

# Altered gap junctional communication and renal haemodynamics in Zucker fatty rat model of type 2 diabetes

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## Abstract

**Aims/hypothesis** We examined the link between altered gap junctional communication and renal haemodynamic abnormalities in diabetes in studies performed on Zucker lean (ZL) and the Zucker diabetic fatty (ZDF) rat model of type 2 diabetes.

**Methods** The abundance of connexin (Cx) 37, 40 and 43 was assessed by western blot and immunohistochemistry. Renal haemodynamics was characterised with GAP peptides, which are Cx mimetics, to inhibit gap junctions as a probe in both strains.

**Results** ZDF rats exhibited higher plasma glucose, 8-epi-prostaglandin F<sub>2</sub>α excretion, renal plasma flow and GFR than ZL rats. In ZDF rat kidney phosphorylation of

Cx43 was enhanced compared with that in ZL rats. Immunohistochemical study revealed that the density of abundance of Cx37 in renin-secreting cells was significantly reduced in ZDF rats. Although renal autoregulation was markedly impaired in ZDF rats, it was preserved in ZL rats. GAP27 for Cx37,43 and for Cx40 impaired renal autoregulation in ZL rats, but failed to induce further alterations in renal autoregulation in ZDF rats.

**Conclusions/interpretation** Our findings indicate that ZDF rats have glomerular hyperfiltration with impaired autoregulation. They also demonstrate enhanced phosphorylation of Cxs and reduced production of Cxs in ZDF rat kidney, especially of Cx37 in renin-secreting cells. Finally, our data suggest that an impairment of gap junctional communication in juxtaglomerular apparatus plays a role in altered renal autoregulation in diabetes.

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Oxidative stress · Renin–angiotensin system ·  
Tubuloglomerular feedback

## Abbreviations

Cx	Connexin
Cx <sup>37,43</sup> GAP27	GAP27 for Cx37,43
Cx <sup>40</sup> GAP27	GAP27 for Cx40
Cx <sup>43</sup> GAP26	GAP for Cx43
EDHF	Endothelium-derived hyperpolarising factor
MAP	Mean arterial pressure
RPF	Renal plasma flow
TGFback	Tubuloglomerular feedback
ZL	Zucker lean
ZDF	Zucker diabetic fatty

## Introduction

Gap junctions comprising various connexins (Cx) are abundantly produced throughout the cardiovascular system, providing direct electrical and chemical communication between adjacent cells to coordinate physiological responses such as cardiac rhythm and the regulation of vasomotor tone [1–3]. In the kidney, extensive coupling by gap junctions, involving Cx37 and Cx40, enables intercellular communication between the renin-secreting cells of the afferent arteriole, the endothelium and the mesangial cells, which, together with the macula densa, form the juxtaglomerular apparatus [4–7]. Cx40 also appears to be essential for the correct targeting of renin-secreting cells to the terminal regions of the renal arterial tree and for the pressure-induced control of renin release in mice [8–10]. In addition, our studies have shown that Cx37 and Cx40 in the juxtaglomerular apparatus play an important role in transducing purinergic tubuloglomerular feedback (TGF<sub>fb</sub>) response signal and controlling the renin-angiotensin system [11, 12].

Perturbations in Cx production involving transcriptional and post-transcriptional mechanisms are increasingly recognised to accompany pathophysiological conditions such as ischaemic heart disease, heart failure, atrial fibrillation, hypertension and diabetes [1, 2, 13, 14]. However, data using animals in which a specific Cx has been deleted suggest that compensatory changes may occur in the remaining Cxs [2]. On the other hand, fine control of Cx structure through post-translational modifications such as phosphorylation may constitute additional mechanisms through which the function of gap junction could be disrupted. Indeed, the phosphorylation of Cx43 modulates channel gating, turnover and distribution [15]. Interestingly, high glucose treatment decreases gap junction function in microvascular endothelial cells and aortic smooth muscle cells either by reducing production or increasing phosphorylation of Cx43 [16, 17]. These observations raise the possibility that dysfunction of gap junctions from altered Cxs and also varied abundance of Cxs contribute to diabetic nephropathy, a disease characterised by reduced afferent arteriolar tone and glomerular hyperfiltration [18–20].

In the present study, we assessed the alterations of renal autoregulation in a rat model of type 2 diabetes by determining the levels of Cx37, Cx40 and Cx43, and investigating the role of these Cxs in renal haemodynamics using GAP peptides, which block gap junctions through mimicry of extracellular sequences for specific Cxs [21, 22]. Our findings that enhanced phosphorylation of Cxs and reduced abundance of Cx37 in renin-secreting cells are associated with little involvement of Cx37 and Cx40 in renal autoregulation in diabetes seem to agree with our previous studies, in which conductance of vascular gap

junctions was subject to pathophysiological modulations [23]. A recent report that peptidomimetic molecules prevent closure of cardiac Cx43 gap junctions points to a possible basis for the development of new therapeutic tools for diabetic nephropathy [24].

## Methods

### Animals

All experiments were performed using 8-week-old male Zucker diabetic fatty (ZDF) and Zucker lean (ZL) rats (Charles River, Kanagawa Japan) to assess early changes in diabetes. Experimental protocols followed Principles of Laboratory Animal Care and were approved by the Ethics Committee of Saitama Medical University and Australian National University. Animals were housed separately in metabolism cages and had free access to tap water and rat chow consisting of 24% protein, 14% fat, 5% ash, 3% fibre and 46% nitrogen-free extract (Quick Fat; Nihon Clea, Tokyo, Japan) in a temperature-controlled room with a 12 h light/dark cycle until experiments began. No insulin treatment was performed.

### Immunohistochemistry

ZL ( $n=4$ ) and ZDF ( $n=4$ ) rats were killed with an overdose of pentobarbital. Kidneys were removed and cut into 3 to 4 mm transverse slices, which were fixed in ice-cold acetone for 20 min at 4°C and then washed in PBS for 30 min. The slices were cryoprotected overnight in 30% (wt/vol.) sucrose in PBS, embedded in Tissue Tek OCT (Sakura Fintek, Torrance, CA, USA) and coronal sections (30  $\mu$ m) cut on a cryostat. Staining of Cx37, Cx40, Cx43 and renin were carried out as described previously [25, 26]. For more details, see also Electronic supplementary material (ESM), [Immunohistochemistry](#).

### Morphological analysis

The image analysis program Image J (National Institutes of Health, Bethesda, MD, USA) was used to generate single projections for each series of images. These single images were subsequently used to analyse Cx and renin staining. The distance from the glomerulus of renin-secreting cells along the afferent arteriole was measured and occurrence of additional groups of renin-stained cells in the same afferent arteriole was noted in a binary manner. The incidence of renin-secreting cells in the efferent arteriole was similarly noted. Efferent arterioles were identified by lack of Cx staining [11] and also by the irregular arrangement of smooth muscle cells.

Cx37 staining was quantified in two regions of the afferent arteriole after background subtraction using the threshold image function of Image J. The first region colocalised with renin staining and region was 40  $\mu\text{m}$  proximal to this area along the same afferent arteriole. The density of Cx staining within renin-secreting cells was defined by Cx staining in the total area coincident with renin staining, after subtraction of Cx staining in the area represented by the endothelium. Staining density in renin-secreting cells was normalised for variation in intensity between preparations against abundance relative to endothelial cell density in the proximal region in each preparation.

#### Western blotting

ZDF ( $n=4$ ) and ZL ( $n=4$ ) rats were anaesthetised with pentobarbital (50 mg/kg i.p.) and decapitated. Both kidneys were removed, immersed in liquid nitrogen and kept frozen at  $-80^\circ\text{C}$  until the assay. As described previously,  $\beta$ -actin was used as a housekeeping protein [27, 28]. For more details please see ESM, [Western blotting](#).

#### Haemodynamics

Animal preparations were performed, as detailed previously [5, 11, 12, 29] and in ESM, [Haemodynamics](#). Under anaesthesia, the right jugular vein was cannulated with polyethylene tubing (PE50). The animals were infused at a rate of 1.2 ml/h with isotonic saline solution containing 6% (wt/vol.) BSA during surgery and thereafter with isotonic saline solution containing 1% (wt/vol.) BSA, 7.5% (wt/vol.) Inutest (Laevosan-Gesellschaft, Linz/Donau, Austria) and 1.5% (wt/vol.) para-aminohippuric acid (Merck Sharp Dohme, West Point, PA, USA) to enable calculation of GFR and effective renal plasma flow (RPF). The left femoral artery was catheterised with PE50 filled with heparinised saline (100 U/ml) to allow blood sampling and continuous arterial pressure measurements. An adjustable clamp was placed on the aorta above the left renal artery to control left renal arterial pressure. We used GAP peptides because they interrupt gap junctions through mimicry of extracellular sequences for specific Cxs as the decoy. The left adrenal artery was cannulated with extended PE10 to infuse heparinised saline or GAP peptides (5 mg administered intra-arterially, and followed by 0.2 mg/min) at a rate of 0.6 ml/h [30, 31], and the solution for transjugular infusion was adjusted to 2% (wt/vol.) BSA and infused at a rate of 0.6 ml/h to make water load similar. After completion of surgery, 1 h of equilibration was allowed before initiating experimental protocols.

To test whether Cxs play a role in renal autoregulation, the effects of GAP27 for Cx37,43 ( $\text{Cx}^{37,43}\text{GAP27}$ ), GAP27 for Cx40 ( $\text{Cx}^{40}\text{GAP27}$ ) and GAP26 for Cx43 ( $\text{Cx}^{43}\text{GAP26}$ )

(Severn Biotech, Worcester, UK) were examined. This series of studies was performed on ZL and ZDF rats (six rats per group). Two consecutive 20 min control clearances were carried out. The aortic clamp was tightened to reduce renal arterial pressure by approximately 20 mmHg before initiating two consecutive 20 min clearance periods. Subsequently, the aortic clamp was released. Saline was exchanged by infusion for GAP peptide, which was infused into the adrenal artery throughout the remaining experimental periods. Since our previous studies have shown that  $\text{Cx}^{37,43}\text{GAP27}$  or  $\text{Cx}^{40}\text{GAP27}$  induced an increase in blood pressure [12], the aortic clamp was slightly tightened to prevent this increase and hence maintain renal arterial pressure at the control level. To obtain maximal effects of GAP peptides [11], at least another 60 min equilibration period was allowed before initiating two consecutive 20 min clearance periods. Subsequently, the aortic clamp was further tightened to reduce renal arterial pressure by approximately 20 mmHg and two consecutive 20 min clearance studies were carried out.

#### Renin–angiotensin

In additional experiments, plasma renin activity was measured separately in eight groups of rats (ZDF and ZL rats in the absence or presence of three GAP peptides,  $n=4$ –5 per group). Surgical procedures were the same as the above, except that the ureter was not cannulated. After 1 h of equilibration, GAP peptides were infused into the adrenal artery as detailed above. In the case of  $\text{Cx}^{37,43}\text{GAP27}$  or  $\text{Cx}^{40}\text{GAP27}$ , the aortic clamp was used to maintain renal arterial pressure at baseline level. After 1 h, 2 ml blood sample was taken from the femoral artery in chilled tubes containing EDTA. Plasma was kept deep-frozen until the assay [11, 29].

#### Statistics

Data are expressed as means  $\pm$  SEM. Statistical analysis was performed using ANOVA and Student's *t* test with or without Bonferroni's correction for multiple comparisons when appropriate. We used  $\chi^2$  tests for analysis of renin abundance in efferent arterioles and for identification of multiple groups of renin-secreting cells along the afferent arterioles. A value of  $p<0.05$  was considered statistically significant.

## Results

#### General animal condition

ZDF rats had higher body weight ( $n=18$  for each group) and plasma glucose than ZL rats ( $p<0.05$ ) (Table 1)

**Table 1** Animal conditions and baseline renal haemodynamics

Characteristic	ZL	ZDF
Body weight (g)	272±4	364±5*
Kidney weight (g)	0.96±0.02	1.19±0.03*
Mean BP (mmHg)	98±1	100±2
Plasma glucose (mmol/l)	4.9±0.2	12.8±0.9*
RPF (ml min <sup>-1</sup> [g kidney wt] <sup>-1</sup> )	3.51±0.07	3.85±0.06*
GFR (ml min <sup>-1</sup> [g kidney wt] <sup>-1</sup> )	0.94±0.03	1.15±0.03*
Urine volume (μl/min)	7±1	19±2*

Values are mean±SEM

\* $p<0.05$

although they were not fed with Purina 5008 (Purina Mills, Richmond, IN, USA). ZDF rats had diabetes at the time of the experiments although blood pressure was not elevated. Kidney weight in ZDF rats was also greater than in ZL rats ( $p<0.01$ ). Neither of the rat groups had any evidence of hydronephrosis at this age. Packed cell volume was similar between ZL (48±1%) and ZDF rats (49±1%). ZDF rats excreted more albumin (190±18 μg/day) than ZL rats (15±3 μg/day;  $p<0.01$ ). Creatinine clearance (2.70±0.19 vs 1.86±0.16 ml/min;  $p<0.01$ ) and 8-epi-prostaglandin F2α excretion (2.2±0.2 vs 1.5±0.2 ng/day;  $p<0.05$ ) were elevated in ZDF rats. Urine flow, RPF and GFR were significantly higher in ZDF than ZL rats (Table 1).

#### Western blotting

The levels of Cx37, Cx40 and Cx43 did not differ between the kidneys of ZDF and ZL rats at the age examined (Fig. 1a–c). However, a significant higher amount of Cx43 was phosphorylated in ZDF rats (Fig. 1d). We were not able to perform similar analysis on phosphorylated Cx37 and Cx40, as specific antibodies for each were not available.

#### Immunohistochemistry

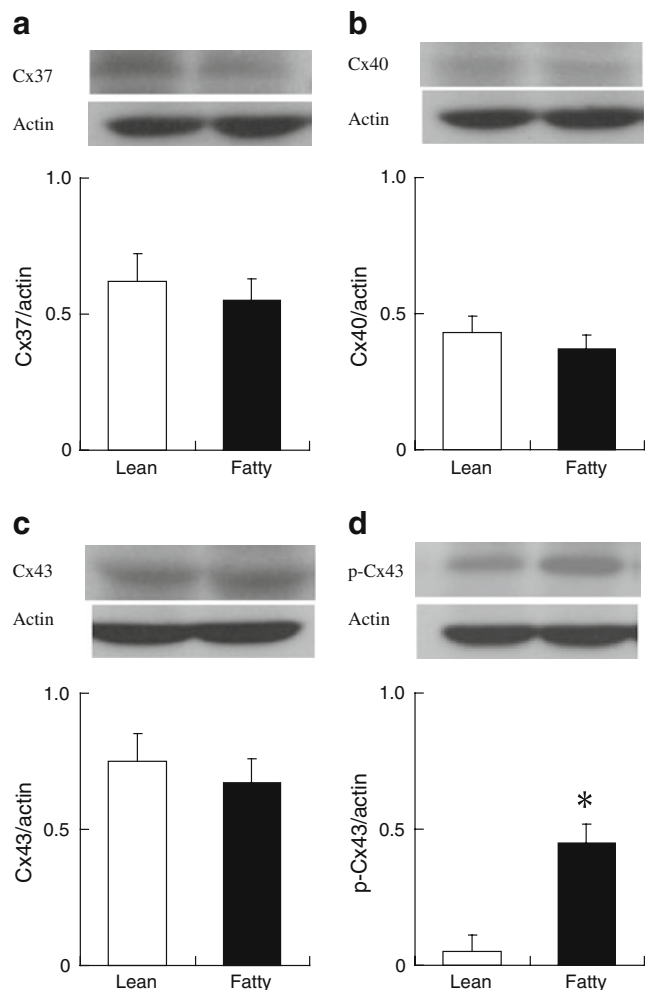
**ZL rats** Cx37 was strongly produced in the endothelium of the afferent arterioles and larger arteries, and weakly produced in the efferent arterioles. In the glomerulus, Cx37 was found only at the vascular pole (ESM Fig. 1). Labelling for renin demonstrated that Cx37 was also produced in renin-secreting cells, but not in extraglomerular mesangial cells (Fig. 2a).

Cx40 was similarly strongly produced in the endothelium of the afferent arterioles and larger arteries, and in renin-secreting cells (ESM Fig. 1). In contrast to Cx37, Cx40 was present throughout the glomerulus and in the extraglomerular mesangium, but was not found in the efferent arterioles (Fig. 2b).

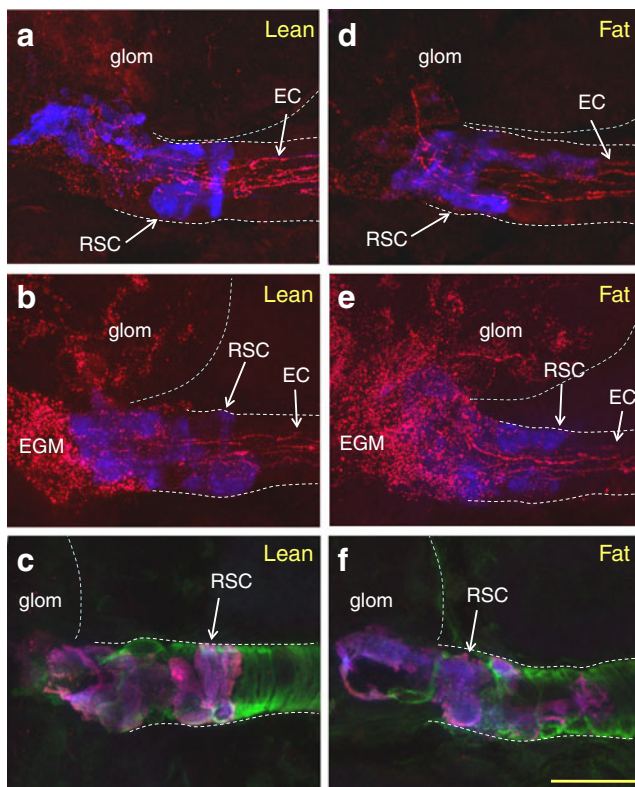
Cx43 was produced weakly in the endothelium of the afferent arterioles and throughout the glomerulus. In renin-secreting cells, Cx43 staining colocalised with the intracellular staining for renin, but was not located in a punctate form in the cell membrane (Fig. 2c).

In addition to the renin-secreting cells located along the afferent arterioles at the entrance to the glomerulus, renin-secreting cells were also found at sites more distant from the glomerulus in some nephrons (ESM Fig. 1). Renin staining was also found surrounding the exit of some efferent arterioles.

**ZDF rats** When compared with the staining in ZL rats, the abundance patterns for Cx37, Cx40 and Cx43 were similar in the nephrons of ZDF rats (Fig. 2d–f), although the density of Cx37 staining in renin-secreting cells appeared to be reduced (Fig. 2a, d). The abundance of renin in the terminal afferent arterioles also appeared to be reduced in



**Fig. 1** Western blot analysis of (a) Cx37, (b) Cx40, (c) Cx43 and (d) phosphorylated Cx43 (p-Cx43) in Zucker rat kidneys as labelled. \* $p<0.05$  for difference between ZL and ZDF rats



**Fig. 2** High power fields of (a, d) Cx37, (b, e) Cx40 and (c, f) Cx43 abundance in the juxtaglomerular apparatus of lean and fat Zucker rats. Renin-secreting cells (RSC) (blue) show punctate presence of Cx37 (a, d) and Cx40 (b, e). Cx40 was also prominent in the glomerulus (glom) and extraglomerular mesangium (EGM) in ZL (b) and ZDF rats (e). Cx43 was produced in the cytoplasm of the RSCs in ZL (c) and ZDF (f) rats. Scale bar 20  $\mu\text{m}$ . EC, endothelial cells; green, myosin

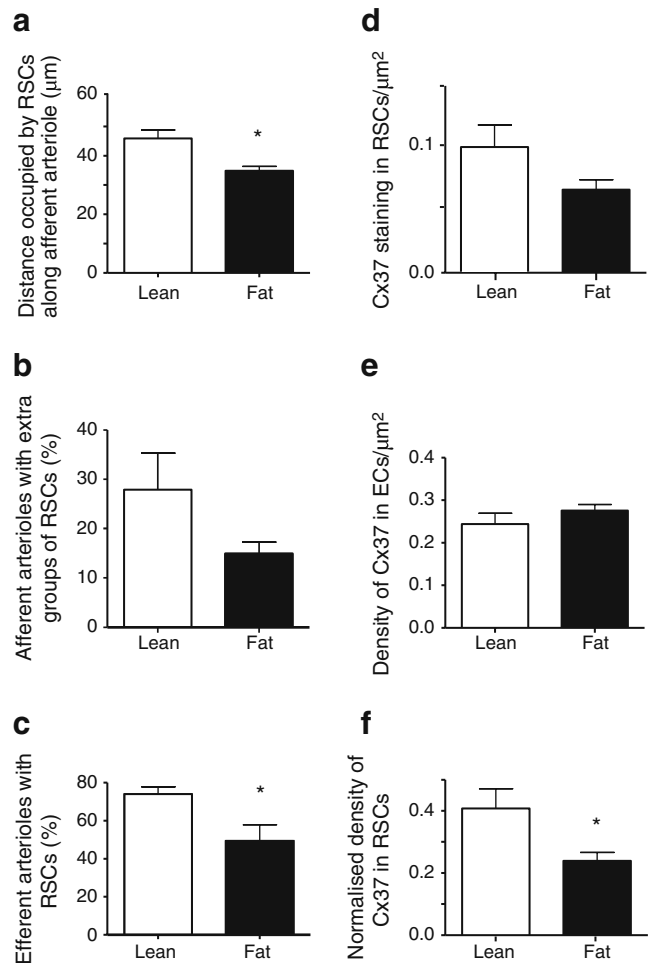
ZDF rats (Fig. 2a–f). Thus, the following morphological analysis was performed.

#### Morphological analysis

Measurement of the distance from the glomerulus of renin-secreting cells along the afferent arteriole showed that this was significantly shorter in the afferent arterioles from ZDF rats than in those from ZL rats ( $p < 0.05$ ) (Fig. 3a). Furthermore, the percentage of afferent arterioles with additional groups of renin-secreting cells along the afferent arterioles tended to be lower in the arterioles of ZDF rats than in those of ZL rats, although this did not reach statistical significance ( $p = 0.06$ ) (Fig. 3b). However, the percentage of efferent arterioles with renin-secreting cells around their origin from the glomerulus was significantly lower in ZDF rats than in ZL rats ( $p < 0.05$ ) (Fig. 3c).

While it was not possible to quantify the density of Cx40 staining in renin-secreting cells due to potential overlap with extraglomerular mesangial cells, this was done for

Cx37, where no production was detected in the extraglomerular mesangium. Expressed as positive staining above background per unit area, levels of Cx37 tended to be reduced in the renin-secreting cells of ZDF compared with ZL rats (Fig. 3d). To normalise for variation in staining between different staining runs, a region of endothelial staining removed from the renin-secreting cells was quantified. While content of Cx37 in the endothelial cells



**Fig. 3** Quantification of distribution and Cx37 abundance in renin-secreting cells. **a** The distance from the glomerulus of renin-secreting cells (RSC) along the afferent arterioles was significantly smaller in ZDF than ZL rats. Arterioles ZL  $n = 39$ , ZDF  $n = 37$ . **b** The percentage of afferent arterioles exhibiting more than one group of renin-secreting cells tended to decrease in ZDF rats (71 arterioles in four ZL rats, 70 arterioles in four ZDF rats). **c** The percentage of efferent arterioles with renin-secreting cells around their origin was significantly lower in ZDF than in ZL rats (44 arterioles in four ZL rats, 52 arterioles in four ZDF rats). **d** The density of Cx37 staining in renin-secreting cells tended to decrease in ZDF compared with ZL rats. **e** The density of Cx37 staining was similar in the endothelium (EC) of afferent arterioles of ZL vs ZDF rats. **f** The density of Cx37 staining, normalised to endothelial staining (e), was significantly less in ZDF than in ZL rats. **d–f** Arterioles ZL  $n = 11$  in four rats, ZDF  $n = 17$  in four rats.  $*p < 0.05$

of the afferent arterioles of ZL and ZDF rats was not significantly different (Fig. 3e), the density of abundance of Cx37 in the renin-secreting cells, normalised to this endothelial content, showed a significant reduction in ZDF compared with ZL rats (Fig. 3f).

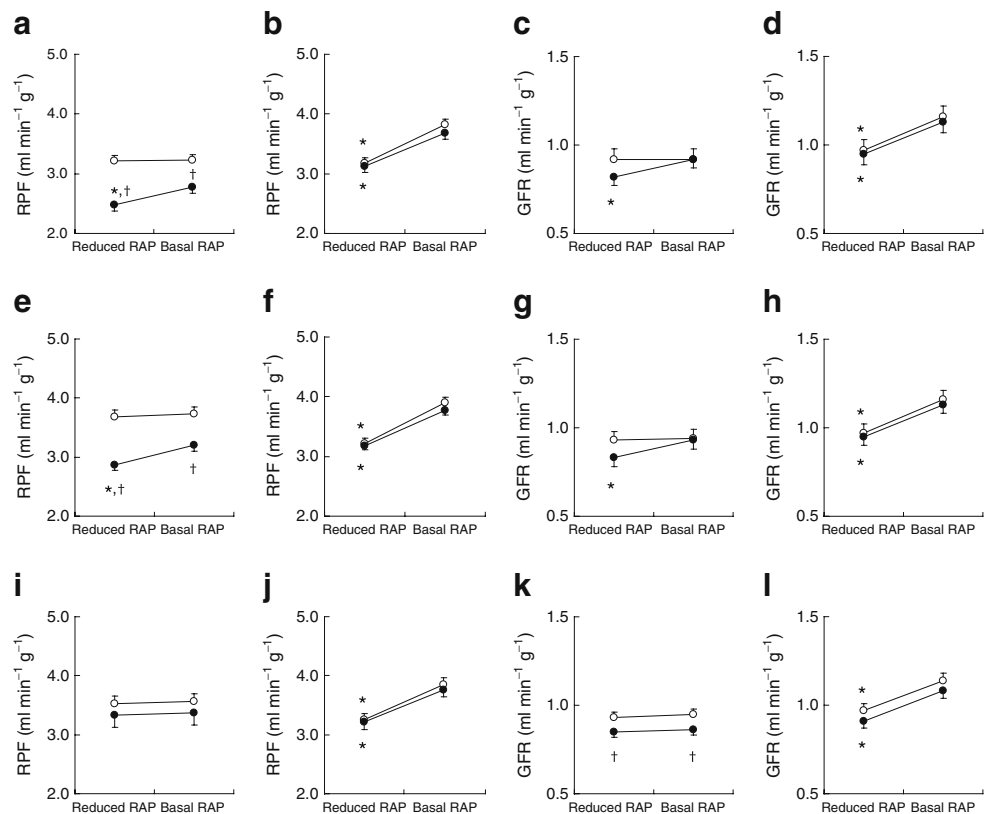
### Renal haemodynamics

In ZL rats, changes in mean arterial pressure (MAP) from  $98 \pm 2$  to  $82 \pm 2$  mmHg failed to alter RPF and GFR (Fig. 4a, c). During intra-renal infusion of  $Cx^{37,43}$ GAP27, alterations of MAP from  $98 \pm 2$  to  $79 \pm 1$  mmHg decreased RPF and GFR in ZL rats.  $Cx^{37,43}$ GAP27 elevated filtration fraction in ZL rats ( $28 \pm 2$  to  $33 \pm 2\%$ ;  $p < 0.05$ ). MAP was elevated to  $116 \pm 2$  mmHg ( $p < 0.01$ ) following full release of the aortic clamp. Autoregulatory indices for RPF ( $5 \pm 8$  to  $54 \pm 10\%$ ;  $p < 0.01$ ) and GFR ( $1 \pm 9$  to  $58 \pm 11\%$ ;  $p < 0.05$ ) were also increased by  $Cx^{37,43}$ GAP27 in ZL rats. Autoregulatory efficiency was already impaired in ZDF rats under basal conditions (Fig. 4b, d). The reduction of MAP from  $100 \pm 2$  to  $82 \pm 2$  mmHg markedly decreased RPF and GFR in these rats. However, subsequent administration of  $Cx^{37,43}$ GAP27 did not induce further decrements in autoregulation of ZDF rats, while alterations of MAP from  $98 \pm 2$  to  $80 \pm 1$  mmHg decreased RPF and GFR in this rat group.

Under control conditions, RPF and GFR were autoregulated in the range of MAP from  $101 \pm 2$  to  $82 \pm 1$  mmHg in ZL rats (Fig. 4e, g). Following intra-renal infusion of  $Cx^{40}$ GAP27, autoregulation of RPF and GFR was reduced to between  $98 \pm 2$  and  $80 \pm 1$  mmHg in ZL rats. Subsequent release of the aortic clamp increased MAP to  $112 \pm 2$  mmHg ( $p < 0.01$ ). Thus,  $Cx^{40}$ GAP27 increased filtration fraction from  $25 \pm 1$  to  $29 \pm 1\%$  ( $p < 0.05$ ) and autoregulatory indices of RPF ( $4 \pm 10$  to  $54 \pm 13\%$ ;  $p < 0.05$ ) and GFR ( $1 \pm 6$  to  $54 \pm 8\%$ ;  $p < 0.01$ ) in ZL rats. In ZDF rats, however, the reduction of MAP from  $102 \pm 2$  to  $82 \pm 2$  mmHg markedly decreased RPF and GFR even under basal conditions (Fig. 4f, h). Subsequent administration of  $Cx^{40}$ GAP27 did not induce further decrements in autoregulation of ZDF rats. In the presence of  $Cx^{40}$ GAP27, the reduction of MAP from  $98 \pm 2$  to  $80 \pm 2$  mmHg reduced RPF and GFR in ZDF rats.

As shown in Fig. 4i, k, RPF and GFR were well autoregulated in the range of MAP from  $101 \pm 2$  to  $80 \pm 1$  mmHg in ZL rats. Following intra-renal infusion of  $Cx^{43}$ GAP26, autoregulation of RPF and GFR was still preserved between  $98 \pm 1$  and  $80 \pm 1$  mmHg. Thus,  $Cx^{43}$ GAP26 decreased GFR ( $p < 0.05$ ), but did not alter autoregulatory indices for RPF and GFR in ZL rats. There was no significant change in MAP after release of the aortic clamp. In ZDF rats, reduction of MAP, under basal

**Fig. 4** GAP effects on autoregulation of RPF (a, b, e, f, i, j) and GFR (c, d, g, h, k, l) by kidney weight (in g) in ZL and ZDF rats. a–d Effects of  $Cx^{37,43}$ GAP27, (e–h)  $Cx^{40}$ GAP27 and (i–l)  $Cx^{43}$ GAP26. White circles, control; black circles treated with the respective GAP peptide. \* $p < 0.05$  and † $p < 0.05$  for difference within each group and between groups, respectively. RAP, renal arterial pressure



conditions, from  $99 \pm 2$  to  $79 \pm 2$  mmHg altered PRF and GFR (Fig. 4j, l). The administration of  $Cx43$ GAP26 to ZDF rats resulted in decrements of RPF and GFR in response to a decrease in MAP from  $99 \pm 1$  to  $80 \pm 1$  mmHg.  $Cx43$ GAP26 failed to alter GFR in ZDF rats.

### Renin–angiotensin

As shown in Fig. 5, plasma renin activity was similar between ZDF rats ( $14 \pm 2$  ng ml<sup>-1</sup> h<sup>-1</sup>) and ZL rats ( $12 \pm 2$  ng ml<sup>-1</sup> h<sup>-1</sup>) under control conditions. In ZL rats,  $Cx37,43$ GAP27 ( $28 \pm 3$  ng ml<sup>-1</sup> h<sup>-1</sup>;  $p < 0.05$ ) and  $Cx40$ GAP27 ( $25 \pm 3$  ng ml<sup>-1</sup> h<sup>-1</sup>;  $p < 0.05$ ) increased plasma renin activity, whereas  $Cx43$ GAP26 did not. However, these trends were strikingly weakened in ZDF rats. Although  $Cx40$ GAP27 tended to increase plasma renin activity, no GAP peptide induced any significant alteration in plasma renin activity in ZDF rats (by ANOVA).

### Discussion

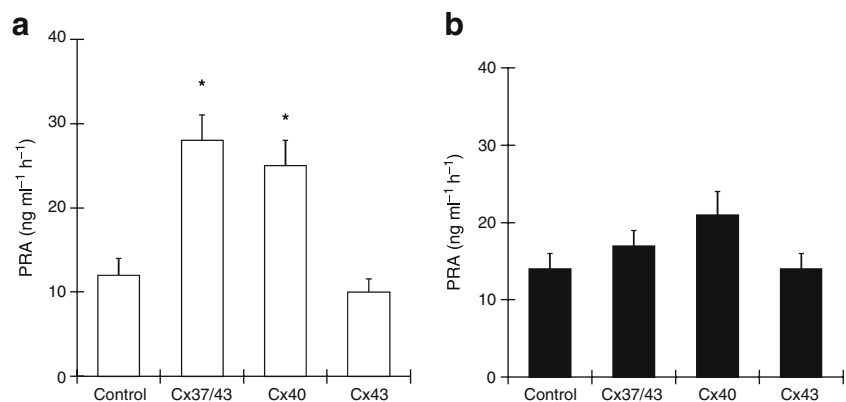
Diabetes in ZDF rats resembles type 2 diabetes in humans, with animals becoming obese and acquiring diabetes with hyperinsulinaemia as they grow. At 8 weeks old, ZDF rats already had diabetes when fed with Quick Fat diet. The results may vary in long-lasting or advanced diabetes. Caution is required when extrapolating the present findings to clinical situations. In addition, we did not observe the hydronephrotic changes previously reported in 10-week-old ZDF rats [32]. We did, however, observe glomerular hyperfiltration, a characteristic of early diabetic nephropathy, in conscious and anaesthetised ZDF rats. Moreover, while blood pressure was similar between the two strains in the present study, 8-epi-prostaglandin F2 $\alpha$  excretion was increased in ZDF rats, suggesting that oxidative stress is higher. Metabolic memory including advanced glycation endproducts and epigenetic alterations could be important,

too [33]. Thus, ZDF rats are an appropriate animal model for investigating mechanisms in the development of diabetic nephropathy.

An increase in salt transport at the macula densa initiates TGF $\beta$  signals that constrict the afferent arteriole [34]. Thomson et al. have proposed the tubular hypothesis for diabetic glomerular hyperfiltration [19]. Hyperglycaemia elicits a large increase in proximal tubular reabsorption associated with glucose reuptake, thereby reducing salt delivery to the macula densa. Thus, hyperglycaemia by itself would reduce afferent arteriolar tone by attenuating TGF $\beta$  tone [20]. Previous findings have indicated that most of autoregulatory tone comes from TGF $\beta$  in the range of arterial pressure between 100 and 80 mmHg under our experimental condition [5, 11, 12]. Consistent with the above hypothesis, the present data indicate that renal autoregulation was markedly impaired in ZDF rats at spontaneous levels of mean BP, reducing preglomerular vascular tone and participating in glomerular hyperfiltration. However, our results seem different from those of Griffin et al. [35], who found that renal autoregulation was preserved in Zucker spontaneously hypertensive rats, an offspring from the breeding of Zucker obese and spontaneously hypertensive rats. Genetic differences between ZL and ZDF rats, such as differences in leptin receptors, might cause the autoregulatory curve to shift toward higher pressure in ZDF rats. In contrast, Brännström et al. [36] report that TGF $\beta$  is enhanced in spontaneously hypertensive rats. Diverse strains in an animal model of diabetes could account for differing results. Alternatively, our preliminary data suggest that a high-salt diet only partially improves renal autoregulation in ZDF rats with restored distal delivery (T. Takenaka, T. Inoue, T. Miyazaki and H. Suzuki, unpublished observations). Collectively, these data suggest that TGF $\beta$  signal transduction is also impaired in ZDF rats.

Our recent investigations on Wistar rats demonstrated that Cx43 is produced in glomerular podocytes, as well as

**Fig. 5** GAP peptides alter plasma renin activity (PRA) in (a) ZL and (b) ZDF fatty rats. Cx37/43, Cx40 and Cx43 show experimental groups treated with  $Cx37,43$ GAP27,  $Cx40$ GAP27 and  $Cx43$ GAP26. \* $p < 0.05$  for difference from the control



in afferent arteriolar endothelial cells, and that  $Cx43$ GAP26 reduced GFR without affecting renal autoregulation [11]. Our data obtained here from ZL rats show comparable trends, supporting the notion that inhibition of Cx43 elicits preglomerular vasoconstriction. Also in the present study, blockade of Cx43 did not have any significant effects on GFR in ZDF rats, although western blotting showed a significant increase in phosphorylation of Cx43 in ZDF rat kidney. This phosphorylation of Cx43, like that induced by high glucose in vascular smooth muscle cells, would interfere with gap junction expression, distribution, degradation and function [15, 17, 37]. Indeed, Satriano et al. [38] have reported reduced levels of Cx43 in kidney from a streptozotocin-induced rat model of type 1 diabetes. Sawai et al. [39] noted that Cx43 abundance was distributed non-uniformly in human glomerular podocytes of type 2 diabetes patients and that these abnormalities in Cx predict poor renal prognosis. In the present study, ZDF rats excreted more albumin than ZL rats. Taken together, these results suggest that Cx43 phosphorylation may lead to the malfunction of gap junctions, consisting of Cx43 in podocytes as well as endothelial cells, that are presumably involved in albuminuria in diabetes.

On the other hand, our recent data revealed that Cx37 and Cx40 are localised in glomerular arteriolar endothelial cells and juxtaglomerular granular cells in control rats [11], and are involved in purinergic TGF $\beta$ back signal transduction [12]. The present findings that  $Cx40$ GAP27 and  $Cx37,43$ GAP27, but not  $Cx43$ GAP26 diminished renal autoregulation in ZL rats support the idea that gap junctions comprising Cx37 and Cx40 transduce TGF $\beta$ back signals. Although our immunohistochemical studies did not reveal differences in Cx40 content between ZL and ZDF rats, we did demonstrate that the density of abundance of Cx37 in renin-secreting cells was decreased in ZDF rats, at least in part accounting for abnormal renal autoregulation in these animals. In addition, hyperglycaemia by itself or oxidative stress activates protein kinase C and mitogen-activated protein kinase, both of which could phosphorylate Cxs [40–42]. In the present study, plasma glucose and 8-epi-prostaglandin F $2\alpha$  excretion, an indicator of oxidative stress, were higher in ZDF rats. Thus it is likely that Cx37 and Cx40 are also phosphorylated in ZDF rats, similarly to Cx43. Interestingly, phosphorylated Cxs are prone to be ubiquitinated to degrade [37]. Reductions of Cx40 and weakened effects of endothelium-derived hyperpolarising factor (EDHF) have been demonstrated in the mesenteric artery of Zucker obese rats [43]. Cxs play an important role in myogenic response [44], one of the mechanisms mediating renal autoregulation [45]. The present results raise the possibility that myogenic response is also attenuated in diabetes. In combination with reduced abundance and enhanced phosphorylation of Cxs, the finding that  $Cx40$ GAP27 and  $Cx37,43$ GAP27 failed to

induce further changes in renal autoregulation in ZDF rats constitutes novel evidence that gap junctions composed of Cx37 and Cx40 were already malfunctioning in ZDF rats, suggesting that Cxs form a link between biochemical alterations and pathophysiological derangements in diabetic nephropathy.

Angiotensin II has been considered a positive modulator of TGF $\beta$ back [7, 33, 46]. We have currently provided evidence that TGF $\beta$ back is diminished in ZDF rats, suggesting that low adenosine prevails in juxtaglomerular apparatus [47]. The present finding that plasma renin activity was similar between ZDF and ZL rats may account at least in part for the acute effects of angiotensin inhibition on glomerular hypertension in diabetes [48] and make it unlikely that renin–angiotensin system played a role in deteriorated TGF $\beta$ back in ZDF rats. Cx40-deficient mice lack the pressure control of renin release and TGF $\beta$ back contribution to renal blood flow autoregulation [10, 49]. Cx37 and Cx40 are involved in control of renin release and TGF $\beta$ back in Wistar rats [11, 12]. In the present study, while  $Cx40$ GAP27 and  $Cx37,43$ GAP27 increased plasma renin activity and filtration fraction in ZL rats, they exerted insignificant effects in ZDF rat. GAP peptides induce renal vasoconstriction also by inhibiting EDHF actions [31]. Consequently, the present finding that intra-renal infusion of  $Cx40$ GAP27 or  $Cx37,43$ GAP27 failed to elicit changes in the renin–angiotensin system in ZDF rats complement the notion that diabetes modifies the communication through gap junctions composed of Cx37 and Cx40, impairing signal transduction within the juxtaglomerular apparatus.

Abnormal Cxs in diabetes may cause redistribution of renin-secreting cells in the kidney. Kurtz et al. performed an elegant study showing that renin-positive cells are absent in the vessel wall and found in extraglomerular mesangium, glomerular tuft and periglomerular interstitium in Cx40-deficient mice [8]. In the present study, the distance from the glomerulus of renin-secreting cells along the afferent arteriole in ZDF rats was shorter than in ZL rats, but both strains exhibited scanty renin staining in extraglomerular mesangium. Furthermore, our data demonstrate that ectopic renin staining in efferent arterioles was more frequent in ZL rats than ZDF rats. Acquired modification of Cxs and gap junction dysfunction may produce consequences different from congenital Cx deletion. We previously reported that Cx37 content was accentuated in diabetic mice kidney [50]. Thus, species difference and diabetes seem to be involved [14]. Further studies are clearly required to elucidate how diabetes alters Cx37 initially in rat renin-secreting cells.

In summary, the present findings indicate that ZDF rats manifested characteristics of diabetes including hyperglycaemia and enhanced oxidative stress. Our results also demonstrate the derangement of Cxs in ZDF rats, which could result in malfunction of gap junctions in juxtaglo-



merular apparatus. Finally, the present data suggest that impairment of gap junction plays a role in altered renal autoregulation in diabetes.

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