).

Albumin and creatinine assays

Urinary albumin was measured using a Nephrot rat albumin ELISA kit (Cedarlane, Hornby, ON, Canada). Briefly, standards and urine samples were incubated in microwells coated with anti-rat albumin for 20 min at

room temperature followed by incubation with rat albumin-horseradish peroxidase conjugate for another 30 min. Unbound reactants were removed by washing and the bound conjugate was reacted with tetramethylbenzidine substrate solution; the chromogenic reaction was stopped with dilute sulphuric acid and the colour intensity was determined using a microplate reader at 450 nm.

Plasma and urinary creatinine were determined on a CX-7 autoanalyser; the assay is based on mixing the samples with alkaline picrate reagent and monitoring the rate of complex formation, which is directly proportional to creatinine concentration, bichromatically at 520 and 560 nm.

Renal haemodynamic studies

Renal haemodynamic studies were done as previously described (Omer *et al.* 1995). Briefly, rats were anaesthetized by i.p. injection of 1 g/kg urethane; no anaesthetic supplementation was required during the course of the experiment. Polyethylene catheters were inserted into the jugular vein for infusions and into the carotid artery for blood sampling and for monitoring the arterial pressure by means of a Statham pressure transducer (P23) on a Grass polygraph (Quincy, MA, USA). A catheter was placed into the urinary bladder for continuous urine collection. Following a 30 min period of stabilization after the completion of cannulations, a continuous i.v. infusion of [^3H]inulin (2 $\mu\text{Ci/kg}$ per h) and [^{14}C]PAH (2 $\mu\text{Ci/kg}$ per h) was started at a rate of 1.2 ml/h. Following a 2 h period of equilibration, two 15 min urine samples were collected into pre-weighed Eppendorf tubes; control blood samples (300 μl) were collected from the arterial cannula at the midpoint of each urine sampling; blood loss was replaced by an equivalent volume of physiological saline. Following these control sample collections, infusion of L-NA was started at 0.25 $\mu\text{mol/kg}$ per min for 30 min; a urine sample was collected corresponding to the last 15 min of L-NA infusion and a blood sample was collected at the midpoint of urine collection. This procedure was repeated at an L-NA infusion rate of 0.5 $\mu\text{mol/kg}$ per min and then at 1 $\mu\text{mol/kg}$ per min. Following the final blood collection, kidneys were removed and their weights recorded. Plasma and urine [^3H]inulin and [^{14}C]PAH concentrations were determined by counting radioactivity on an LKB 1219 Rackbeta scintillation counter (Wallac, Turku, Finland); clearance of [^3H]inulin and [^{14}C]PAH was calculated to determine GFR and RPF respectively (Bank & Aynedjian 1993, Omer *et al.* 1995). Filtration fraction (FF) was calculated as a quotient of GFR and RPF. Urinary sodium was measured by an IL-443 flame photometer (Cole-Parmer, Vernon Hills, IL, USA) (Omer *et al.* 1995).

Plasma $\text{NO}_2^-/\text{NO}_3^-$

Plasma $\text{NO}_2^-/\text{NO}_3^-$ was measured using the Griess reagent (Moshage *et al.* 1995, Verdon *et al.* 1995). Briefly,

samples were incubated with nitrate reductase in the presence of NADPH to reduce all nitrate to nitrite. After the enzyme incubation, samples were treated with Griess reagent and quantified by measuring absorbance at 540 nm using a plate reader; known concentrations of NaNO_2 and NaNO_3 were utilized as standards in each assay. The NO measured in this way reflected the sum of NO_2^- and NO_3^- in the original sample.

NOS activity

In addition to animals used for haemodynamic studies, five or six rats from each group were anaesthetized with i.p. injections of 40 mg/kg sodium pentobarbitone. Blood was collected by means of cardiac puncture; kidneys, heart, aorta, placentas and uterus were removed, snap frozen in liquid nitrogen and stored at -80°C for the assay of NOS activity. Tissue NOS activities were determined as previously described (Salter *et al.* 1991, Hardy *et al.* 1996). Briefly, tissues were homogenized by means of a Polytron (Brinkmann, Rexdale, Ontario, Canada) at setting 7 in a buffer containing 320 mM sucrose, 50 mM Tris-HCl, 1 mM dithiothreitol, 100 $\mu\text{g}/\text{ml}$ polymethylsulphonyl fluoride, 10 $\mu\text{g}/\text{ml}$ leupeptin, 100 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor, 2 $\mu\text{g}/\text{ml}$ aprotinin and 60 mM L-valine, which was adjusted to pH 7.4 with HCl. The homogenate was centrifuged at 12 000 g for 20 min and the supernatant was used for the assay of NOS activity.

An aliquot of the supernatant (35–75 μg protein) was incubated in the absence or the presence of 2 mM L-NA in 400 μl assay buffer containing 50 mM Hepes, 1 mM dithiothreitol, 1.2 mM MgCl_2 , 2 mM CaCl_2 , 100 μM [^3H]L-arginine, 1 mM NADPH, 15 μM tetrahydrobiopterin, 1 μM flavine adenine dinucleotide and 1 μM calmodulin for 10 min at 37°C in a shaking water bath. The reaction was terminated by adding the following: 1 ml ice-cold 100 mM Hepes (pH 5.5) containing 10 mM EGTA and 1.5 ml 1:1 H_2O :Dowex-50 (200–400, 8% crosslinked, Na^+ form). The Na^+ form of Dowex-50 was prepared by washing four times the H^+ form of resin with 1 M NaOH and then washing with H_2O until the pH was <7.5 (Salter *et al.* 1991, Hardy *et al.* 1996). The resin incubate mix was allowed to settle for 10 min and then centrifuged at 3500 g for 10 min. The synthesis of [^3H]citrulline was estimated by counting the radioactivity of the supernatant. Radioactivity in the presence of 10 mM EGTA in the buffer yielded Ca^{2+} -independent NOS activity. Total NOS activity was calculated as the L-NA-sensitive formation of [^3H]citrulline and Ca^{2+} -dependent NOS activity was the difference between the total NOS activity and that in the presence of EGTA. The effect of different concentrations of L-NA on NOS activity was determined in three separate experiments each in triplicate using rat cerebellum, which contains high NOS activity (Moncada *et al.* 1991); it was found that the total NOS activities (pmol citrulline/mg protein per min) using

100 μM , 1 mM and 2 mM L-NA were 136 ± 4 , 139 ± 4 and 135 ± 3 respectively and did not significantly differ from each other.

Vascular functions

Thoracic aortas were isolated and placed in oxygenated (95% O_2 and 5% CO_2) Krebs buffer (pH 7.4) of the following composition (mM): NaCl 117, KCl 4.7, CaCl_2 2.5, MgSO_4 1.18, KH_2PO_4 1.2, NaHCO_3 25, dextrose 11 and EDTA 0.03 (Chemtob *et al.* 1992). The buffer was gassed with a mixture of 95% O_2 and 5% CO_2 . Four rings (approximately 4 mm long and 1.5–2 mm diameter) from each aorta were set up in 25 ml organ baths at 37°C at an applied tension of approximately 2 g, which was monitored on a Grass polygraph by Grass force-displacement transducers (FT037) (Quincy). The endothelium was preserved in two rings and removed from two rings. Preparations were allowed to equilibrate for 1 h with changes in Krebs buffer every 15 min. Following stabilization, two endothelium-intact rings and one endothelium-denuded ring were contracted with 100–300 nM phenylephrine (producing approximately 75% of the maximal contraction); concentration–relaxation response curves to ACh were determined in duplicate on the two endothelium-intact rings and to SNP on the one endothelium-denuded ring. One endothelium-denuded ring was used to construct concentration–contraction response curves to phenylephrine. EC_{50} values of these three agents were derived from the regression line of log molar concentration–percentage response curves.

Statistics

Data means were analysed by one-way ANOVA followed by Benferroni test for significance. A probability of <0.05 was assumed to denote a significant difference. Data are presented as means $\pm$ S.E.M.

Results

General effects of streptozotocin diabetes

Effects of diabetes of 14–15 days duration on different variables are presented in Table 1. Diabetes was associated with a decrease in body weight and an increase in kidney weight and plasma glucose. Basal values of urinary volume (UV), GFR, RPF, FF and plasma $\text{NO}_2^-/\text{NO}_3^-$ were higher in diabetic than in control animals and higher in pregnant diabetic than in virgin diabetic rats. The mean arterial pressure (MAP) was lower in pregnant than in virgin rats. Diabetes led to a significant increase in urinary albumin and creatinine excretion but these variables did not differ between virgin and pregnant rats (Table 1). Diabetes of a shorter duration (7 days) also decreased body

Table 1 Different variables in virgin control (VC), virgin diabetic (VD), pregnant control (PC) and pregnant diabetic (PD) rats 14–15 days following injection of streptozotocin or the vehicle. Data are means ± S.E.M. of 7–9 experiments

Variables	VC	VD	PC	PD
Body weight (g)	253 ± 10	234 ± 4 ^{a,b}	285 ± 6	263 ± 5 ^a
Kidney weight (g)	2.1 ± 0.1	3.0 ± 0.2 ^{a,b}	2.2 ± 0.1	2.9 ± 0.1 ^a
Plasma glucose (mmol/l)	7.4 ± 1.3	37 ± 3.1 ^{a,b}	7.8 ± 1.3	35.3 ± 2.9 ^a
Placental weight (mg)	—	—	450 ± 13	516 ± 16 ^a
Mean arterial pressure (mmHg)	122 ± 2	114 ± 3 ^{a,b}	104 ± 2 ^d	106 ± 2 ^{b,d}
Renal plasma flow (ml/kg per min)	10.8 ± 0.8	15.8 ± 0.9 ^{a,c}	13.1 ± 0.3 ^d	25.1 ± 1.6 ^{a,d}
Glomerular filtration rate (ml/kg per min)	2.6 ± 0.35	7.1 ± 0.11 ^{a,b}	4.3 ± 0.20 ^d	8.9 ± 0.31 ^{a,d}
Filtration fraction	0.23 ± 0.03	0.45 ± 0.02 ^{a,b}	0.30 ± 0.02	0.38 ± 0.01 ^{a,d}
Plasma NO ₂ ⁻ /NO ₃ ⁻ (μmol/l)	243 ± 10	720 ± 50 ^{a,c}	600 ± 40 ^d	1930 ± 230 ^{a,d}
Urinary volume (μl/min per kg)	21 ± 5	112 ± 39 ^{a,b}	44 ± 9 ^d	124 ± 32 ^{a,d}
Urinary Na ⁺ (μmol/kg per min)	0.76 ± 0.21	12.8 ± 1.1 ^{a,b}	0.56 ± 0.18	11.6 ± 1.8 ^{a,d}
Urinary albumin (ng/min per kg)	130 ± 20	1220 ± 160 ^{a,b}	220 ± 80	1225 ± 150 ^{a,d}
Urinary creatinine (nmol/min per kg)	106 ± 23	214 ± 46 ^a	170 ± 32	236 ± 37 ^{a,d}

^aDifferent ($P<0.05$) from the corresponding controls; ^bdifferent ($P<0.05$) from the corresponding values for PC; ^cdifferent ($P<0.05$) from the corresponding value for PD; ^ddifferent ($P<0.05$) from the corresponding values for VC rats (ANOVA).

Figure 1 Effects of L-NA on MAP of nondiabetic and diabetic virgin (a) and 14–15 days pregnant (b) rats. *Different ($P<0.05$) from the corresponding values in control rats; effects of L-NA were significant at all doses in virgin and pregnant control and pregnant diabetic rats but only at 1 μmol/kg per min in virgin diabetic rats. #Different ($P<0.05$) from the corresponding values for the non-diabetic controls. #Different ($P<0.05$) from the basal values for the same group of rats. Data are means ± S.E.M. of seven or eight experiments.

weight and increased plasma glucose and urine output; body weights (g), plasma glucose (mmol/l) and UV (μl/kg per min) were respectively: 259 ± 7 , 6.8 ± 0.3 and 19 ± 6 for virgin controls ($n=7$); 243 ± 4 , 34.9 ± 3 and 126 ± 12 for virgin diabetic rats ($n=6$); 270 ± 2 , 7.3 ± 0.6 and 31 ± 9 for control pregnant rats ($n=6$); and 240 ± 2 , 22.7 ± 1.3 and 136 ± 11 for diabetic pregnant rats ($n=5$).

Effects of L-NA on renal haemodynamics 14–15 days after induction of diabetes

L-NA caused a dose-dependent increase in MAP in all groups of rats; the relative increases in MAP in virgin diabetic rats was less than in the other three groups of rats (Fig. 1a and b). L-NA did not exert a significant effect on GFR in virgin control rats but caused a dose-dependent decrease in GFR in pregnant controls and in both virgin

Figure 2 Effects of L-NA on GFR, RPF and FF of nondiabetic and diabetic virgin (a, c and e) and 14–15 days pregnant (b, d and f) rats. *Different ($P<0.05$) from the corresponding values for the nondiabetic controls. #Different ($P<0.05$) from the basal values in the same group of rats. Data are means ± S.E.M. of seven or eight experiments.

and pregnant diabetic rats. The L-NA-induced decrease in GFR was greater in diabetic rats than in nondiabetic animals (Fig. 2a and b). Infusion of L-NA caused a dose-dependent decrease in RPF in all groups of rats; the effect was more marked in the diabetic than in control

Figure 3 Effects of L-NA on plasma $\text{NO}_2^-/\text{NO}_3^-$ in nondiabetic and diabetic virgin (a) and 14–15 days pregnant (b) rats. *Different ($P < 0.05$) from the corresponding values for the nondiabetic controls. #Different ($P < 0.05$) from the basal values for the same group of rats. Data are means $\pm$ S.E.M. of five or six experiments.

animals (Fig. 2c and d). The maximal decrease in RPF following L-NA infusion was $37 \pm 3\%$ in virgin diabetic rats and significantly less than in pregnant diabetic animals ($64 \pm 4\%$). L-NA increased FF in control virgin rats but not in the other three groups of animals (Fig. 2e and f).

UV and sodium excretion

Basal excretion of water and sodium was significantly greater in 14 days diabetic than in nondiabetic rats (Table 1). L-NA exerted dose-dependent antidiuretic and antinatriuretic effects in all groups of rats and the maximal decrease in both variables following $1 \mu\text{mol/kg}$ per min was approximately 50% of the basal (data not shown).

Plasma $\text{NO}_2^-/\text{NO}_3^-$

Diabetes of 2 weeks duration was associated with an increase in plasma $\text{NO}_2^-/\text{NO}_3^-$. Infusion of L-NA did not exert a significant effect on plasma $\text{NO}_2^-/\text{NO}_3^-$ of nondiabetic virgin rats (Fig. 3a); the effect in control pregnant rats (Fig. 3b) was observed only at the highest dose of L-NA ($1 \mu\text{mol/kg}$ per min) studied. In contrast to nondiabetic rats, all doses of L-NA caused a dose-dependent reduction in plasma $\text{NO}_2^-/\text{NO}_3^-$ in both

virgin and pregnant diabetic rats (Fig. 3a and b); these effects were significantly greater in pregnant diabetic than in virgin diabetic animals.

NOS activity

Diabetes of 14 days duration led to an increase in calcium-dependent NOS activity in the kidney, heart, aorta and uterus of both virgin and pregnant rats; the placental NOS activity was not modified by diabetes (Table 2). Diabetes also led to an increase in total NOS activity in the heart, aorta and uterus (data not shown).

Effects of L-NA on renal haemodynamics 7 days after induction of diabetes

The effects of diabetes of a shorter duration (7 days) in both virgin and pregnant rats (Fig. 4) were similar to those described above for diabetes of 14–15 days duration. L-NA at a dose of $0.5 \mu\text{mol/kg}$ per min (producing near maximal effects in diabetic rats of 14–15 days duration) caused a significant increase in MAP (Fig. 4a) in all the four groups of rats but the increase was less in virgin diabetic rats than in the other three groups of animals. L-NA caused a decrease in GFR in virgin diabetic, control pregnant and diabetic pregnant rats but not in control virgin rats (Fig. 4b). L-NA decreased RPF in all the four groups of rats (Fig. 4c) but increased FF only in control virgin rats (Fig. 4d).

Vascular responses

The maximal endothelium-dependent vasorelaxant effects of ACh (% of phenylephrine-induced tone) on aortic rings from virgin control, virgin diabetic, pregnant control and pregnant diabetic rats were $90 \pm 3\%$ ($n=7$), $85 \pm 3\%$ ($n=6$), $89 \pm 4\%$ ($n=5$) and $80 \pm 6\%$ ($n=5$) respectively and did not significantly differ from each other. ACh was a more potent relaxant on rings from diabetic virgin and pregnant rats than on preparations from nondiabetic

Table 2 Calcium-dependent NOS activity (Ca^{2+} -dependent [^3H]citrulline production, pmol/mg protein) in different tissues of virgin control (VC), virgin diabetic (VD), pregnant control (PC) and pregnant diabetic (PD) rats. The duration of pregnancy and diabetes at the time of tissue collections was 14–15 days. Data are means $\pm$ S.E.M. ($n=5$ or 6)

	VC	VD	PC	PD
Tissue				
Kidney	1.79 ± 0.15	2.65 ± 0.17^a	1.84 ± 0.14	2.52 ± 0.16^a
Heart	0.39 ± 0.06	0.98 ± 0.08^a	0.91 ± 0.25^b	$2.22 \pm 0.15^{a,c}$
Aorta	0.91 ± 0.14	2.73 ± 0.05^a	1.22 ± 0.22	$3.85 \pm 0.17^{a,c}$
Uterus	0.80 ± 0.10	2.63 ± 0.19^a	1.58 ± 0.12^b	2.71 ± 0.14^a
Placenta	—	—	2.25 ± 0.17	2.95 ± 0.19

^aDifferent ($P < 0.05$) from the corresponding controls; ^bdifferent ($P < 0.05$) from the corresponding virgin controls; ^cdifferent ($P < 0.05$) from the corresponding virgin diabetic rats (ANOVA).

Figure 4 Effects of L-NA at a dose of 0.5 µmol/kg per min on the MAP, GFR, RPF and FF of control virgin (CV), 7 days diabetic virgin (DV), control pregnant (CP) and 7 days diabetic pregnant (DP) rats. Data are means $\pm$ S.E.M. of five to seven experiments. *Different ($P < 0.05$) from the corresponding values in the control period before starting the infusion of L-NA.

animals; vasorelaxant EC_{50} of ACh on rings from control virgin, diabetic virgin, control pregnant and diabetic pregnant rats ($n=5-7$) were 130 ± 28 nM, 37 ± 8 nM, 140 ± 33 nM and 44 ± 8 nM respectively. NO donor SNP caused 100% relaxation of all preparations and there was no difference in its vasorelaxant potency on aortic rings from the four groups of rats (Fig. 5c and d). The maximal phenylephrine-induced increases in the tone of aortic rings from virgin control, virgin diabetic, pregnant control and pregnant diabetic rats ($n=5-7$) were 1002 ± 98 mg, 1150 ± 20 mg, 1170 ± 225 mg and 970 ± 42 mg respectively and did not differ significantly from each other; as well, there was no difference in the vasoconstrictor potency of phenylephrine on the preparations from the four groups of rats (Fig. 5e and f).

Figure 5 Concentration-response curves to ACh, SNP and phenylephrine (PE) on rings of thoracic aorta from control virgin (CV), diabetic virgin (DV), control pregnant (CP) and diabetic pregnant (DP) rats. The duration of diabetes and pregnancy was 14–15 days. Data are means $\pm$ S.E.M. of five or six aortic rings from separate animals.

Discussion

NO modulates renal haemodynamics (Tolins *et al.* 1990, Danielson & Conrad 1995) and contributes to the renal hyperfiltration during diabetes (Bank & Aynedjian 1993, Tolins *et al.* 1993, Komers *et al.* 1994). The renal complications of diabetes are worsened by pregnancy (Kitzmiller & Combs 1996). The present studies were therefore done to determine if the role of NO in diabetes-associated renal hyperfiltration is altered by pregnancy. For this purpose we used both virgin and pregnant diabetic rats as the model and the role of NO in hyperfiltration was assessed using L-NA, which is an effective inhibitor of NOS (Musch & Busse 1990, Moncada *et al.* 1991, Hardy *et al.* 1996).

In conformity with other data (Bank & Aynedjian 1993, Tolins *et al.* 1993, Komers *et al.* 1994), diabetes of both 1 and 2 weeks duration produced hyperfiltration, which was virtually abolished by L-NA. However, L-NA was much less effective in increasing MAP in virgin diabetic rats than in control virgin animals (Figs 1a and 5a). Although a decrease in the pressor response to NOS inhibitors in male rats has previously been reported by other workers (Kiff *et al.* 1991, Bank & Aynedjian 1993, Komers *et al.* 1994), the reason for a decrease in the hypertensive effect of L-NA in virgin but not in pregnant diabetic rats observed in the present study (Figs 1b and 4a) is not clear; this could be caused by several factors including a heterogeneity in

the role of NO in modulating the tone of different vascular beds (Kiff *et al.* 1991). An overall decrease in the role of NO in resistance vessels during diabetes (Poston & Taylor 1995) could explain the relatively poor hypertensive response to L-NA in virgin diabetic animals; this inference implies that somehow hormonal changes accompanying pregnancy tend to alter the role of NO, for which there is some evidence in the literature (Moroi *et al.* 1998).

Several studies have reported that the inhibition of NO synthesis ameliorates the diabetes-associated increase in GFR and RPF (Bank & Aynedjian 1993, Tolins *et al.* 1993, Komers *et al.* 1994); this inference is supported by our data that the renal haemodynamic responses to the inhibition of NO synthesis by L-NA were more marked in both 7 days and 14–15 days diabetic (Figs 2 and 4) than in nondiabetic rats. Indeed the effects of the low dose of L-NA (0.25 µmol/kg per min) on GFR was apparent only in diabetic animals (Fig. 2a) as has been previously reported (Komers *et al.* 1994), although higher doses caused a small but significant decrease in GFR in control pregnant rats (Figs 2b and 4b). Other workers also found that relatively low doses of L-NAME (2 µg/min per rat) and L-NMMA (100 µg/min per rat) did not reduce GFR in conscious virgin rats although a significant decrease in GFR was observed in pregnant rats (Danielson & Conrad 1995). On the other hand, a bolus high dose of 10 mg/kg (approximately 35 µmol/kg) of L-NAME was found to reduce GFR in conscious virgin rats (Baylis *et al.* 1990, 1993). It would thus seem that the ineffectiveness of L-NA to reduce GFR in virgin controls and do so only at relatively high doses in control pregnant (both 7 and 14–15 days) rats, reflects differences in experimental conditions and doses of NOS blockers between the present study using urethane-anaesthetized rats and conscious rats used by others (Baylis *et al.* 1990, 1993, Danielson & Conrad 1995).

In contrast to effects in nondiabetic animals discussed above, both low and high doses of L-NA markedly decreased GFR in animals diabetic for 14–15 days (Fig. 2a and b) or 7 days (Fig. 4b). L-NA decreased RPF in all groups of rats although the magnitude of this effect was significantly greater in diabetic than in nondiabetic rats (Fig. 2). These data suggest that NO might play a greater role in modulating RPF than GFR. L-NA-induced increase in FF in nondiabetic but not in diabetic rats might be because of disproportionate changes in GFR and RPF and because of high basal values of FF in diabetic animals (Fig. 2e and f). Overall the effects of L-NA on renal haemodynamics are strongly suggestive of a role for increased NO synthesis in hyperfiltration associated with diabetes. Since the overall renal haemodynamic effects of L-NA were greater in diabetic pregnant than in diabetic virgin rats, it would appear that the contribution of NO in renal hyperfiltration was augmented by pregnancy. An increase in NO synthesis as reflected by high plasma $\text{NO}_2^-/\text{NO}_3^-$ levels and tissue (kidney, heart, aorta)

NOS activity during diabetic pregnancy (Table 1) is in accordance with this suggestion.

Diabetes increased basal urinary flow and sodium excretion rates (Table 1) in conformity with other reports (Komers *et al.* 1994). L-NA exerted antinatriuretic and antidiuretic effects in all groups of animals; these data are consistent with the observed decrease in GFR, although our data do not exclude the possibility of effects of NO on other segments of the nephron. We did not observe any pressure natriuresis as reported by others (Johnson & Freeman 1992, Komers *et al.* 1994). This discrepancy between our findings and those of others (Komers *et al.* 1994) could be related to the dose and method of administration of L-NA; we administered lower doses (0.25–1 µmol/kg per min) of L-NA by continuous infusion compared with a bolus dose of 37 µmol/kg L-NAME used by others (Komers *et al.* 1994).

We found that 2 weeks of diabetes slightly but significantly increased the endothelium-dependent vasorelaxant effect of ACh but did not modify responses to SNP and phenylephrine (Fig. 5). A lack of change in the vasorelaxant effect of NO donor SNP and the vasoconstrictor effect of phenylephrine is in conformity with other reports (Durante *et al.* 1988); these data suggest that the vascular reactivity is not modified by diabetes of 2 weeks duration. However, the increase in vasorelaxant potency of ACh on aortic rings from diabetic rats is at variance with the generally observed dysfunction of endothelial cells in diabetes of relatively long duration (Poston & Taylor 1995, Sobrevia & Mann 1997); the most likely explanation for this discrepancy appears to be the duration of diabetes. Of particular relevance to the present study is the report that diabetes of 12 days duration increased the vasodilator effects of ACh on renal vessels (Bhardwaj & Moore 1988). It is very likely that increased NO release during early stages of diabetes contributes to endothelial dysfunction with the progression of the diabetic state.

Our experimental protocol does not permit any definitive conclusion regarding the source of the increased role of NO in renal haemodynamics during diabetes and diabetic pregnancy. The assay of NOS activity in different tissues suggests that several organs might contribute to the increase in plasma NO during diabetes. For example, NOS activity was greater in the kidneys, hearts, aortas and uteri of the diabetic than of nondiabetic rats and greater in pregnant diabetic than in virgin diabetic animals (Table 2).

It has been suggested that increased synthesis of NO by vascular beds and the placenta contributes to the elevated plasma levels and urinary excretion of $\text{NO}_2^-/\text{NO}_3^-$ during normal pregnancy (Conrad *et al.* 1993, McLaughlin & Conrad 1995). Current data suggest that peripheral vasodilatation during pregnancy largely results from endothelial NO (Deng *et al.* 1996, Hines & Mifflin 1997) and pregnancy is associated with increased expression of endothelial NOS in the rat aorta (Goetz *et al.* 1994); in general our data support these inferences. However, a lack

of difference in NOS activity in the placentas of diabetic and nondiabetic rats would suggest that the placenta is not the source of the observed increase in the role of NO in renal haemodynamics during diabetic pregnancy.

In summary, the findings of the present study support our hypothesis that the state of diabetes during pregnancy accentuates the role of NO in renal hyperfiltration.

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