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Differential connexin expression in preglomerular and postglomerular vasculature: Accentuation during diabetes

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Differential connexin expression in preglomerular and postglomerular vasculature: Accentuation during diabetes.

Background. Gap junctions may play an important role in regulating renal blood flow and glomerular responses. We have therefore made a comprehensive analysis of connexin expression in the renal vasculature of control and diabetic mice since elevated glucose has been reported to down-regulate connexin 43 in vascular cells in vitro.

Methods. Connexin distribution was determined with immunohistochemistry using subtype-specific and cell type–specific antibodies. Diabetes was induced with streptozotocin (120/80 mg/kg, intraperitoneally) in C57BL/6 mice.

Results. Connexins 37, 40, and 43 were expressed in endothelial cells of the renal, lobar, arcuate, and interlobular arteries and afferent arterioles, although connexin 43 was weak in the renal and arcuate arteries. Connexin 37 was detected in the media of arcuate, interlobular arteries and afferent arterioles and connexins 37 and 40 were found in renin-secreting cells. Both connexins 37 and 40 were expressed in extraglomerular mesangial cells, connexin 40 was abundantly expressed in intraglomerular mesangial cells, but connexin 37 was limited to mesangial cells at the vascular pole. In contrast, only connexin 43 was detected in endothelial cells of efferent arterioles and there was no connexin staining in the media. In diabetes, connexin 40 was expressed in smooth muscle cells along afferent arterioles, glomerular connexin staining was more extensive and connexin 43 was detected in renin-secreting cells. In contrast connexin 43 expression in endothelial cells of efferent arterioles was markedly reduced.

Conclusion. The renal vasculature and mesangial cells are well coupled on the preglomerular side but there is little evidence that the coupling extends into the efferent arteriole. This pattern of cell coupling is accentuated during diabetes.

Regulation of renal blood flow (RBF) and glomerular filtration rate (GFR) depend not only on renal resistance vessels, which include the arcuate and interlobular arteries, afferent and efferent arterioles, and descending vasa recta [1], but also on a feedback mechanism termed tubuloglomerular feedback response [2]. During the latter response, changes in salt concentration in the distal tubule are sensed by the macula densa cells and this produces vasoconstriction of the afferent arteriole leading to decreased glomerular flow and filtration rate [2]. While nitric oxide may be instrumental in the initiation of the tubuloglomerular feedback response [3], gap junctions are likely to play an important role in the transfer of information through the intervening mesangial cells to the afferent arteriole [4, 5]. In order to coordinate responses in adjacent nephrons, the vasoconstrictor response needs to be conducted upstream into and along interlobular arteries. Such conduction of vasomotor responses along renal vessels has been observed in the split hydronephrotic rat kidney [6] and coupling between neighboring nephrons has also been demonstrated in normal and hypertensive rats [7, 8]. Studies of the mechanisms underlying the conduction of vasomotor responses in other vascular systems have provided strong evidence in favor of a crucial role for electric coupling through gap junctions in the endothelium and smooth muscle in these phenomena [9–11].

At the ultrastructural level, gap junctions have been shown to exist between endothelial cells, smooth muscle cells, and between the two cellular layers in interlobular arteries and afferent arterioles [12, 13].Gap junctions have also been found among and between intraglomerular mesangial cells and extraglomerular mesangial cells [14–16] and to form in vitro between renin-secreting cells, smooth muscle, and endothelial cells [17]. Accordingly, nine connexin subtypes are reported to be expressed at the mRNA level: connexins 26, 30.3, 31, 32, 37, 40, 43, 45, and 46 [18, 19]. However, a comprehensive analysis of connexin protein expression, other than connexin 40 [20–22], has not been made in the renal vasculature. Furthermore, no reports are available regarding changes in connexin expression during diabetes mellitus, although inhibition of gap junctional intercellular communication and down-regulation of connexin 43 expression by high glucose has been described in cultured microvascular endothelial cells and aortic smooth muscle cells [23, 24]. The aim of the present study was therefore to investigate the expression of the vascular connexin proteins, connexins

Key words: renal vasculature, connexin protein expression, gap junction.

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37, 40, and 43 in the renal vasculature and juxtaglomerular apparatus of wild-type C57BL/6 mice and to study whether any changes occur to connexin expression during diabetes.

METHODS

Tissue preparation

All procedures were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purpose, under a protocol approved by the Animal Experimentation Ethics Committee of the Australian National University.

Male C57BL/6 mice aged 10 to 16 weeks (N = 20) were deeply anaesthetized (5 mg/kg xylazine and 25 mg/kg ketamine, intraperitoneally). For cryosections, fresh kidneys were removed into dissecting buffer containing (mmol/L): 3 3-N-morpholinopropanesulfonic acid (MOPS); 1.2 NaH_2PO_4; 145 NaCl; 4.7 KCl; 2 $CaCl_2$; 1.2 $MgSO_4$; 2 pyruvate; 0.02 ethylenediaminetetraacetic acid (EDTA) (sodium); 4.6 glucose; and 0.15 albumin. The kidney capsule was removed and the kidney sliced transversely into 1 mm blocks and fixed in cold acetone at 4°C for 30 minutes. This fixation regime produced optimal staining for all three antibodies used in this study. Tissue blocks were then washed with phosphate-buffered saline (PBS) (2 × 15 minutes), immersed in 30% sucrose in PBS at 4°C overnight, and embedded in Cryo-M-Bed (Bright Instrument Company, Cambridge, UK). Coronal sections were cut (10 μm and 30 μm), mounted on slides coated with silane (2%, Sigma-Aldrich, Sydney, NSW, Australia), dried for 30 minutes over phosphorus pentoxide, and stored frozen.

For whole-mount preparations of larger renal arteries, including renal, lobar, and arcuate arteries, mice were perfused with normal saline containing 0.1% bovine serum albumin (BSA)/0.1% $NaNO_2$ via the left ventricle at a pressure of 60 mm Hg to maximally dilate blood vessels. Dilated blood vessels were then fixed in situ by perfusion with 2% paraformaldehyde in 0.1 mol/L PBS for 10 minutes. Kidneys with attached renal arteries were removed into PBS. Renal arterial trees were carefully dissected up to the interlobular arteries, pinned in a Sylgard (Dow Corning, Midland, MI, USA) coated Petri dish and the renal, lobar, and arcuate arteries were cut open longitudinally.

Immunohistochemistry

Frozen slides were brought to room temperature and all the following procedures were done at room temperature. Tissues were preincubated for 30 minutes in a blocking solution containing 2% BSA (or 2% of the serum of the secondary antibody) and 0.2% Triton in PBS, followed by 48 hours incubation in sheep antirat connexin 37 and connexin 40 [25] (1:250), rabbit antirat connexin 43 (Zymed Laboratories, Inc., San Francisco, CA, USA) (1:250 for cryosections and 1:100 for whole mounts of renal, lobar, and arcuate arteries) in the same solution in a humidified chamber. These three antibodies have been previously tested for specificity against the relevant connexin subtype using Western blotting and immunohistochemistry of COS-7 cells transfected with plasmid DNA encoding either connexins 37, 40, and 43 [26]. Tissues were rinsed sequentially with normal PBS for 10 minutes, high-salt PBS (450 mmol/L NaCl in PBS) for 5 minutes, and normal PBS (2 × 15 minutes), then incubated in Cy3-conjugated antigoat (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) (1:600) or antirabbit (Jackson ImmunoResearch Laboratories, Inc.) (1:100) antibodies in 0.3% Triton in PBS for 2 hours.

In order to determine the cellular site of connexin expression, sections were double labeled with either rabbit antimyosin (U. Gröschel-Stewart, Technische Universitat, Darmstadt, Germany) (1:250) for 1 hour, or rabbit antihuman von Willebrand factor (anti-VWF) (Dako, Glostrup, Denmark) (1:300) for 1 hour, or rabbit antineuronal nitric oxide synthase (anti-nNOS) (Zymed Laboratories) (1:100) overnight to label vascular smooth muscle cells, endothelial cells, and macula densa cells, respectively. Subsequent detection was achieved with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit (Dako) (1:40) antibody in 0.3% Triton in PBS for 1 hour. Tissues were mounted in buffered glycerol and viewed with a confocal laser scanning microscope and Argon/Kryton laser (TCS 4D) (Leica Instruments, Vienna, Austria). Series of images were collected in the Z axis at 1 μm intervals to encompass entire blood vessels and recombined to a single image. For double-labeled sections, either single images or image series were combined in two colors using Adobe Photoshop as indicated. For comparisons of staining between diabetic and control mice, sections were mounted on the same slides and for any one connexin antibody, comparative images were obtained using the same pinhole and voltage settings. In order to determine the extent of nonspecific staining, tissue sections were treated with solutions from which the primary antibody was omitted or solutions containing primary antibody which had been preincubated for 1 hour at room temperature with the appropriate antigenic peptide.

Morphologic identification and analysis

Afferent arterioles were distinguished from efferent arterioles by their larger size, connection to larger interlobular arteries, and absence of branching before entering the glomerulus [27]. Extraglomerular mesangial cells were identified by their position between afferent arterioles and efferent arterioles and close relationship to macula densa cells labeled with anti-nNOS [2]. Measurement of diameter of renal arterioles was based upon myosin staining.

Quantification of connexin staining was made on confocal images of the afferent arterioles (connexins 37, 40,

and 43), efferent arterioles (connexin 43), and glomeruli (connexin 37 and 40) using the software program, Analytic Imaging Station 3.0 (AIS, Imaging Research, St Catharines, Ontario, Canada) and the grain counting function in which a background level of staining could be defined. The amount of connexin staining was expressed as the percentage of the scan area occupied by staining, (i.e., levels greater than background). Scanned areas were defined by the edges of the arterioles or the glomeruli. Using this method, it was not possible to distinguish between endothelial staining and smooth muscle staining. In the case of analysis for connexin 40 within afferent arterioles, the staining in two different areas was measured. Afferent arteriole 1 refers to regions of afferent arterioles within 40 μm of the glomerulus, while afferent arteriole 2 refers to regions of afferent arterioles further than 40 μm from the glomerulus. In the case of analyses of afferent arterioles for connexin 37 and connexin 43, no distinction was made as preliminary data showed that staining in these two areas was indistinguishable.

All data were expressed as means ± SEM. Student *t* test was used to compare paired or unpaired data and statistical significance was set at $P < 0.05$.

Diabetes protocol

Wild-type adult male C57BL/6 mice (8 to 10 weeks old) were housed at room temperature with a 12-hour light/dark cycle and allowed free access to standard mice chow and drinking water. Diabetes was induced by intraperitoneal injections of streptozotocin [2-deoxy-(3-methyl-3-nitrosoureido)-D-glucopyranose] (Calbiochem, Darmstadt, Germany) in 0.2 mol/L acetate-buffered saline (pH 4.4) on two consecutive days (120 mg/kg body weight). After 48 hours, if diabetes had not developed, another two injections of streptozotocin (80 mg/kg body weight) were given over 2 days. Capillary blood glucose concentration was assessed using a Medisense blood glucose electrode sensor (Abbott Laboratories, Victoria, Doncaster, Australia). Diabetes was defined as a nonfasting capillary blood glucose concentration ≥18 mmol/L on 2 consecutive days. Gender- and age-matched C57BL/6 mice injected with acetate buffer only were used as control.

After establishment of diabetes, nonfasting capillary blood glucose concentration and body weight were monitored at least twice a week throughout the study period. If weight loss was greater than 1 mg/day, an intraperitoneal supplement of 0.9% saline was given. We defined 1 to 2 weeks, 4 to 6 weeks, and 8 to 10 weeks after establishment of diabetes as early, intermediate, and late stages of diabetes. At each diabetic stage, four control and four diabetic mice were deeply anaesthetized and their kidneys were processed for immunohistochemistry. The mice were killed by exsanguination.

RESULTS

Size difference in arteriolar diameter

The diameter of afferent arterioles from superficial glomeruli was significantly greater (16.81 ± 1.08 μm) ($N = 5$) ($P < 0.05$) than that of efferent arterioles (13.43 ± 1.11 μm) ($N = 5$). However, the diameter of afferent arterioles from juxtamedullary region was similar (17.62 ± 0.65 μm) ($N = 4$) ($P > 0.05$) to that of muscular efferent arterioles (16.73 ± 0.63 μm) ($N = 4$).

Connexin staining in control kidneys

No significant difference in Connexin staining was noted in any of the renal structures studied during the period of these experiments from 10 to 16 weeks of age in control mice.

Extra- and intrarenal arteries

In the endothelium of larger renal arteries, including the renal artery and lobar artery, bright, punctate staining for connexin 37 (Fig. 1A) and connexin 40 (Fig. 1B) was found while staining for connexin 43 was weak and irregular (Fig. 1C). None of the three connexins were detected in the smooth muscle cells of these arteries. The expression of connexins 37, 40, and 43 was similar in the endothelial cells of the arcuate and interlobular arteries, being intense for connexin 37 (Figs. 2A and 3A), and connexin 40 (Figs. 2B and 3B), and weak for connexin 43 (Figs. 2C and 3C). In contrast to the renal and lobar arteries, in the smooth muscle cells of both the arcuate and interlobular arteries, connexin 37 could be detected, although there was no evidence for connexins 40 or 43 (Figs. 2 and 3).

Juxtaglomerular apparatus

In the afferent arterioles, double-labeled with antimyosin, bright, punctate staining of connexin37 (Fig. 4A and B), connexin 40 (Fig. 4C and D), and connexin 43 (Fig. 4E and F) was detected along the endothelial cells. In each case staining was continuous to the entrance to the glomerulus (Fig. 5A, B, and E), underlying all of the smooth muscle staining. In contrast, neither connexin 37 (Fig. 4A) nor connexin 40 (Fig. 4C) was detected in the endothelium of the efferent arterioles. However, we consistently detected connexin 43 staining (Figs. 4E and 5F) in the endothelium of the efferent arterioles. This staining was equal to or slightly less extensive than that of the afferent arterioles (see Fig. 10C). Connexin 43 staining was also detected around the superficial cortical peritubular capillaries (Fig. 4E). No differences in connexin 37, connexin 40, and connexin 43 staining were detected between arterioles supplying the superficial glomeruli and the juxtamedullary glomeruli.

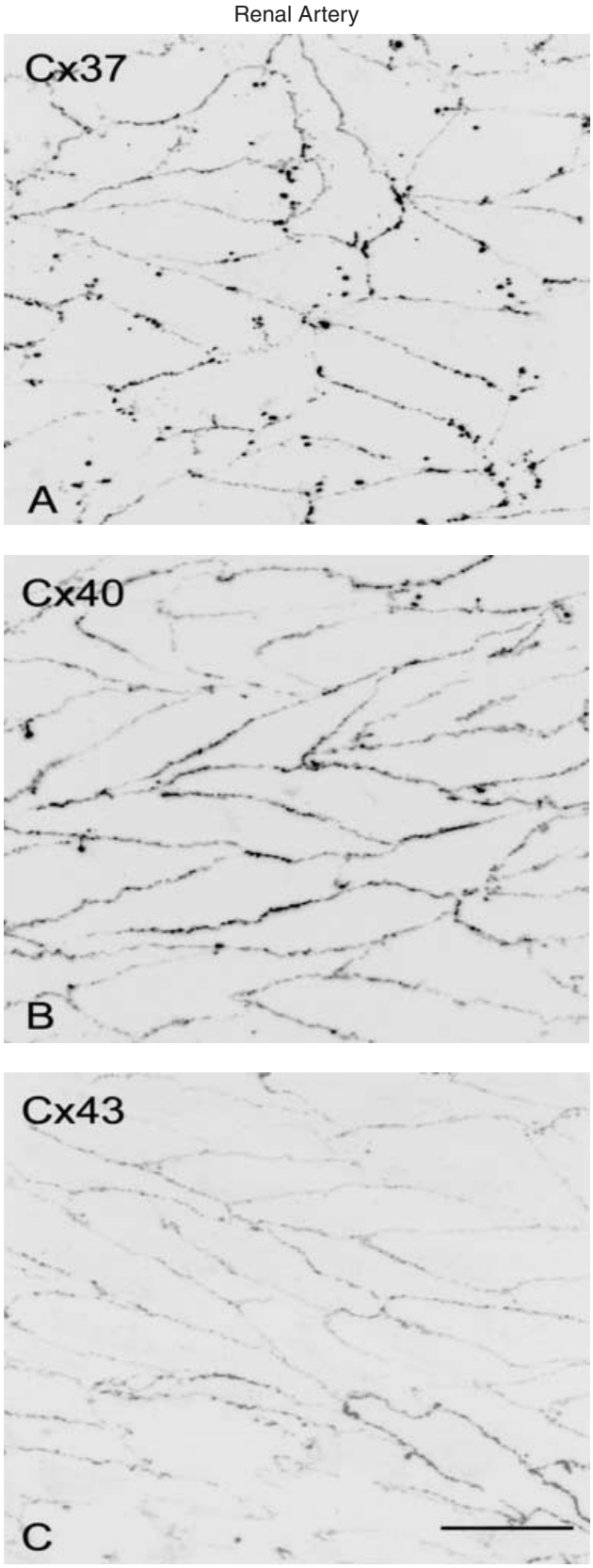

Fig. 1. Connexin (Cx) expression in the renal artery. Cx37 (*A*), Cx40 (*B*), and Cx43 (*C*) were found in the endothelium of renal artery. The longitudinal axis of the vessels runs left to right. No connexins were detected in the smooth muscle cells (scale bar represents 25 μm).

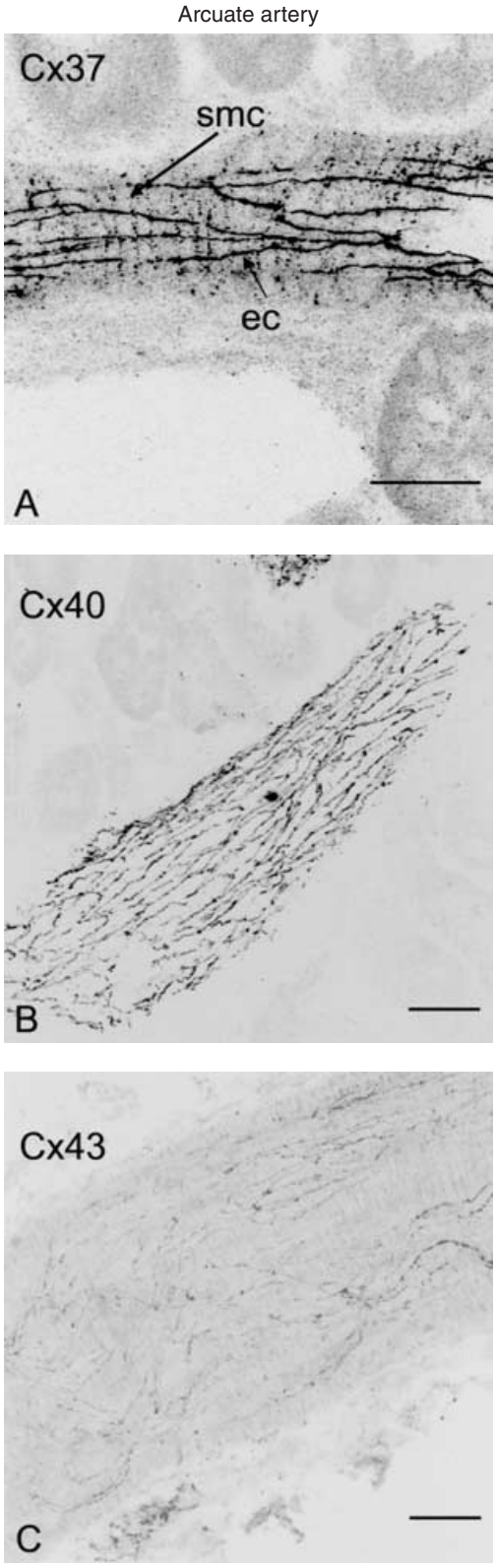

Fig. 2. Connexin (Cx) expression in the arcuate artery. Cx37 (*A*), Cx40 (*B*), and Cx43 (*C*) were found in the endothelium of the arcuate artery. The longitudinal axis of the vessels runs left to right. Connexin 37 was also expressed along the edges of the smooth muscle cells (A). In (C), the arcuate artery was cut open along its length. Abbreviations are: ec, endothelial cells; smc, smooth muscle cells [scale bar represents 25 μm in (A), 50 μm in (B and C)].

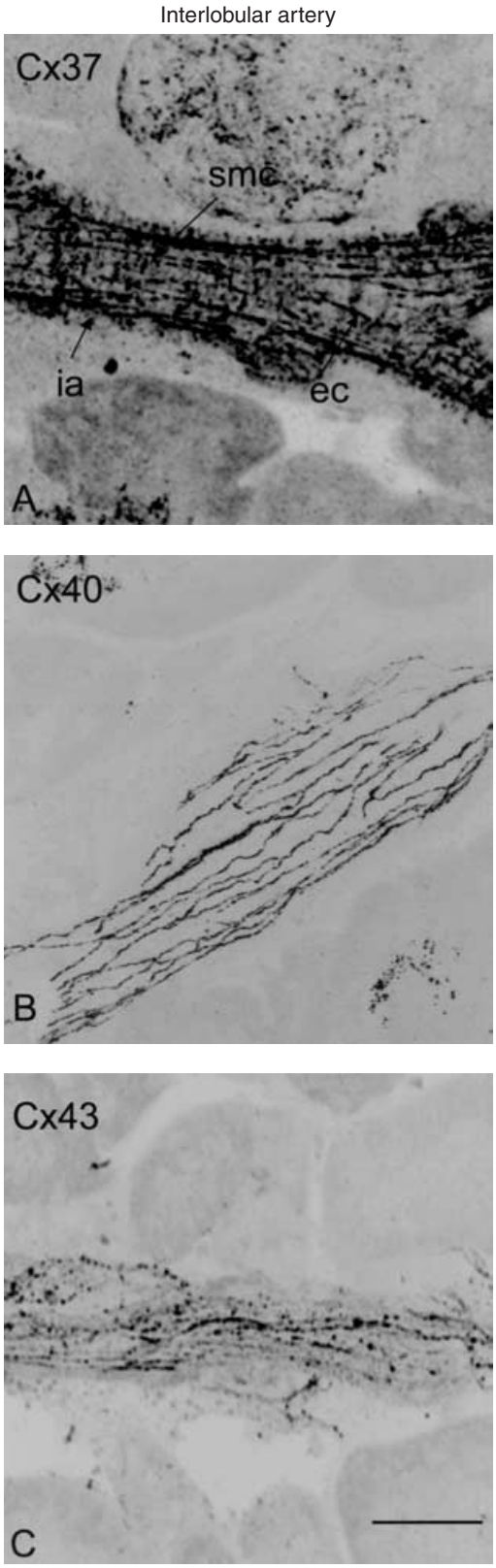

Fig. 3. Connexin (Cx) expression in the interlobular artery. Punctate staining of Cx37 was found in the endothelium and the smooth muscle cells (*A*), while Cx40 (*B*) and Cx43 (*C*) were restricted to the endothelium. The longitudinal axis of the vessels runs left to right. Abbreviations are: ec, endothelial cells; smc, smooth muscle cells; ia, interlobular artery (scale bar represents 25 μm).

In the smooth muscle cells, connexin 37 staining was detected in more than 50% of the afferent arterioles (26/48 of 31 mice) (Fig. 5C). In the section of the afferent arteriole adjacent to the glomerulus, the site of the renin-secreting cells of the juxtaglomerular apparatus, abundant connexin 37 (Fig. 5A) and connexin 40 (Fig. 5B and D) staining was detected in more than 98% of the afferent arterioles examined (52/53 afferent arterioles of 31 mice), while no connexin 43 (Fig. 5E) was ever detected. Connexin 40 was generally not found in the smooth muscle cells of the afferent arterioles, although in some preparations staining appeared to extend for up to 40 μm beyond the region occupied by the renin-secreting cells (Figs. 5D and 10B). Connexin 43 was never detected in the smooth muscle cells of afferent arterioles or renin-secreting cells (Fig. 5E). In the efferent arterioles, there was no evidence for any connexin 37, 40, or 43 expression in the smooth muscle (Figs. 4A and C and 5F). Connexin 43 staining was detected in occasional tubules within the cortex (Fig. 8E), while connexin 37 and connexin 40 were not detected in any of the tubular structures.

When the macula densa was double-labeled with anti-nNOS, extensive staining of connexin 37 (Fig. 6A) and connexin 40 (Fig. 6B) was observed in the adjacent extraglomerular mesangial cells. Staining of connexin 37 and connexin 40 in the extraglomerular mesangial cells extended into the glomeruli, forming a continuous staining pattern between the extraglomerular mesangial cells and the glomerulus (Fig. 6A to C). While connexin 37 expression was restricted to the vascular pole (Figs. 6A and 8A), connexin 40 was present throughout the glomerulus (Figs. 6B and C and 8C). When the afferent and efferent arterioles were labeled with antimyosin, intense connexin 40 staining could be found between them both outside and inside of the glomerulus (Fig. 6C). No myosin staining was found within the glomerulus (Fig. 6C) and no connexin 43 was detected in either the extraglomerular mesangial cells or inside the glomerulus (Fig. 6D). When endothelial cells were labeled with anti-VWF, staining of connexin 37 (Fig. 6E) and connexin 40 (Fig. 6F) inside the glomerulus did not overlap with anti-VWF staining, suggesting that connexin 37 and connexin 40 staining inside the glomerulus was on intraglomerular mesangial cells surrounding the capillaries. Both connexin 37 and connexin 40 were detected in the vascular bundles, while connexin 43 was not seen (not shown).

Table 1 provides a semiquantitative summary of connexin expression in the kidney of normal C57BL/6 mice. While comparisons of the extent of connexin expression can be made readily between different renal vessels for any one antibody, comparison of staining between antibodies is more difficult due to complications which may arise from differential binding efficiencies.

For each of the connexin antibodies, no staining was found if the primary antibody was omitted or if the primary antibody had been preincubated with the antigenic

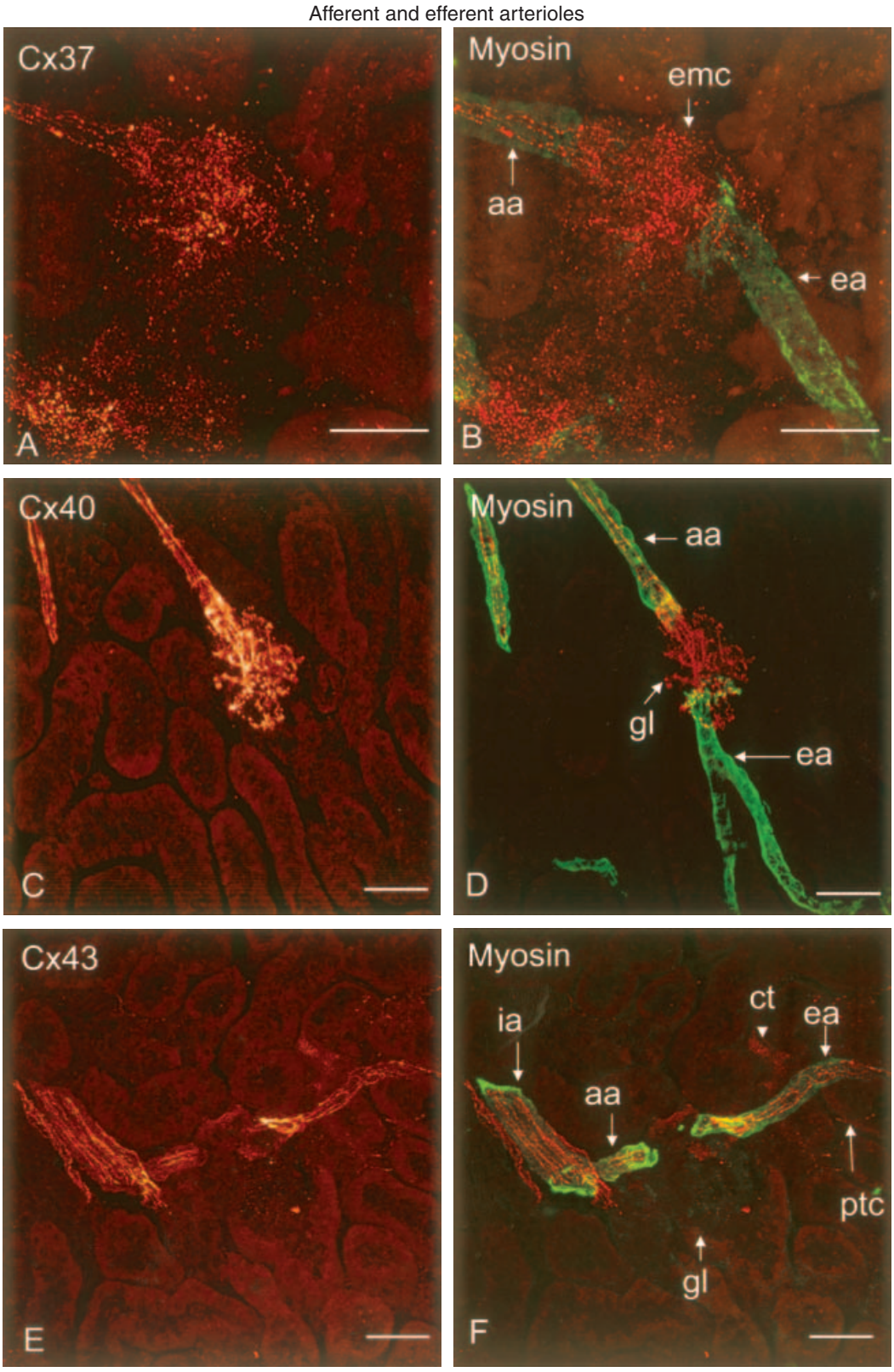

Fig. 4. Connexin (Cx) expression in renal arterioles. When arterioles were labeled with antimyosin (*B*, *D*, and *F*), Cx37 (*A*) and Cx40 (*C*) were detected in the afferent arteriole (aa) but not in the efferent arterioles (ea). In contrast, Cx43 was detected in both arterioles (*E*) compare (F). Cx43 was also detected outside the interlobular artery (ia), around the cortical tubules (ct, arrowhead), and peritubular capillaries (ptc). Gl is glomerulus [scale bar represents 25 μm in (A and B) and 50 μm in (C to F)].

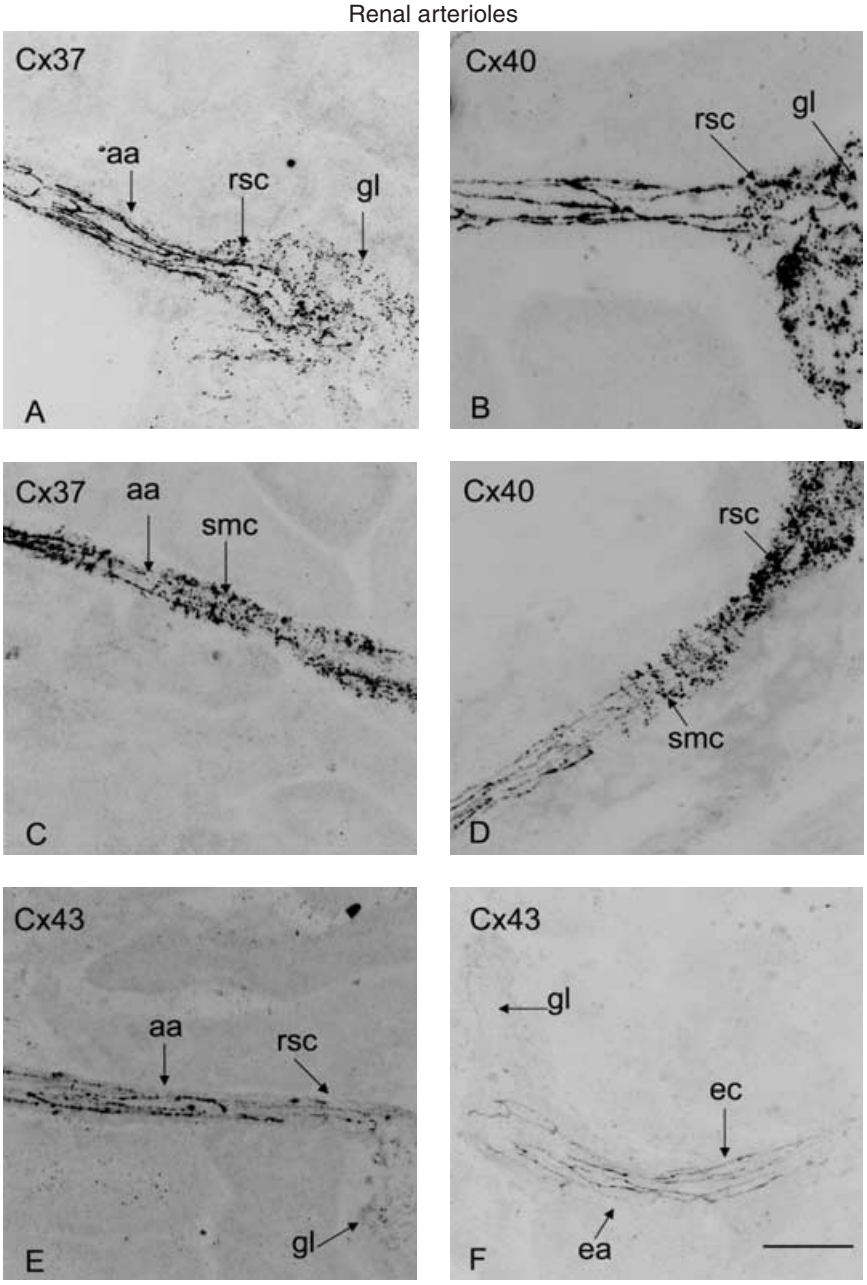

Fig. 5. Connexin (Cx) expression in renal arterioles. In afferent arterioles, Cx37 (*A*), Cx40 (*B*), and Cx43 (*E*) were detected in endothelial cells (ec). In the region of the renin-secreting cells (rsc) adjacent to glomerulus (gl), Cx37 (A), and Cx40 (B and *D*) were detected, but not Cx43 (E). In the smooth muscle cells (smc), Cx37 (*C*) was detected and occasionally in the smooth muscle cells adjacent to the renin-secreting cells Cx40 was found (D), while no Cx43 was found either in the afferent (aa) or efferent arterioles (ea) (E and *F*) (scale bar represents 25 μm).

peptide (Fig. 9A, C, and E). Antimyosin staining was used to mark the location of renal vasculature in these preparations (Fig. 9B, D, and F). As connexin 43 staining was often weaker than connexin 37 and connexin 40, acetone-fixed cardiac muscle was used in addition as a positive control and incubation with the anticonnexin 43 antibody produced extensive and intense staining (Fig. 9G). Incubation of the antibody against connexin 43 with peptide completely eliminated this staining in the heart (Fig. 9H).

Connexin staining in diabetes

In the kidney of mice, 8 to 10 weeks after induction of streptozotocin-induced diabetes, connexin 37 staining of both endothelial cells and smooth muscle cells of the renal vasculature was similar to that of controls (Fig. 7A vs. Figs. 3A, 5A, and 5C). Quantification of connexin 37 showed no significant difference between afferent arterioles in control and diabetic mice ($P > 0.05$) (Fig. 10A). However, inside the glomerulus, there was a significant expansion of connexin 37 from the vascular pole in control (Fig. 8A) to the capillary loops of the glomerulus ($P < 0.05$) (Figs. 8B and 10A) in diabetes.

Connexin 40 staining was detected in the endothelial cells of the afferent arterioles but not in the efferent arterioles (Fig. 7C) of diabetic mice in a similar fashion to that seen in control (Fig. 4C). However, connexin 40

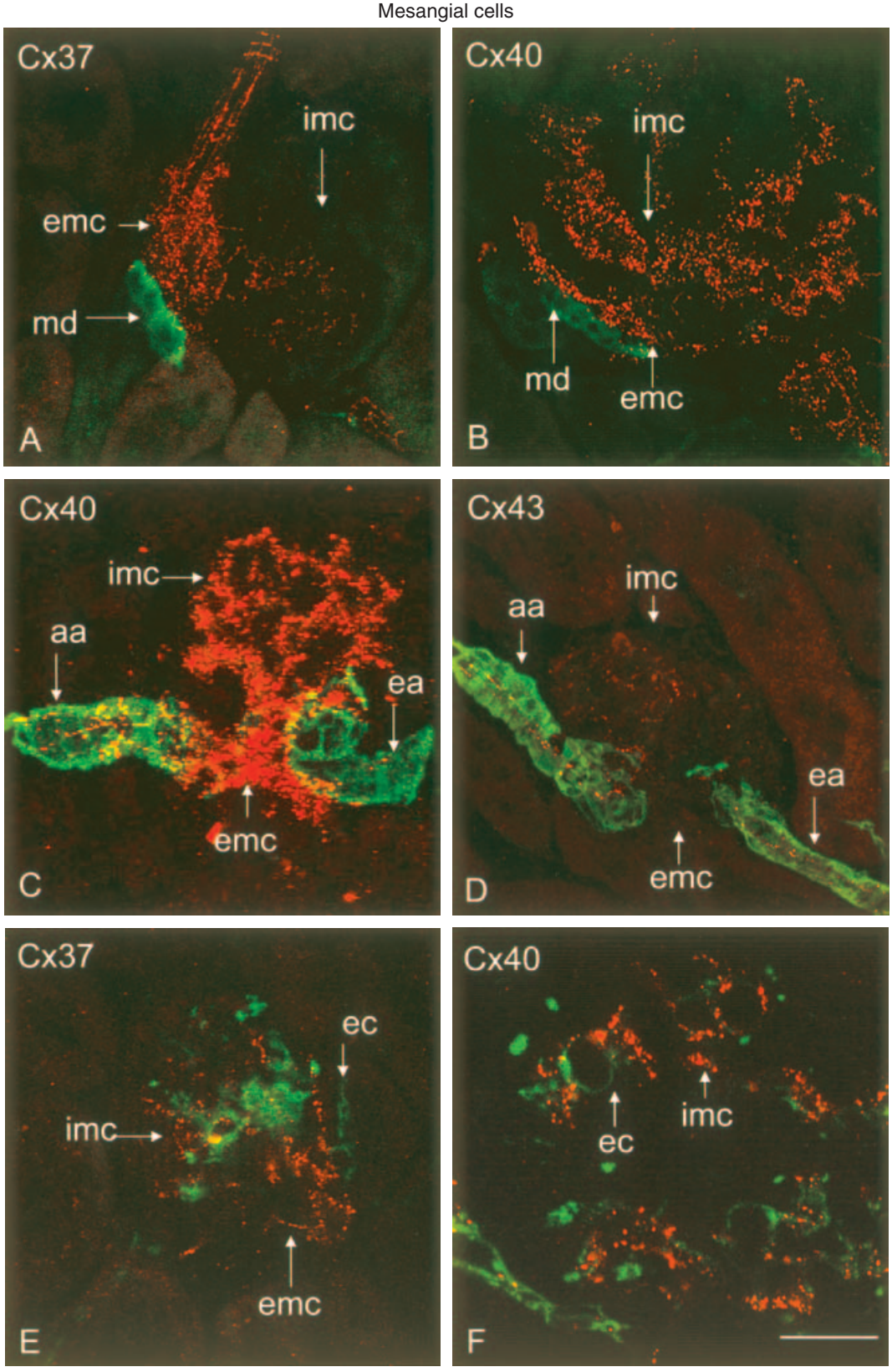

Fig. 6. Connexin (Cx) expression in extra- and intraglomerular mesangial cells. Extraglomerular mesangial cells (emc) lying adjacent to the macula densa (md) labeled with antineuroal nitric oxide synthase (anti-nNOS) (*A* and *B*) expressed Cx37 (A) and Cx40 (B). When afferent (aa) and efferent arterioles (ea) were labeled with antimyosin (*C* and *D*), extraglomerular mesangial cells (emc) could be seen to express Cx40 but not Cx43. When endothelial cells (ec) were labeled with anti-von Willebrand factor (anit-VWF), Cx37 (*E*) and Cx40 (*F*) were shown to be expressed on intraglomerular mesangial cells (imc) (scale bar represents 25 μm).

Table 1. Distribution of connexin 37, 40, and 43 in murine renal vasculature

Vasculature	Connexin subtype	Connexin 37	Connexin 40	Connexin 43
Renal artery	Endothelial cells	+++	+++	+
	Smooth muscule cells	–	–	–
Lobar artery	Endothelial cells	+++	+++	+
	Smooth muscle cells	–	–	–
Arcuate artery	Endothelial cells	+++	+++	+
	Smooth muscle cells	++	–	–
Interlobular artery	Endothelial cells	+++	+++	++/+
	Smooth muscle cells	++	–	–
Afferent arteriole	Endothelial cells	+++	+++	++
	Smooth muscle cells	++	+/–	–
	Renin-secreting cell	++	+++	–
Efferent arteriole	Endothelial cells	–	–	++/+
	Smooth muscle cells	–	–	–
Vascular bundles	Endothelial cells	++	++	–
	Smooth muscle cells	–	–	–
Mesangial cells	Intraglomerular	++/+	+++	–
	Extraglomerular	+++	+++	–

Abbreviations are: +++, intense, punctate staining; ++, moderate, punctate staining; +, weak, irregular staining; –, no staining.

staining was detected between the smooth muscle cells in more than 50% of the afferent arterioles (27/53 of three mice) in diabetes (Fig. 7B to D). Quantification of connexin 40 staining in regions of afferent arterioles further than 40 μm from the glomerulus showed a significant increase in diabetes ($P < 0.05$) (Fig. 10B) (afferent arterioles 2), while staining in regions of afferent arterioles within 40 μm was not significantly different between control and diabetic mice ($P = 0.07$) (Fig. 10B) (afferent arterioles 1). This increase in smooth muscle staining was detected only after 4 weeks of diabetes and was most pronounced after 8 weeks of diabetes. Inside the glomerulus, connexin 40 staining along the capillary loops in control (Fig. 8C) appeared to extend peripherally in diabetes (Fig. 8D). However, quantification of connexin 40 staining showed a trend to an increase in size of the glomeruli and the area stained, but no significant change in the proportional area occupied by connexin 40 staining ($P > 0.05$) (Fig. 10B).

A decrease in connexin 43 staining was found in the endothelium of the efferent arterioles in diabetes (Figs. 7E and 10C). This was first detected in early stages of diabetes (2 weeks) in superficial glomeruli and became more widespread after 8 week of diabetes. At 10 weeks of diabetes, clear but irregular connexin 43 staining was first detected between the renin-secreting cells (Fig. 7F). An increase of connexin 43 staining in the renal tubules of the cortex (Fig. 8F) was also detected in diabetic mice compared to that seen in that of controls (Fig. 8E). Both the number of labeled tubules and the intensity of the staining were increased during diabetes.

DISCUSSION

The present study has demonstrated that connexin 37, connexin 40, and connexin 43 are extensively expressed in endothelial cells and smooth muscle cells of preglomerular renal vasculature and extra- and intraglomerular mesangial cells in C57BL/6 mice. In contrast, connexin 43 is the only connexin expressed in the postglomerular vasculature and this was entirely confined to the endothelial cells. Our results thus provide strong evidence that the preglomerular vasculature is much better coupled than previously thought [18, 20–22], in contrast to the postglomerular vasculature where only weak endothelial coupling could be found.

In endothelial cells of the renal arteries and afferent arterioles, connexin 37 was strongly expressed. This is consistent with previous studies showing connexin 37 in endothelial cells of mouse aorta and renal artery [28–30], rat renal arteries, caudal artery, and coronary artery [18, 25, 31]. However, it is in contrast to Seul and Beyer [22] who failed to detect connexin 37 in renal vasculature of adult B6, SJL f2 mice. This discrepancy might be explained by different fixation protocols, different antibodies, or different mouse strains. Connexin 40 was also abundantly expressed in the endothelial cells of the preglomerular renal arteries and arterioles, in agreement with previous studies in mouse and rat vasculature [22, 25, 31]. In contrast, connexin 43 expression was weak and irregular in renal, lobar, and arcuate arteries. While connexin 43 was expressed in rat aorta, carotid artery, superior mesenteric artery, and medium-sized renal arteries [21, 32], it was not found in the mouse aorta [28], suggesting considerable species differences. Interestingly, as the vessel size decreased, staining for connexin 43 increased in the endothelium of renal vessels. Thus endothelial cells of the renal vasculature, especially the interlobular arteries and afferent arterioles, are well coupled to each other via connexin 37, connexin 40, and connexin 43. This provides an efficient communication route through which vascular responses could be conducted along the smaller renal arteries leading to changes in renal vascular resistance, RBF, and filtration rate.

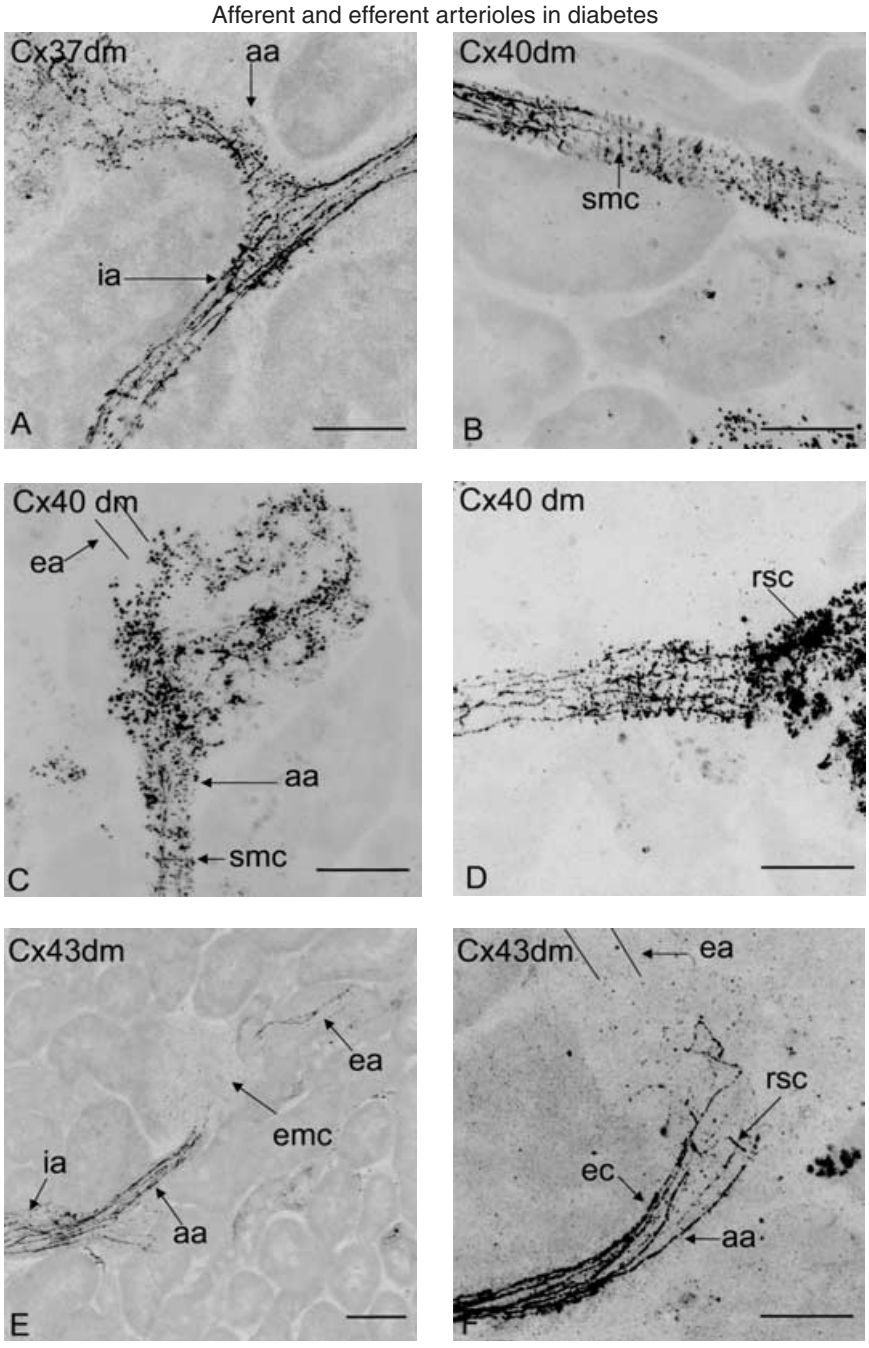

Fig. 7. Changes of connexin (Cx) expression in diabetic arterioles. No difference in Cx37 was detected between control (see Figures 3A and 5A and C) and diabetes (10 weeks' duration (*A*) in the afferent arterioles (aa) and interlobular artery (ia). Cx40 was detected only in the afferent arteriole but not in the efferent arteriole (ea) (*C*) in diabetes. Cx40 was frequently detected in the smooth muscle cells (smc) of the afferent arterioles in diabetes (*B*, C, and *D*). A decrease or absence of Cx43 in the efferent arteriole was noted in diabetes (*E* and *F*), although Cx43 was detected in the renin-secreting cells (rsc) in diabetes (F). dm is diabetes mellitus [scale bar represents 25 μm in (A to D and F) and 50 μm in (E)].

In contrast to the abundance of cell coupling in the endothelium of afferent arterioles, only connexin 43 was expressed in the efferent arterioles. Such restricted connexin expression in the endothelium is atypical of that generally described in arterioles and arteries [18, 21, 22, 33, 34], but accords with the differential expression of connexin 40 found previously in the afferent and efferent renal arterioles of mice [22]. Inside the glomerulus, when endothelial cells were labeled with anti-VWF, we could not detect any connexin staining in the endothelium of the glomerular capillary loops suggesting that there is little or no coupling between endothelial cells of the afferent arterioles and those of the efferent arterioles. While Seul and Beyer [22] made a similar suggestion, this was based on an absence of staining in the endothelium of the distal portion of the afferent arteriole near the entrance to the glomerulus. In contrast we found that connexins 37, 40, and 43 were expressed in the endothelial cells of the afferent arterioles within the juxtaglomerular region of the glomerulus and suggest that an interruption in coupling between the preglomerular and postglomerular vasculature may occur within the glomerulus itself.

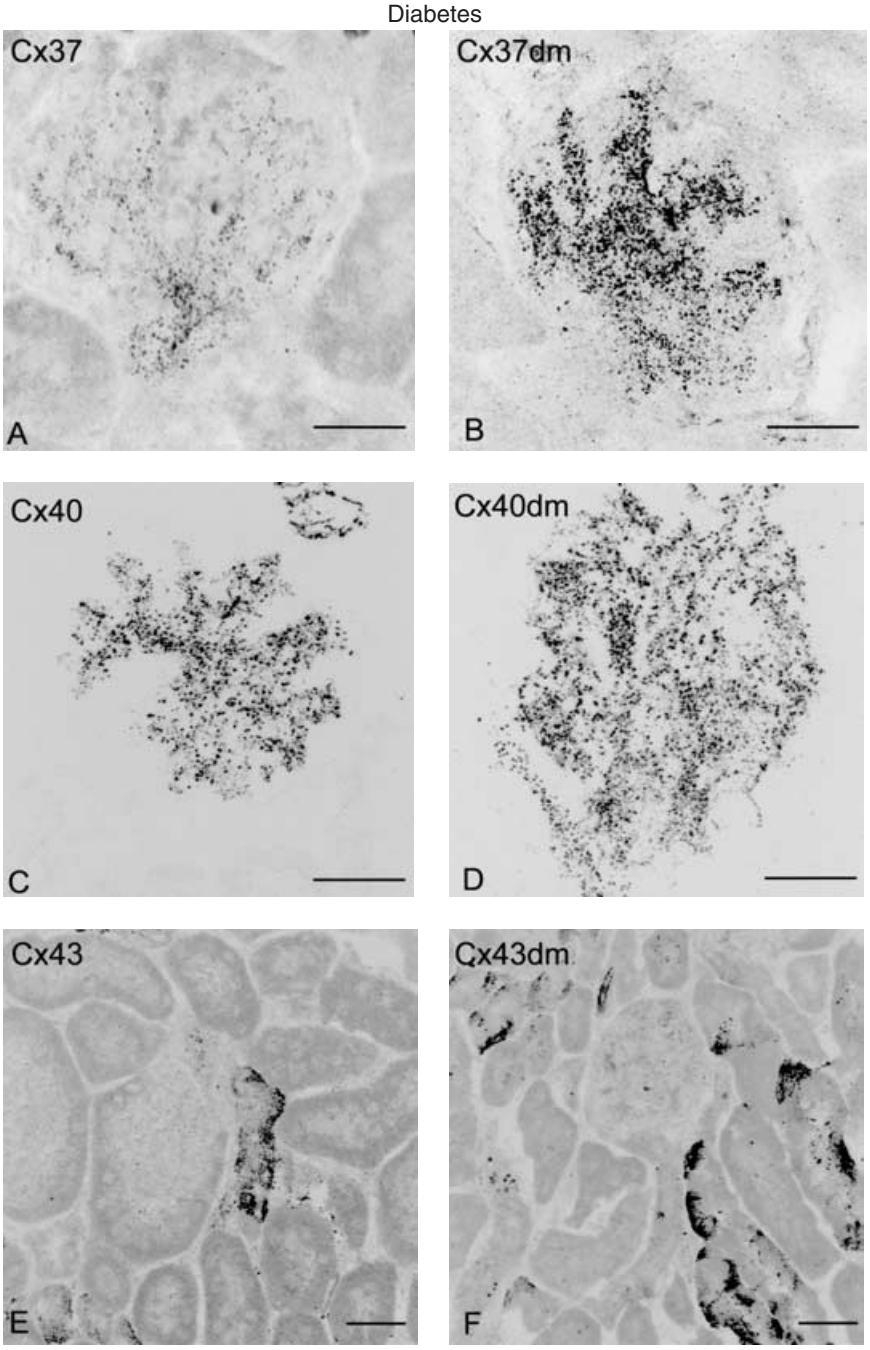

Fig. 8. Changes of connexin (Cx) expression in glomeruli and cortical tubules during diabetes for 10 weeks. An intraglomerular expansion of Cx37 was detected in diabetes (*B*) compared to that in control, where it is restricted to the vascular pole (*A*). More intensive and extensive Cx40 expression was found inside the glomerulus in diabetes (*D*) compared to that in control kidneys (*C*). The glomerulus (A to D) was orientated with the vascular pole at the bottom. An increase in Cx43 was noted in the cortical tubules in diabetes (*F*) compared to that seen in control (*E*) [scale bar represents 25 μm in (A to D) and 50 μm in (E and F)].

In the smooth muscle cells of the arcuate artery, interlobular artery and afferent arteriole and in the renin-secreting cells, connexin 37 was clearly expressed although it was absent from the media of the renal and lobar arteries. Connexin 37 has previously been described in the media of large rat arteries, like the caudal and coronary arteries [25, 31]. In contrast, Arensbak et al [18] failed to detect connexin 37 protein expression in preglomerular arteries although they successfully demonstrated mRNA for connexin 37 in cultured preglomerular smooth muscle cells. Our results thus provide the first direct evidence of connexin 37 expression in the smooth muscle cells of small renal arteries and afferent arterioles and in the renin-secreting cells. We found no evidence for connexin 43 expression in the media of renal arteries or arterioles as in rat muscular arteries [32], while connexin 40 was found in the renin-secreting cells and occasionally in the adjacent smooth muscle cells of the afferent arteriole, consistent with previous studies [18, 21, 22]. In the efferent arterioles, none of the three connexins was

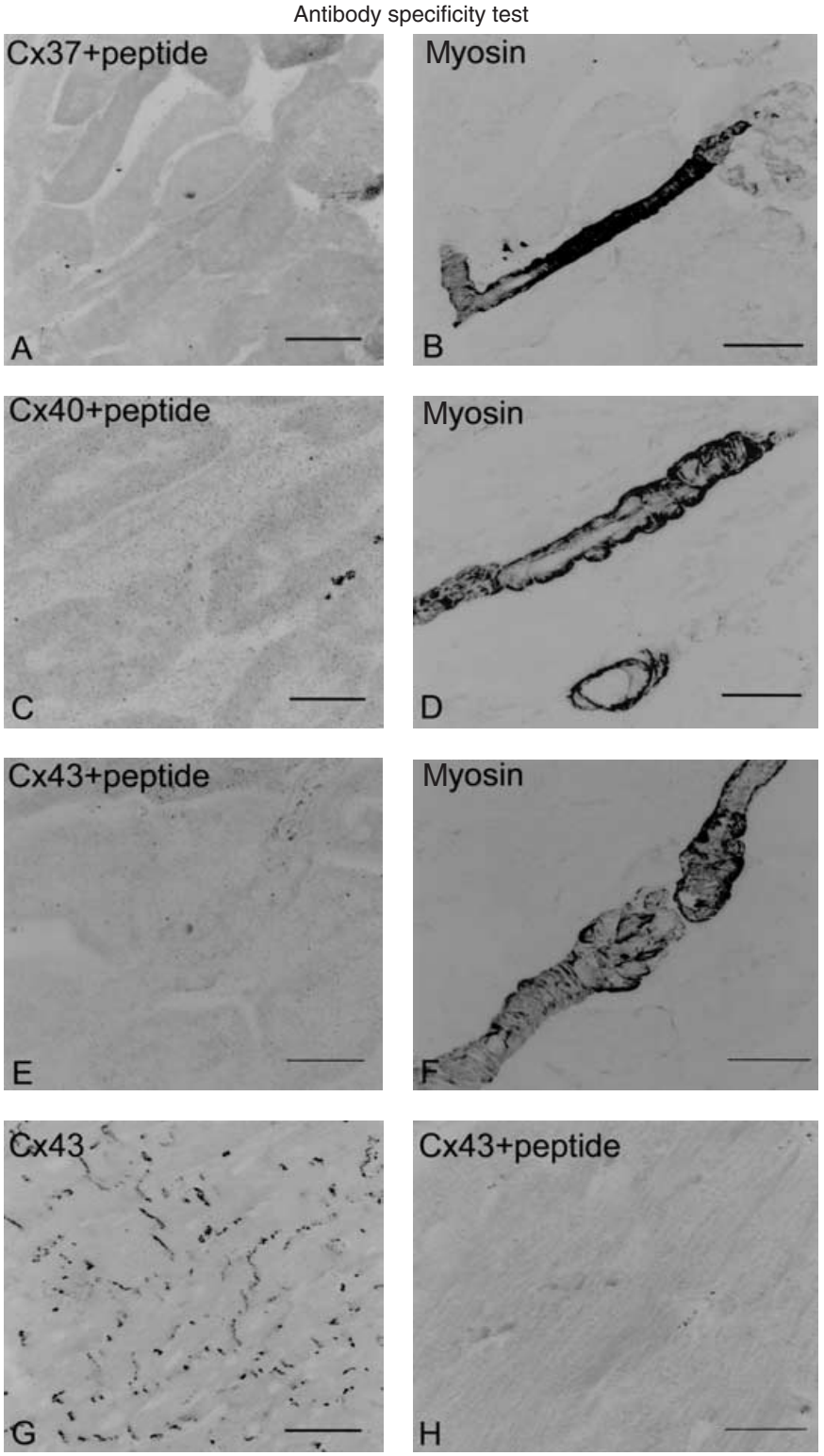

Fig. 9. Connexin (Cx) antibody specificity test. Antibodies against Cx37, Cx40, and Cx43 were preincubated with their corresponding antigenic peptides before incubation with the tissue (*A*, *C*, and *E*, respectively) and antimyosin (*B*, *D*, and *F*) was used to label the renal vasculature. The staining for Cx37, Cx40, and Cx43 seen in Figures 4 and 5 was completely abolished (A, C, and E). Extensive Cx43 staining found in cardiac muscle (*G*) was also completely abolished after peptide block (*H*) [scale bar represents 25 μm in (A to F) and 50 μm in (G and H)].

detected in the smooth muscle cells, consistent with the absence of gap junctions in electron microscopic studies [12].

Both extra- and intraglomerular mesangial cells exhibited intense expression of connexin 37 and connexin 40, while connexin 43 was not found. While this is consistent with connexin 40 being previously described in mesangial cells [18], our results provide the first evidence that multiple connexins are expressed at the gap junctions demonstrated amongst mesangial cells in electron microscopic

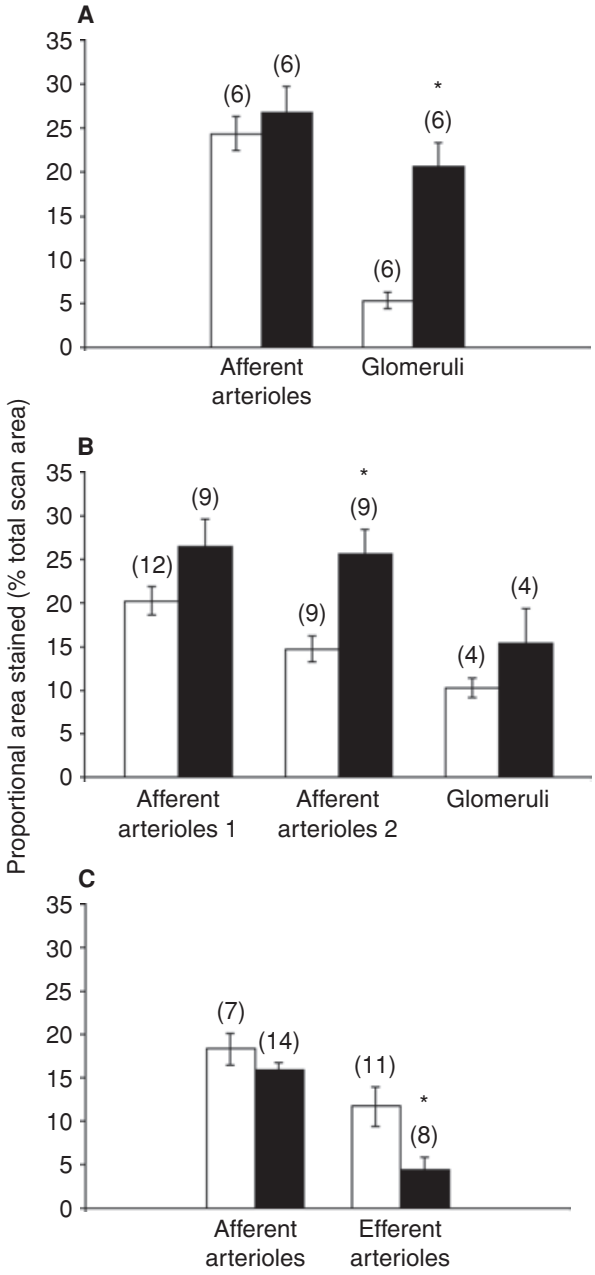

Fig. 10. Quantification of connexin (Cx) staining in renal vasculature during diabetes. (*A*) A significant increase in the proportional area of Cx37 staining was found in glomeruli from diabetic mice. $^*P < 0.05$, compared to control mice. However, there was no change in the Cx37 staining in the afferent arterioles. (*B*) A significant increase in Cx40 staining was detected in afferent arterioles of diabetic mice when measured in areas further than 40 μm from the glomeruli. $^*P < 0.05$, afferent arterioles 2. No significant difference was found in Cx40 staining in regions of afferent arterioles within 40 μm of glomeruli ($P > 0.05$, afferent arterioles 1), nor was there any difference in the proportional area of staining within the glomeruli. (*C*). A significant decrease in Cx43 was detected in the efferent arterioles from diabetic mice. $^*P < 0.05$. However, there was no change in the staining in the afferent arterioles. Symbols are: (□), control; (■), diabetes (4 to 10 weeks). Numbers in parentheses represent the number of arterioles or glomeruli measured from at least four different mice in each case.

studies [14–16]. These connexins provide a mechanism by which both extra- and intraglomerular mesangial cells can behave as a functional syncytium [35]. Electrophysiologic studies have shown that mesangial cells are essential for the transfer of information from the macula densa to afferent arterioles [4, 5]. The abundant connexin expression in the afferent arteriole and mesangial cells suggests a direct communication route for the tubuloglomerular feedback response without the need for further chemical mediators.

Extensive connexin expression in the afferent arterioles and interlobular arteries provides a mechanism for the coordination of RBF and GFR seen between adjacent nephrons [7, 36], through the ability of afferent arterioles and interlobular arteries to conduct vasomotor responses [6, 8]. Studies in other microcirculatory vessels have provided evidence that both the endothelial and smooth muscle layers can provide pathways for the conduction of vasodilation and vasoconstriction [9]. In contrast, the limited connexin expression in efferent arterioles ensures that afferent and efferent arterioles can be differentially regulated. This is consistent with differences in the innervation type and density of afferent and efferent arterioles [37]. Interestingly, the differential connexin subtype expression between afferent and efferent arterioles is matched by different anatomical and functional properties of the smooth muscle cells suggestive of a greater role for voltage dependent mechanisms in the vessels with the greater incidence of cell coupling [38–41].

GFR is governed by a variety of factors, including nerves, hormones, glomerular morphology, and tubuloglomerular feedback response [42]. During the latter response, the resistance of afferent and efferent arterioles change in opposite directions in response to alterations in salt concentration in the distal tubules [43]. In the early stages of diabetes there is a marked increase in GFR which is associated with reduced resistance of the afferent arterioles and diminished responsiveness to vasoconstrictor stimuli, while responses of the efferent arterioles are unchanged [44, 45]. In streptozotocin-induced diabetes mellitus, we found a marked increase in the extent of connexin 40 expression in the smooth muscle cells of the afferent arterioles and the appearance of connexin 43 in the renin-secreting cells. In contrast, there was a substantial decrease in connexin 43 expression in the endothelial cells of the efferent arterioles. These data suggest that coupling within the preglomerular vasculature is increased while coupling within the postglomerular vasculature is decreased.

Increased connexin expression in the preglomerular vasculature might account for the observed hyperfiltration due to a greater ability to conduct vasodilatory responses through both the endothelium and the smooth muscle to coordinate adjacent nephrons. Indeed, during the early stages of diabetes there appears

to be an increased activation of adenosine triphosphate (ATP)-dependent potassium channels in afferent arterioles under basal conditions and a decreased activity of vasoconstrictors [45]. On the other hand, a decreased coupling of the postglomerular vasculature might result in increased intraglomerular capillary pressure, leading to capillary damage. Within the glomerulus itself the observed increase in coupling amongst mesangial cells might contribute to a further increase in GFR in diabetes. Whether these changes in connexin expression actually result in the pathophysiology of the diabetic kidney or are the results of adaptation to glomerular hyperfiltration awaits further experimentation.

CONCLUSION

We have for the first time demonstrated the presence of connexin 37 coupling smooth muscle cells of smaller renal arteries and arterioles and among the renin-secreting cells and mesangial cells of C57BL/6 mice. We have also demonstrated that there are more connexins expressed in the preglomerular renal vasculature and juxtaglomerular apparatus than previously thought, while the postglomerular vasculature is poorly coupled. Our data of connexin expression in the kidneys of diabetic mice show that these patterns of cellular coupling are further accentuated in diabetes.

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