Programming of growth, insulin resistance and vascular dysfunction in offspring of late gestation diabetic rats

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ABSTRACT

ODM (offspring of diabetic mothers) have an increased risk of developing metabolic and cardiovascular dysfunction; however, few studies have focused on the susceptibility to disease in offspring of mothers developing diabetes during pregnancy. We developed an animal model of late gestation diabetic pregnancy and characterized metabolic and vascular function in the offspring. Diabetes was induced by streptozotocin (50 mg/kg of body weight, intraperitoneally) in pregnant rats on gestational day 13 and was partially controlled by twice-daily injections of insulin. At 2 months of age, ODM had slightly better glucose tolerance than controls (P < 0.05); however, by 6 months of age this trend had reversed. A euglycaemic–hyperinsulinaemic clamp revealed insulin resistance in male ODM (P < 0.05). In 6–8-month-old female ODM, aortas had significantly enhanced contractility in response to KCl, ET-1 (endothelin-1) and NA (noradrenaline). No differences in responses to ET-1 and NA were apparent with co-administration of L-NNA (N\textsubscript{G}-nitro-L-arginine). Relaxation in response to ACh (acetylcholine), but not SNP (sodium nitroprusside), was significantly impaired in female ODM. In contrast, males had no between-group differences in response to vasoconstrictors, whereas relaxation to SNP and ACh was greater in ODM compared with control animals. Thus the development of diabetes during pregnancy programmes gender-specific insulin resistance and vascular dysfunction in adult offspring.

INTRODUCTION

There is increasing evidence that adverse factors in the perinatal environment predispose an individual to disease later in life. This concept of ‘developmental programming of adult onset diseases’ has primarily focused on maternal undernutrition and/or poor fetal growth and the later development of adult-onset diseases. [1–3]. However, other maternal conditions, including diabetes, produce an adverse environment for the developing fetus, resulting in increased risk of obesity, hypertension, insulin resistance and dyslipidaemia in the offspring [4–6]. There is a paucity of understanding of the mechanisms that underlie the adverse long-term metabolic and cardiovascular programming that occurs after exposure to maternal diabetes.

Previous studies in animals have demonstrated that maternal diabetes promotes alterations in metabolic function and vascular reactivity in offspring [7–15]; however, these studies have primarily used animals made diabetic either prior to pregnancy [8,13,14] or early in pregnancy [7,10]. The effect of the development of diabetes during pregnancy programmes gender-specific insulin resistance and vascular dysfunction in adult offspring.
occurs with gestational diabetes, on long-term metabolic and cardiovascular function is not known. We hypothesized that offspring of maternal rats made diabetic during the last third of gestation would demonstrate altered growth, glucose metabolism and vascular reactivity. To address this hypothesis, we examined glucose tolerance, insulin sensitivity and aortic reactivity to vasodilatory and vasorelaxing agents in 6–8-month-old offspring of rats made diabetic during the last week of pregnancy.

**MATERIALS AND METHODS**

**Animals**

All procedures were performed within the regulations of the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of the University of Iowa. Pregnant Sprague–Dawley rats (Charles River Laboratories) were injected intraperitoneally with either STZ [streptozotocin; 50 mg/kg of body weight in 10 mmol/l citrate buffer (pH 3.5)] or an equivalent volume of citrate buffer alone on gestational day 13. STZ-injected rats had blood glucose measured twice daily via tail-nicking, using the LifeScan OneTouch Ultra Blood Glucose Monitoring System. Hyperglycaemia was partially controlled and ketosis was avoided by subcutaneous injection of insulin (Humulin; Eli) in the morning and insulin-glargine (Lantus; Sanofi-Aventis) each evening to maintain blood glucose between 100 and 400 mg/dl. Control rats were injected with saline twice daily. Dams were allowed to delivery spontaneously. All pups were cross-fostered to standard rat chow on day 21.

**Glucose tolerance and insulin sensitivity**

Glucose tolerance was assessed in all offspring at 2 and 6 months of age after starving for 3–5 h by measuring blood glucose at baseline, and 15, 30, 60 and 120 min after intraperitoneal injection of 2 g of dextrose/kg of body weight (20 % solution). Testing was typically performed in the early afternoon (12.00–14.00 hours). Serum insulin was measured at baseline and 30 min after the injection using a rat insulin ELISA kit (Crystal Chem). An ivGTT [intravenous GTT (glucose tolerance test)] was performed in a subset of the 5-month-old offspring after overnight starvation by measuring central venous blood glucose and serum insulin at baseline, and at 1, 2, 3, 5, 10, 15, 25, 40 and 60 min after 0.5 g of dextrose/kg of body weight infused via the left carotid artery. In a separate subset of offspring, insulin sensitivity was measured in overnight starved offspring by euglycaemic–hyperinsulinaemic clamp, infusing insulin at 20 milli-units·kg⁻¹·body weight·min⁻¹ via a right internal jugular catheter and adjusting the dextrose infusion to maintain blood glucose at a target level of approx. 90 mg/dl. Euglycaemic–hyperinsulinaemic clamps and ivGTTs were performed as terminal procedures under pentobarbital anaesthesia, utilizing acute catheterization of the left carotid artery and right jugular vein.

**Vascular reactivity vessel preparation and arteriography mounting**

Offspring used for vascular reactivity studies (n = 14 female, and n = 14 male) were products of diabetic (n = 5) or control (n = 5) female rats. At 6–8 months of age, offspring were killed and descending thoracic aorta segments were harvested, cleansed of adherent connective tissue and sectioned into 2-mm-long rings. Two ring segments were denuded of endothelium (‘rubbed’) by inserting forceps tips into the lumen and gently rolling the vessel ring. Aortic rings were mounted in individual 18-ml isolated organ chambers (Radnoti Glass Technology) and were connected to an isometric force transducer. Contractile responses were recorded with a MacLab 8E (ADInstruments) and were stored on a Power Macintosh 8600 computer. Passive stretch was set at 90 % of the tension required to obtain peak responses to KCl (2.5 g; determined in preliminary studies), and the rings were allowed to equilibrate in bicarbonate-buffered PSS (physiological salt solution) at 37 °C for 60 min before the start of experimentation. The composition of the PSS was as follows: 130 mmol/l NaCl, 4.7 mmol/l KCl, 1.18 mmol/l KH₂PO₄, 1.17 mmol/l MgSO₄·7 H₂O, 14.9 mmol/l NaHCO₃, 1.6 mmol/l CaCl₂·H₂O, 5.5 mmol/l dextrose and 0.03 mmol/l CaNa₂-EDTA (pH 7.30). PSS was aerated with a mixture of 95 % O₂/5 % CO₂.

**Experimental protocols for vascular function**

Separate baths were used to assess the cumulative concentration responses to AngII (angiotensin II; 10⁻¹¹–10⁻⁷ mol/l), ET-1 (endothelin-1; 10⁻¹¹–10⁻⁷ mol/l), NA (noradrenaline; 10⁻¹¹–10⁻⁷ M) and 5-HT (5-hydroxytryptamine; 10⁻¹¹–10⁻⁷ mol/l). Arteries were re-equilibrated with washes of PSS before the measurement of vasoconstrictor responsiveness in the presence of l-NNa (N⁰₂-nitro-l-arginine; 10⁻⁵ mol/l). Separate baths were used to assess cumulative concentration–vasorelaxant responses to SNP (sodium nitroprusside; 10⁻⁹–10⁻⁵ mol/l) or ACh (acetylcholine; 10⁻¹⁰–10⁻⁷ mol/l) after preconstriction with NA (10⁻⁵ mol/l). All PSS reagents and vasoactive compounds were acquired from Sigma with the exception of ET-1 (Alexis).

**Morphometric analysis**

Distal segments of thoracic aorta were incubated for 10 min in PSS containing 10⁻⁵ mol/l SNP, fixed in Pen-Fix (Richard Allen Scientific) and paraffin-embedded.
Vascular changes in diabetic offspring

Figure 1 Daily blood glucose levels in pregnant dams with diabetes induced by the injection of STZ (●) on day 13 of gestation and sham controls (○).

Glucose values represent average daily values and are means ± S.E.M. P < 0.05 between groups for all data points. The mean values in the control groups ranged from 97.8 to 99.4 mg/dl, with an S.E.M. range of 2.73–4.08 mg/dl.

Figure 2 Weight distribution of 1-day-old male (A) and female (B) rat pups of diabetic or control mothers.

White distribution, pups from diabetic mothers; grey distribution, pups from control mothers. The distribution is shown as a normalized frequency plot. Tick marks representing the weights of each pup are shown below the x-axis. n, number of animals in each group.

RESULTS

Dams injected with STZ had peak blood glucose levels on gestational days 15 and 16 and remained hyperglycaemic throughout the remainder of gestation (Figure 1). The number of pups/litter were similar (P = 0.54) in the STZ group (12.6 ± 1.1) and the control group (13.4 ± 0.5). Likewise, there were no group differences in the gestational day of delivery. A total of 87 male [ODM (offspring from diabetic mothers), n = 45; CON (control) offspring, n = 42] and 38 female [ODM, n = 18; CON offspring, n = 20] offspring from seven diabetic and seven control dams were used for the present study. Differences in male and female numbers probably resulted from culling of the smallest pups at birth without attention to gender.

Birthweight and growth

Perinatal weights of CON offspring had a bell-shaped distribution (Figure 2). In contrast, ODM had an increased number of high- and low-birthweight pups. There was no difference in mean birthweights between male ODM and CON pups; however, the variance in birthweight among male ODM was 3.7-fold higher than among the CON group (P < 0.0001). A similar pattern was also observed in females. Weight gain in male ODM was initially similar to CON offspring, began to lag behind at approx. 2 months of age, and remained slightly less at 140 days (absolute weights at 140 days, 630 ± 7 compared with 611 ± 9 g in CON offspring and ODM respectively; P < 0.05). No differences in growth were detected among female offspring. For male ODM, birthweight was a positive predictor of weight at young ages, but transitioned to a negative predictor of weight persisting through at least 140 days (r = −0.36, P < 0.05; n = 44). This negative correlation was not observed in the CON offspring; however a similar trend was apparent among the female ODM. Animal weights at the time of metabolic and vascular testing, outlined below, were not significantly different between groups at any time point (Table 1).

Glucose tolerance and insulin sensitivity

Glucose tolerance was measured at 2 and 5 months of age. Glucose values were lower in 2-month-old male ODM at 30 min compared with CON offspring (225 ± 8 compared with 248 ± 7 mg/dl respectively; P < 0.05), but no other differences between the groups were identified at any time point (Figure 3). Plasma insulin values at 0 and 30 min during the GTT were not different between experimental groups (results not shown).

Results from the ivGTTs (performed only in male offspring at 6 months of age) demonstrated no difference in glucose levels between ODM and CON offspring (Figure 4). Results from the euglycaemic–hyperinsulinaemic clamp demonstrated significant insulin resistance among male ODM, as they required 30% less glucose at
Table 1 Animal weights at the times of metabolic and vascular testing
Values are means ± S.E.M. ipGTT, intraperitoneal injection of dextrose for glucose tolerance; Clamp, euglycaemic–hyperinsulinaemic clamp. Euglycaemic–hyperinsulinaemic clamp and ivGTT were not performed in females.

<table>
<thead>
<tr>
<th>Group</th>
<th>ipGTT (2 months)</th>
<th>ipGTT (6 months)</th>
<th>Clamp</th>
<th>ivGTT</th>
<th>Vascular function</th>
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<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CON offspring</td>
<td>243 ± 4</td>
<td>362 ± 8</td>
<td>—</td>
<td>—</td>
<td>422 ± 17</td>
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<tr>
<td>ODM</td>
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<td>371 ± 10</td>
<td>—</td>
<td>—</td>
<td>444 ± 18</td>
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<tr>
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<tr>
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<td>358 ± 5</td>
<td>642 ± 10</td>
<td>636 ± 21</td>
<td>712 ± 37</td>
<td>719 ± 29</td>
</tr>
</tbody>
</table>

Figure 3 Blood glucose levels during intraperitoneal GTTs in female and male CON offspring (●) and ODM (■)
(A) Females at 2 months (n = 17 in ODM, and n = 19 in CON offspring); (B) males at 2 months (n = 42 in ODM, and n = 37 in CON offspring); (C) females at 5 months (n = 17 in ODM, and n = 19 in CON offspring); (D) males at 5 months (n = 17 in ODM, and n = 19 in CON offspring). Values are means ± S.E.M.

Vascular function in female ODM
Aortic segments from female ODM had similar maximal responses to KCl (90 mmol/l) compared with CON offspring, although responses at lesser concentrations were slightly but significantly increased (P < 0.05; Figure 5A). Vasoconstrictive responses to 5-HT, NA and ET-1 were significantly enhanced in aorta from ODM relative to CON offspring (Figures 5B–5D); responses to AngII were not different (Figure 5E).

As expected, removal of the endothelium increased the constrictive responses to 5-HT, NA and ET-1 in both groups (except for CON offspring response to ET-1) (Figures 5B–5D). However, the differences in the responses to 5-HT and NA between ODM and CON offspring were lost, suggesting the endothelium buffers vasoconstriction to a greater extent in CON offspring compared with ODM. In the presence of the l-NNA, aorta from CON offspring but not ODM had increased vasoconstrictive response to NA (Figure 5F) and ET-1 (Figure 5G). As a result, the vasoconstrictive response of aorta from ODM and CON offspring to NA was similar in the presence of l-NNA, whereas the response to ET-1 remained greater in ODM.

Relaxation was investigated by examining responses to the endothelium-independent vasodilator SNP and the endothelium-dependent vasodilator ACh. In endothelium-intact aorta, relaxation in response to

steady-state (120–140 min of insulin infusion) than the CON offspring to maintain euglycaemia (17.0 ± 0.8 compared with 24.3 ± 1.5 mg·kg⁻¹·min⁻¹ of body weight·min⁻¹ for ODM and CON offspring respectively; P < 0.05).

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Vascular changes in diabetic offspring

Figure 4  Blood glucose (upper panel) and plasma insulin (lower panel) levels during ivGTTs in 6-month-old male CON offspring (●) and ODM (■). Values are means ± S.E.M. (n = 6 per group). No differences in any time point or in the total area under the curve, as determined by the trapezoidal rule, were detected.

SNP was similar in both groups (Figure 6A). However, responses to ACh were markedly different between the groups, with relaxation being significantly greater in aortas from CON offspring compared with ODM (Figure 6B). In endothelium-denuded aortas (‘rubbed’), responses to SNP were decreased in both groups; however, the attenuation was significantly greater in ODM than CON offspring.

Vascular dimensions
Sections of distal thoracic aorta were used to examine external vessel diameter, luminal diameter and media thickness. No significant differences between female ODM and CON offspring were identified for any of these parameters (Figure 7).

Vascular function in male ODM
No significant differences between male groups in the contractile responses to KCl, 5-HT, AngII or ET-1 were observed (Figures 8A–8D). The response to NA was significantly greater in intact aorta from ODM compared with CON offspring (Figure 8E), similar to that seen in the females. In endothelium-denuded vessels, contractile responses to 5-HT, ET-1 and NA were similar in the two groups (Figures 8B, 8D and 8E), whereas responses to AngII were significantly enhanced in ODM compared with CON offspring (Figure 8C). The presence of l-NNA resulted in increased contractile responses to NA in CON offspring, but had no effect on ET-1 in either group (Figures 8F and 8G).

Vasorelaxation responses to SNP and ACh were also significantly different between groups. Specifically, intact aorta from male ODM had greater relaxation in response to SNP and ACh compared with CON offspring (P < 0.05; Figures 9A and 9B). This finding is in sharp contrast to that displayed in female ODM, where decreased sensitivity to ACh and SNP were observed in ODM relative to CON offspring. Removal of the endothelium abolished the between-group differences in response to SNP.

Vascular dimensions
Male ODM had significantly decreased aorta wall diameter and wall area compared with CON offspring (P < 0.05; Figure 7). No differences were detected in aorta lumen area between groups.

DISCUSSION
Exposure to diabetes in utero is a significant risk factor for the development of components of the metabolic syndrome, including glucose intolerance, insulin resistance and hypertension [4,16]. The majority of previous studies evaluating the effects of diabetes during pregnancy have studied offspring of mothers made diabetic before or relatively early in gestation. We have developed a rat model of diabetes with maternal hyperglycaemia limited to the last third of pregnancy, at a time beyond the embryonic development period (http://embryology.med.unsw.edu.au/OtherEmb/Rat.htm). Our model results in a spectrum of birthweights, with ODM maintaining relatively normal glucose tolerance, but males developing insulin resistance. Female, but not male, ODM also had altered vascular reactivity, suggesting that gender-specific cardiovascular dysfunction can be induced in offspring of mothers displaying a ‘gestational-onset’ form of diabetes.

Our model of late gestation maternal hyperglycaemia produced both microsomic and macrosomic pups. The heavier pups tended to come from mothers with mild hyperglycaemia, whereas more severe hyperglycaemia predicted underweight pups (r = −0.54, P = 0.0001). Postnatal weight gain among male ODM was slightly reduced, consistent with a previous study using STZ to induce more severe gestational diabetes [12]. Pregestational maternal diabetes induced by STZ has produced both large [17,18] or small [9,11,19,20] offspring. In these studies, adult size correlated with birthweight [9,17–19,21]. In contrast in our present study, birthweight inversely correlated with adult size.

In humans, in utero exposure to diabetes is associated with a long-term risk of developing Type 2 diabetes [5] and/or insulin resistance syndrome [4]. Likewise, the male offspring in our present study developed insulin resistance by 6 months of age, despite initially improved glucose tolerance at 2 months of age. Similar results have
been observed by other investigators in the offspring of STZ-induced diabetic mothers [12]. In contrast, offspring of mothers with hyperglycaemia induced by glucose infusion experience impaired glucose tolerance due to the progressive loss of glucose-stimulated insulin secretion [22]. The reasons for this discrepancy are unclear, although the infusion model provides excess glucose-based calories, potentially reducing normal dietary intake of other nutrients by the mothers.

The STZ model of maternal diabetes has been used by other investigators to examine cardiovascular consequences in the offspring. Holemans et al. [10] reported that the sensitivity of mesenteric arteries to NA was enhanced in female offspring of mothers made diabetic on day 1 of pregnancy with STZ, whereas sensitivity and maximum relaxation to ACh, but not SNP, were reduced. Our present findings in female ODM of impaired relaxation in response to ACh, but not SNP, are consistent with those of Holemans et al. [10] and suggest exposure to maternal hyperglycaemia even late in gestation results in offspring with endothelial dysfunction. Because relaxation in response to SNP was similar in the two groups, it is less likely that there are intrinsic differences in vascular smooth muscle sensitivity to NO. The presence of

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Figure 6 Concentration–response curves for aorta vasodilation in female CON offspring (○) and ODM (■) produced by SNP (A) and ACh (B)

Endothelium-intact aorta from ODM and CON offspring (solid line), and rubbed aorta from ODM and CON offspring (broken line). Values are means ± S.E.M. (n = 7 for each group). *P < 0.05 between intact and rubbed within same treatment group (ANOVA); ¶P < 0.05 between intact aorta from ODM and CON offspring, or between rubbed aorta from ODM and CON offspring (treatment group effect, ANOVA).

endothelial dysfunction is suggested further by the findings that between-group differences in vasoconstrictive responses were attenuated by removal of the endothelium and that l-NNA enhanced vasoconstrictive responses to ET-1 and NA in CON offspring by not ODM.

The gender-specific differences in vascular responsiveness in ODM, with females being primarily affected, are distinct from findings of other investigators. Rocha et al. [14] found that the in vivo response of mesenteric microvessels to ACh and bradykinin, but not SNP, were significantly attenuated in male offspring of dams made diabetic prior to mating. Male offspring from dams treated with STZ on day 7 of pregnancy also had increased vascular responsiveness to phenylephrine but not SNP [7]. In contrast, we found that intact aorta from male ODM and CON offspring had similar contractile responses to KCl, AngII, 5-HT or ET-1. In addition, aorta from male ODM had significantly greater relaxation in response to ACh and SNP compared with control animals, suggesting an enhancement of smooth-muscle-mediated vasodilation. Studies in other models of adverse fetal environments also demonstrate gender differences in the pathophysiological responses, with female offspring having a lower incidence of hypertension and vascular dysfunction [23]. A role for sex hormones interacting with other regulatory mechanisms, including the renin–angiotensin system, has been suggested. Reasons for the discrepant findings in the gender affected between our present study and others are unclear, but may be related to underlying differences in the models and the timing of the in utero insult.

Vessel morphometry, specifically aorta diameter, luminal diameter and media thickness, were not different between female groups. Similarly, Holemans et al. [10] found no differences in vessel internal diameter; however, in male offspring, aortic wall diameter and wall area were significantly decreased in ODM compared with controls. This finding suggests that the diabetic

Figure 7 Abdominal aorta lumen area and wall area and diameter in female (left-hand panels) and male (right-hand panels) CON offspring (filled bars) and ODM (open bars)
Values are means ± S.E.M. *P < 0.05 compared with CON offspring.
environment in utero resulted in alterations in vascular smooth muscle development in males. The effects of in utero hyperglycaemia on vascular smooth muscle cell proliferation, have to our knowledge, not been explored.

Blood pressure was not measured in the animals used in the present study. Thus it is possible that the alterations in vascular reactivity are secondary changes due to hypertension, rather than primary effects of an altered in utero environment. Holemans et al. [12] found that female ODM had normal blood pressures, whereas male offspring of dams made diabetic prior to mating or on day 7 of pregnancy were hypertensive by 2–3 months of age [10]. Rocha et al. [14] also found male offspring of dams made diabetic prior to mating were hypertensive by 3 months of age relative to controls.

Several additional limitations of the present study exist. Because we used STZ to induce maternal diabetes, it is possible that the agent crossed the placenta and had direct effects on the fetus; however, we believe this to be unlikely since insulin levels were similar in young offspring regardless of exposure to STZ. Schroeder et al. [15] also found that administration of STZ to pregnant dams at 12–13 days had no significant effects on fetal (day 20–21) insulin values. Our model is also one of maternal insulinopenia and hyperglycaemia and not gestational diabetes, a condition of insulin resistance. Additionally, the vascular reactivity studies were performed in aorta, and the findings may not be reflective of endothelial and vascular smooth muscle function in resistance vessels. Finally, killing the smallest newborns resulted in a bias of studying the larger offspring.
The majority of other studies using the STZ model to investigate cardiovascular or metabolic function in ODM have not cross-fostered offspring after birth. Because milk volume or content may be altered in diabetic rats, programming effects in these studies may be related to postnatal nutrition. As all of the offspring were cross-fostered in the present study, the metabolic and cardiovascular changes we identified in ODM are probably related to the abnormal intrauterine environment, rather than postnatal nutrition. Diabetes increases the levels of many macronutrients in the maternal circulation, including glucose, triacylglycerol (triglyceride), NEFAs (non-esterified fatty acids) and ketone bodies [24,25]. Most metabolic fuels are readily transferred from the maternal circulation across the placenta by passive diffusion, facilitated diffusion or by facilitated transport [26]. Thus the growing fetus is exposed to an oversupply of most circulating fuels during diabetic pregnancy. It is not known which macronutrients may be deleterious to the offspring with regards to long-term health.

Our present results suggest that exposure to a hyperglycaemic milieu during the last third of gestation results in gender-specific metabolic and cardiovascular abnormalities in the offspring. The consequences of being born to a diabetic mother present tremendous health implications. With improved understanding of the cellular and molecular responses of the metabolic pathways and the cardiovascular system to the intrauterine diabetic milieu, it may be possible that early pharmacological interventions can be designed to prevent or attenuate the development of disease later in life.

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