In vivo regulation of endothelium-dependent vasodilation in the rat renal circulation

and the effect of streptozotocin-induced diabetes

Amanda J. Edgley¹,², Marianne Tare¹, Roger G. Evans¹, Con Skordilis¹, Helena C. Parkington¹.

¹. Department of Physiology, Monash University, Australia.
². Department of Medicine, St Vincent’s Hospital, University of Melbourne, Australia.

Corresponding Author at current address:
Dr Amanda Edgley
Department of Medicine, St Vincent’s Hospital
PO Box 2900
Fitzroy
Victoria  Australia
3065
Ph:   +613 9288 3275
Fax: +613 9288 2581
Email: aedgley@medstv.unimelb.edu.au

Running Title: In vivo renal endothelial function and diabetes
**ABSTRACT**

We assessed the relative contributions of endothelium-derived relaxing factors to renal vasodilation *in vivo* and determined whether these are altered in established streptozotocin-induced diabetes. In non-diabetic rats, stimulation of the endothelium by locally administered acetylcholine or bradykinin induced transient renal hyperemia. Neither basal renal blood flow (RBF) nor renal hyperemic responses to acetylcholine or bradykinin were altered by blockade of prostanoid production (indomethacin), or by administration of charybdotoxin (ChTx) plus apamin to block endothelium-derived hyperpolarizing factor (EDHF). In contrast, combined blockade of nitric oxide (NO) synthase (*N*-nitro- L-arginine methylester; L-NAME) and prostanoid production reduced basal RBF and the duration of the hyperemic responses to acetylcholine and bradykinin, and revealed a delayed ischemic response to acetylcholine. Accordingly, L-NAME and indomethacin markedly reduced integrated (area under the curve) hyperemic responses to acetylcholine and bradykinin. Peak increases in RBF in response to acetylcholine and bradykinin were not reduced by L-NAME and indomethacin, but were reduced by subsequent blockade of EDHF. L-NAME plus indomethacin and ChTx plus apamin altered RBF responses to endothelium stimulation in a qualitatively similar fashion in diabetic and non-diabetic rats. The integrated renal hyperemic responses to acetylcholine and bradykinin were blunted in diabetes, due to a diminished contribution of the component abolished by L-NAME plus indomethacin. We conclude that NO dominates integrated hyperemic responses to acetylcholine and bradykinin in the rat kidney *in vivo*. After prior inhibition of NO synthase, EDHF mediates transient renal vasodilation *in vivo*. Renal endothelium-dependent vasodilation is diminished in diabetes due to impaired NO function.

**Abstract word count:** 249

**Keywords:** kidney circulation, acetylcholine, bradykinin, endothelium-derived hyperpolarizing factor, nitric oxide.
INTRODUCTION

The vascular endothelium mediates relaxation of the underlying smooth muscle via the actions of several vasodilators, including nitric oxide (NO), prostanoids (e.g. prostacyclin), and an endothelium derived hyperpolarizing factor (EDHF) (36). The relative importance of these vasodilators varies across different vascular beds (32). While NO is critical in the larger vessels, EDHF assumes greater importance in small resistance arteries (34).

Endothelium derived NO, prostanoids and EDHF have all been implicated in the control of renal vascular tone in vitro. Inhibition of NO synthase (NOS) significantly reduces endothelium-dependent vasodilation in the renal bed, with an additional role of prostanoid revealed by inhibition of cyclooxygenase (COX) (30). Following inhibition of NOS and COX there remains a vasodilator response to endothelial stimulation by acetylcholine (ACh) and/or bradykinin (BK) and this is attributed to EDHF. For example, EDHF has been identified in the isolated perfused kidney of the rat (19, 22), and in afferent (15, 38, 39) and efferent (30) arterioles in vitro. The physiology of EDHF in the kidney has been little studied under in vivo conditions. De Vriese and colleagues have shown that ACh induces hyperemia in the rat kidney in vivo, in the presence of NOS and COX blockade (4). This EDHF component of renal endothelium-dependent vasodilation was abolished by connexin-mimetic peptides, implicating the role of myoendothelial gap junctions in this response (5, 13). Moreover, these peptides induced renal vasoconstriction on their own, suggesting a role for EDHF in the maintenance of basal renal vasodilator tone (5). Nevertheless, a number of important questions remain to be answered. Because studies of EDHF in the renal circulation in vivo have, almost exclusively, been performed under conditions of prior blockade of NOS or both NOS and COX, there is relatively little information regarding the relative contributions of NO,
prostanoids, and EDHF in mediating endothelium-dependent vasodilation in the renal circulation *in vivo*. Therefore, we investigated the relative contributions of various endothelium-derived relaxing factors, to *in vivo* renal hyperemic responses to ACh and BK. These contributions were determined subtractively, by quantification of the hyperemic responses to ACh and BK, before and after sequential administration of N\textsuperscript{\textsubscript{o}}-nitro-\textsuperscript{\textsubscript{L}}-arginine methylester (\textsuperscript{\textsubscript{L}}-NAME) to inhibit NOS, indomethacin to inhibit COX, and the established general inhibitors of EDHF, charybdotoxin (ChTx) plus apamin (4, 41), which have been previously applied in studies of renal vascular function (30).

Cardiovascular complications play a central role in the increased morbidity and mortality associated with diabetes, and dysfunction of the endothelium is a major contributor to these complications. Early after the onset of diabetes the role of endothelial vasodilators may be temporarily upregulated (6). However in established diabetes there is evidence that both the NO (33) and the EDHF (4) components of renal endothelium-dependent vasodilation are blunted. There is little information regarding the relative effects of diabetes on the function of these individual endothelium-dependent relaxing factors under *in vivo* conditions. To address this issue, we investigated the impact of established streptozotocin (STZ)-induced, Type I diabetes on endothelial function in the renal vascular bed of anesthetized rats.
METHODS

Outbred, male Wistar rats ($n = 26$, 350-500 g) were maintained on a standard rat chow (GR2; Barastoc Stockfeeds, Pakenham, VIC, Australia) and allowed free access to water. All experimental procedures were approved by the Monash University Department of Physiology Animal Ethics Committee and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Induction of diabetes

At 8 weeks of age, the rats were anesthetized with 2.0–2.5% isoflurane (Abbott Australasia Pty Ltd, Kurnell, Australia). To induce diabetes, STZ (Sigma, St Louis, MO, USA), dissolved in 100 mmol/L citrate buffer (pH 4.5) was injected into a tail vein of 9 rats at a dose of 60 mg/kg (40). Control rats ($n = 8$) received an injection of citrate buffer alone. Blood glucose levels were measured using an Ames Glucometer® II (Bayer Tarrytown, NY).

Surgical procedures

Eight to nine weeks after injection of STZ or its vehicle, each rat was anesthetized (urethane; 1 g/kg i.p; Sigma, St Louis, MO, USA), and placed on a thermostatically controlled heating pad that maintained body temperature at approximately 37°C. A tracheotomy was performed, and a jugular vein catheter (PE-50) inserted for infusion of maintenance fluids. Bovine serum albumin (2% w/v in 154 mM NaCl, Sigma, St Louis, MO, USA) was administered intravenously (i.v.) throughout surgery (6 ml·kg⁻¹·h⁻¹) and during the remainder of the experiment (2 ml·kg⁻¹·h⁻¹). Arterial pressure was recorded via a catheter, filled with heparinised saline (12 IU/ml), placed in the right carotid artery and connected to a pressure transducer (Cobe, Avarda, CO, USA). Local infusion of vasoactive agents into the renal circulation was achieved using a tapered PE-10 catheter inserted into the left femoral artery and advanced
through the abdominal aorta until its tip was positioned approximately 1 mm into the left renal artery (8). To keep the catheter patent, heparinised saline solution was infused at a rate of 50 µL/min. A transit-time ultrasound flow probe (0.7VB, Transonic Systems, Ithaca, USA) was placed around the renal artery for measurement of renal blood flow (RBF). Once surgical procedures were completed animals were allowed a one-hour stabilization period before the experiment commenced.

To characterise the renal endothelium-dependent vasodilator response three protocols were performed, all following the same experimental paradigm. Protocol 1: In groups of control (n = 8) and diabetic (n = 9) rats parameters were measured under control conditions, after administration of l-NAME and indomethacin, and then after additional treatment with ChTx and apamin. Protocol 2: In a group of control rats (n = 4) parameters were measured under control conditions, following administration of indomethacin, and then after additional treatment with l-NAME. Protocol 3: In a group of control rats (n = 5) parameters were measured under control conditions and then after administration of ChTx plus apamin, and then following recovery from the effects of ChTx and apamin. NOS and cyclo-oxygenase activity remained intact throughout the experiment. For all protocols, mean arterial pressure (MAP), heart rate (HR), RBF and renal vascular conductance (RVC; calculated from RBF/MAP) were recorded. The responses of these variables to local renal arterial bolus administration of ACh (Sigma, St Louis, MO, USA) and BK (AusPep, Melbourne, Australia), were tested 3 times in each rat; first under control conditions and then in the presence of various combinations of blockers that interfere with endothelium-dependent vasodilation (Protocols 1-3).

Stimulation of endothelium-dependent vasodilation

ACh and BK were administered as bolus injections (50 µL over 10 s) via the renal artery catheter as previously described (25). Doses of BK and ACh (15-25
pmol/kg) were chosen that resulted in minimal systemic spillover, as evidenced by the absence of changes in MAP.

**Inhibition of NOS, COX and the calcium-activated potassium channels underlying EDHF**

NOS was inhibited by L-NAME (Sigma, St Louis, MO, USA), administered intravenously as a bolus of 10 mg/kg followed by infusion at 3 mg·kg\(^{-1}\)·h\(^{-1}\) for the rest of the experiment. COX was inhibited by bolus injection of indomethacin (10 mg/kg, i.v.; Sigma, St Louis, MO, USA). Responses to ACh and BK were tested 15 min after administration of L-NAME and/or indomethacin. The \(K_{\text{ca}}\) channel blockers ChTx and apamin (AusPep, Melbourne, Australia) were infused locally into the renal vascular bed at 50 \(\mu\)L/min as previously described (25). The concentrations of these agents in the infusion solution were adjusted according to RBF, to achieve renal blood concentrations of approximately \(3 \times 10^{-8}\) mol/L for ChTx, and \(2.5 \times 10^{-7}\) mol/L for apamin. These concentrations have been shown to be effective *in vitro* (2, 7, 42) and *in vivo* (11, 25). In preliminary experiments use of higher concentrations of toxins were not found to have additional inhibitory effect on RBF. Responses to ACh and BK were tested 5 min after infusions of ChTx and apamin commenced.

**Data acquisition and analysis**

Analog signals of MAP (mmHg), HR (bpm) and RBF (ml/min) were digitized at 500 Hz (Universal Acquisition, University of Auckland, New Zealand). Basal levels of parameters were defined as the average over the 3 min period before responses to ACh and BK were elicited. We measured maximum increases and (when present) reductions in RBF in response to ACh and BK or vehicle (heparinised saline), relative to RBF averaged over the 10 s prior to the bolus injections. We also calculated the area under the curve of the RBF responses evoked by ACh or BK to assess the net response evoked by the agonists. Baseline was set as mentioned above, and the duration of
response in the absence of blockers was used to set the time for integration of subsequent responses recorded in the presence of blockers. Thus, both the hyperaemic and ischemic responses evoked by endothelial stimulation were included in the area under the curve calculations. Responses to the saline vehicle were subtracted from responses to ACh and BK to provide the net responses to these vasodilators. RBF responses were expressed relative to dry kidney weight. Dry kidney weight was determined by placing the left kidney in an oven at 70°C for two weeks before it was weighed.

Statistics

Statistical analyses were performed using Systat (Version 11.0; SPSS, Chicago, IL, USA). Values from \( n \) animals are given as mean ± SE and two-sided \( P \leq 0.05 \) was accepted as statistically significant. Between animal comparisons were made using Student’s unpaired t-test. Within animal comparisons were made using Tukey’s post-hoc test after a preliminary analysis of variance (20). Repeated measures analysis of variance was used to determine whether responses to treatments in diabetic rats differed from those of control rats (21).
RESULTS

Basal hemodynamics under anesthesia in control rats:

Basal MAP and HR, resting RBF (corrected for dry kidney weight) and RVC (corrected for dry kidney weight) for control rats are shown in Table 1.

Relative contribution of NO, prostanoids and EDHF to basal hemodynamics and renal endothelial vasodilator function in control rats in vivo

Effects of inhibiting NOS and COX on basal hemodynamics in control rats:

Administration of L-NAME plus indomethacin induced a pressor response over the first 3 min and this was sustained. At 12-15 min after L-NAME and indomethacin administration, MAP was significantly higher than baseline in control rats (+54 ± 4 mmHg, compared with pre-treatment, \( P < 0.001 \)) (Table 1, Figs. 1&2). HR was not significantly affected by administration of L-NAME and indomethacin (Table 1). L-NAME and indomethacin decreased resting RBF by 10.1 ± 1.5 mL·min\(^{-1}\)·g dry kid wt\(^{-1}\), and RVC by 144 ± 16 µL min\(^{-1}\)·mmHg\(^{-1}\)·g dry kid wt (Table 1, Fig. 2).

Administration of indomethacin alone, in the absence of L-NAME, did not significantly alter basal MAP, HR, RBF or RVC (Table 1, Protocol 2).

Effects of blocking EDHF activity on basal hemodynamics in control rats:

On a background of NOS and COX inhibition, renal arterial infusion of ChTx and apamin (to block the K\(^+\) channels underlying EDHF) did not significantly affect MAP, HR, RBF or RVC (Table 1 Protocol 1; Fig. 2). Similarly, administration of ChTx and apamin, without prior administration of L-NAME plus indomethacin, did not significantly alter basal MAP, HR, RBF or RVC (Table 1, Protocol 3).
Effects of endothelial stimulation on renal blood flow in control rats:

Typical responses to endothelial stimulation by bolus injection of ACh or BK into the renal artery of control rats are represented in Figures 3 (ACh) and 4 (BK). Under basal conditions, injection of ACh or BK (15 - 25 pmol/kg) elicited transient renal hyperemia without appreciable changes in MAP or HR (Figs. 3 and 4). Blockade of NOS with L-NAME and COX with indomethacin did not significantly affect the maximum (peak) increase in RBF in response to ACh or BK (Figure 5), but markedly reduced the overall, integrated response (area under the curve, Fig 6), mainly due to a decrease in the duration of the hyperemia. In the presence of L-NAME and indomethacin a delayed, ischemic response to ACh was revealed (Fig. 3 and Fig. 5) but this did not occur in response to BK (Fig. 4). Whilst the peak hyperemic response to ACh was not significantly changed, the integrated response to ACh was reduced from 72 ± 14 to 1 ± 3 ml/g dry kidney weight (Fig. 6). Similarly, the integrated hyperemic response to BK was reduced from 170 ± 34 to 45 ± 4 ml/g dry kidney weight by L-NAME plus indomethacin (Fig. 6). Administration of ChTx and apamin in the presence of L-NAME and indomethacin markedly blunted the peak increases in RBF in response to both ACh (from 5.0 ± 0.7 to 1.3 ± 0.5 ml min^{-1}·g dry kidney^{-1}) and BK (from 5.6 ± 1.4 to 1.5 ± 0.8 ml min^{-1}·g dry kidney^{-1}, Fig. 5), but failed to significantly alter the integrated hyperemic response to either ACh or BK (Fig. 6). In addition, ChTx and apamin did not appreciably alter the later ischemic response to ACh (Figs. 3 and Fig. 5).

In control rats, indomethacin treatment alone did not significantly alter the peak increases in RBF, or the integrated hyperemic responses, to ACh or BK (Fig. 7). Subsequent administration of L-NAME significantly attenuated the integrated hyperaemic response to ACh (p<0.05; Fig. 7) but not BK (p=0.2, Fig. 7).
In another cohort of control rats, blockade of EDHF activity using ChTx plus apamin, without prior administration of L-NAME plus indomethacin, did not significantly alter the peak increases in RBF, or the integrated hyperemic responses to ACh or BK (Fig. 8).

**Effect of established diabetes on basal cardiovascular parameters and renal endothelial function in vivo**

**Basal parameters: diabetic versus control rats**

*Body and kidney weight, and blood glucose concentration:*

Eight to nine weeks after STZ- or vehicle-administration, STZ-treated rats had significantly lower body weight than control rats (308 ± 11 vs 481 ± 13 g, respectively, $P \leq 0.001$), greater blood glucose concentration (25 ± 1 vs 9 ± 1 mmol/L, respectively, $P \leq 0.001$), and greater kidney weight (431 ± 21 vs 365 ± 8 mg dry weight, respectively, $P < 0.01$).

*Basal hemodynamics under anesthesia:*

Basal MAP and HR were indistinguishable in diabetic and control rats ($P = 0.85$ and 0.79, respectively, Fig. 2). Resting RBF and RVC (both corrected for dry kidney weight) were also not significantly different in diabetic compared with control animals ($P = 0.08$ and $P = 0.1$, respectively, Fig. 2).

**Effects of diabetes on basal hemodynamics and renal endothelial vasodilator function**

*Basal hemodynamics in diabetes*

Similar to observations in control rats, L-NAME plus indomethacin induced an immediate pressor response. In contrast with the effects observed in controls, this was not maintained (Fig. 1), and after 15 min MAP was not higher than baseline in diabetic
rats (+25 ± 10 mmHg, compared with pre-treatment, \( P = 0.09 \)). Similar to controls, HR was not significantly affected by L-NAME plus indomethacin (Fig. 2).

After treatment with L-NAME plus indomethacin, resting RBF was reduced by 9.5 ± 1.2 mL·min\(^{-1}\)·g dry kid wt\(^{-1}\), and RVC was reduced by 118 ± 15 \( \mu \)L min\(^{-1}\)·mmHg\(^{-1}\)·g dry kid wt (Fig. 2), and these effects were similar to those observed in control rats. In the presence of L-NAME plus indomethacin, inhibition of EDHF with ChTx plus apamin did not significantly affect MAP, HR, RBF or RVC in diabetic rats, as was the case in controls (Fig. 2).

*Effects of diabetes on renal blood flow responses to endothelial stimulation:*

Under control conditions, the peak increase in RBF in response to ACh was significantly less in diabetic rats (3.2 ± 0.5 ml·min\(^{-1}\)·g dry kidney\(^{-1}\)) than in control rats (4.9 ± 0.8 ml·min\(^{-1}\)·g dry kidney\(^{-1}\), \( P = 0.04 \), Fig. 5). The peak increase in RBF in response to BK was not significantly different in diabetic (2.9 ± 0.5 ml·min\(^{-1}\)·g dry kidney weight) compared with control rats (4.4 ± 0.5 ml·min\(^{-1}\)·g dry kidney\(^{-1}\), \( P = 0.06 \), Fig. 5). The integrated hyperemic response to BK was significantly less in diabetic rats (79 ± 11 ml·g dry kidney\(^{-1}\)) than control rats (170 ± 34 ml·g dry kidney\(^{-1}\), \( P = 0.015 \), Fig. 6), but that evoked by ACh was similar in diabetic rats (61 ± 16 ml·g dry kidney\(^{-1}\)) and control rats (72 ± 12 ml·g dry kidney\(^{-1}\), \( p = 0.6 \), Fig. 6).

The effects of L-NAME and indomethacin on responses to ACh and BK in diabetic rats were qualitatively similar to those in control rats. Thus, L-NAME and indomethacin did not significantly alter the peak increases in RBF in response to ACh or BK (Fig. 5). As was the case in control rats, a later ischemic response to ACh (but not BK) was revealed in the presence of L-NAME and indomethacin in diabetic rats (Fig. 5). Although the peak of the ischemic response to ACh in diabetic rats was similar to that in
control animals, the duration of the response appeared to be shorter in diabetic rats compared with control rats, so that its contribution to the total integrated renal hemodynamic response in diabetic rats was negligible (Fig. 6). L-NAME and indomethacin markedly blunted the integrated hyperemic responses to both ACh and BK in diabetic animals, with a reduction in the area under the curve of the hyperemic response to ACh from $61 \pm 16$ to $4 \pm 3$ ml·g dry kidney$^{-1}$ while that for BK was reduced from $79 \pm 11$ to $34 \pm 7$ ml·g dry kidney$^{-1}$ (Fig. 6). The effects of ChTx and apamin on responses to ACh and BK in diabetic rats were also broadly similar to those in control rats. In diabetic rats, ChTx plus apamin significantly reduced the peak increase in RBF in response to ACh (from $2.9 \pm 0.6$ to $1.0 \pm 0.3$ ml min$^{-1}$ g dry kidney$^{-1}$; $P = 0.025$, Fig. 5) but not that to BK (from $2.7 \pm 0.5$ to $1.4 \pm 0.3$ ml min$^{-1}$ g dry kidney$^{-1}$; $P = 0.2$, Fig. 5). The magnitude of the later ischemic response to ACh was not significantly altered by ChTx plus apamin administered on a background of NOS and COX inhibition (Fig. 5), nor were the integrated hyperemic responses to ACh or BK (Fig. 6).

Relative contributions of NO plus prostanoids and EDHF to the overall hyperemic responses to ACh and BK in control and diabetic rats

The occurrence of both hyperemic and ischemic components in the response to ACh, and the presence of NO and EDHF components in responses to ACh and BK, prompted a more formal subtractive evaluation of the integrated responses, as previously described by us (35). In this approach, the response remaining in the presence of all blockers, e.g. L-NAME, indomethacin, ChTx and apamin, was designated “residual” and was subtracted from the response in the presence of L-NAME plus indomethacin to determine the contribution of EDHF. The response in the presence of L-NAME plus indomethacin was subtracted from the control response to yield the
component attributable to NO. These calculations were performed on the data for each animal individually and the data for all 8 control animals pooled to give the results shown in Figure 9. Then, the total hyperaemic response in control rats was designated 100%, and the response in the diabetic group was expressed as a percentage of the average response in controls (Fig. 9). When expressed in this manner, the hyperemic responses to ACh and BK in diabetic rats were 72% and 50%, respectively, of those in control rats. This was entirely attributable to a diminished contribution of NO, with no significant change in the EDHF component.
DISCUSSION

Our current study has provided 4 major new findings relevant to our understanding of endothelial function in the kidney. Firstly, whilst systemic blockade of NOS and COX reduced basal renal blood flow dramatically, there was little effect on the magnitude of the peak of the hyperemic responses to ACh and BK in the renal circulation. Subsequent administration of apamin and ChTx, inhibitors of small-, intermediate- and large-conductance Ca\(^{2+}\)-activated K\(^+\) channels, respectively, greatly reduced these peak hyperemic responses. However, when administered to rats with intact NOS and COX, apamin and ChTx had little effect on the magnitude of peak hyperemic responses to ACh and BK. These data indicate that, in vivo, EDHF appears to contribute to the initial phase of endothelium-dependent vasodilation in response to ACh and BK. Secondly, the integrated hyperemic responses (areas under the curves) to BK and to a lesser extent ACh, in contrast to the initial peak hyperemia, were reduced by inhibition of NOS and COX, but not by blockade of COX alone, or combined administration of apamin and ChTx alone. Thus, under resting conditions, NO appears to dominate the integrated hyperemic responses to both ACh and BK. Thirdly, we found that the integrated hyperemic responses to ACh and BK are markedly blunted in diabetes, and that this is attributable to a deficit in NO-mediated vasodilation rather than EDHF-dependent vasodilation. Our fourth finding uncovered important differences in the underlying mechanisms mediating the responses of the renal vasculature to ACh versus BK. In control, and to a lesser extent in diabetic rats, ACh induced renal vasoconstriction after combined blockade of NOS and COX. The revelation of the ischemic response to ACh only after administration of \(\text{l-NAME}\) plus indomethacin indicated that the vasoconstrictor response is normally masked by the prolonged vasodilation induced by NO. In contrast, bradykinin did not induce vasoconstriction
under these conditions. Thus ACh, but not BK, appears to induce vasoconstriction in the renal circulation \textit{in vivo}, through as yet unknown mechanisms.

Under control and diabetic conditions, basal vasodilator tone in the rat renal circulation \textit{in vivo} appears to be dominated by NO, with little net contribution from prostanoids or EDHF. This conclusion is based on the observation that RBF and RVC were markedly reduced by NOS blockade alone, but not COX blockade or apamin plus ChTx. Our conclusion differs from that of De Vriese et al. (5), who found that, on a background of systemic NO synthase blockade, renal arterial administration of the connexin mimetic peptides $^{43}\text{GAP27}$ and $^{40}\text{GAP27}$ reduced RBF in anesthetized rats. The toxins used in our study, ChTx and apamin, block the potassium channels involved in generating the hyperpolarisation in the endothelial cells, which is the first step in the pathway leading to EDHF-mediated smooth muscle hyperpolarization and relaxation (13). The GAP peptides interrupt gap junctional communication and thus the spread of the hyperpolarising current and small signalling molecules between the endothelial cells and smooth muscle cells (13). Thus, the discrepancy between our results and those of De Vriese et al. (5) may indicate that multiple EDHF pathways exist in the renal vascular bed. Alternatively, the GAP peptides may have other actions apart from those at gap junctions (32). So, while our current observations do not support a substantial role for a ChTx- and apamin-sensitive EDHF in the control of basal renal vasodilator tone, further studies, using multiple inhibitors of EDHF, are required to fully interrogate this hypothesis.

Our current observations identify at least two contributions to the hyperemic responses to ACh and BK in the rat kidney \textit{in vivo}. The hyperemia induced by ACh and BK was greatly blunted by apamin and ChTx, but only when these agents were administered after prior blockade of NOS and COX. One possible interpretation of these
observations, is that NO and/or prostanoids normally act to inhibit the release and/or actions of EDHF. The corollary to this hypothesis is that multiple redundant signalling cascades can mediate the initial hyperemic phase of the responses to ACh and BK. In support of this, results of a number of previous studies show that NO inhibits cytochrome P-450 enzymes (41), including the epoxygenases responsible for production of epoxyeicosatrienoic acids, a putative EDHF (37). There is also strong evidence from in vitro studies of vascular reactivity, that NO can inhibit EDHF activity (1, 37). Consistent with this, we found that NOS blockade plus renal arterial infusion of a NO donor, but not NOS blockade alone, can blunt BK and ACh induced hyperemia in the rabbit kidney (29). Collectively, these observations indicate that EDHF makes little contribution to endothelium-dependent vasodilation in the kidney under normal physiological conditions, but may come into play under conditions of reduced NO bioavailability, such as in diabetes, renal disease and hypertension (10).

However, there is an alternative possibility. While ChTx plus apamin administered in the presence on NOS and COX blockade did not influence the integrated hyperemic responses to ACh or BK, the toxins had a clear suppressive effect on the peak amplitudes of the responses (Fig 5). It is noteworthy that the peak occurred early in the responses to ACh and BK. These observations could be explained if the EDHF component occurred early in the response to endothelial stimulation and that it was brief in duration, especially in relation to the component that is elicited by NO. The evidence to date on the time course of EDHF in a variety of arteries in vitro would support this notion (7, 25, 42).

The integrated hyperemic responses to ACh and BK appear to be dominated by NO, as they were markedly attenuated by combined NOS and COX blockade, but not by COX blockade alone or by combined inhibition of small-, intermediate- and large-
conductance Ca\(^{2+}\) activated K\(^{+}\) channels. These observations are in accord with our previous observations in rat mesenteric and hindlimb vascular beds \textit{in vivo} (25). In contrast, EDHF appears to make an independent contribution to endothelium-dependent vasodilation in the mouse hindlimb circulation \textit{in vivo} and is not tonically inhibited by NO (11).

Observations from \textit{in vitro} studies indicate that ACh- and BK-induced renal vasodilation are mediated by the combined actions of NO and EDHF (22, 30, 37, 38). However, the precise nature of the EDHF-mediated mechanisms within the renal circulation may differ for ACh and BK (38, 39). Consistent with our previous observations in the rabbit kidney, in the present study, ACh- and BK-induced renal hyperemia was blunted by NOS blockade (29) but not COX blockade (24). Thus NO, but not prostanoids, contributes to endothelium-dependent vasodilation induced by ACh and BK in both rabbit and rat kidney \textit{in vivo}.

Consistent with findings in this and other vascular beds, we found that the renal endothelium-dependent hyperemic responses to BK and ACh were attenuated in established STZ-induced diabetes (Figs 5 and 6). In contrast, in early diabetes, endothelial vasodilator function has been reported to be upregulated in a variety of vascular beds, including that of the kidney, mainly attributable to enhancement of the NO system (3, 18, 27). Recent studies of isolated blood vessels have provided support for the notion that endothelial dysfunction in rat mesenteric artery in established diabetes may be mediated through dysfunction of EDHF-mediated vasodilation (12, 40). This hypothesis is consistent with observations of De Vriese et al. (4) in the rat kidney \textit{in vivo}, who found that the component of ACh-induced hyperemia resistant to NOS and COX blockade was blunted in STZ-induced diabetes. On the other hand, impaired endothelium-dependent vasodilation in afferent arterioles from diabetic rabbits appears
to be mediated by reduced NO bioavailability (33). Our current observations indicate that attenuation of ACh- and BK-induced renal hyperemia in diabetes is attributable chiefly to diminished NO function. We argue this based on our analysis of the various components that make up the integrated responses of RBF to ACh and BK (Fig 9). This analysis shows that the integrated hyperemic responses to ACh and BK are mainly attributable to NO and/or prostanoids, and that this component, but not that attributable to EDHF, is markedly attenuated in diabetes. Previously, it has been demonstrated that in established STZ diabetic rats blockade of cyclooxygenase activity was essentially without effect on RBF (4). However, under some conditions in the presence of reduced NO bioavailability there may be a compensatory increase in the contribution of vasodilator prostaglandins (16). In contrast to our present findings in the kidney, we have previously found that diabetes-induced attenuation of the hyperemic response to ACh in the hindlimb and mesenteric circulations in vivo is chiefly attributable to diminished EDHF function (26). Based on the results of parallel studies performed on the hindlimb and mesenteric vascular beds (26), we conclude that the effects of diabetes on endothelial function are highly organ specific, and may also reflect regional differences in the identity of EDHF (32).

The reduced contribution of NO in the diabetic kidney could result from a decrease in NO synthesis and/or inactivation of available NO. In blood-perfused afferent and efferent arterioles from STZ induced diabetic rats, the impaired responsiveness of the vessels to NO was reversed by addition of superoxide dismutase, suggesting that enhanced tissue superoxide may result in increased NO breakdown and thus impaired NO responsiveness (23). On the other hand, the plasma concentration of L-arginine has been observed to be reduced in STZ diabetic rats and the impaired relaxation to ACh observed in isolated aortic rings from the same animals was restored with L-arginine
pre-treatment (28). Likewise NO production could be impaired via inhibition/uncoupling of eNOS activity resulting from alterations in levels of tetrahydrobioprotein and/or dimethylarginine (for review see (14, 18).

A novel finding was our observation of an ischemic phase of the response to ACh but not to BK. ACh-induced renal ischemia was particularly prominent after NOS blockade, so that after combined administration of L-NAME, indomethacin, ChTx and apamin, and after administration of L-NAME and indomethacin, but not indomethacin alone, the integrated response to ACh was net ischemia. In the same animals, the integrated response to bradykinin was always net hyperemia. Our current experiments suggest that ACh-induced renal vasoconstriction is not mediated by a product of COX, such as the putative prostaglandin endoperoxide thought to be an endothelium-derived constricting factor (6).

A strength of our in vivo approach is the ability to monitor the global response of the renal circulation, with its multiple vascular territories (e.g. cortex versus medulla) and segments (e.g. pre- versus post-glomerular), in which endothelium-dependent vasodilator mechanisms clearly differ (9, 39). Another strength of the in vivo approach is the ability to study the effects of diabetes under conditions of an intact metabolic and hormonal milieu. However, the in vivo approach limits our ability to identify the precise mechanisms underlying the renal vascular responses to ACh and BK. Nevertheless, our current observations have allowed quantification of the relative contributions of various endothelium-derived relaxing factors to the control of RBF in vivo, and how these are altered in diabetes.

In this study, diabetic animals were not supplemented with insulin. However, the results of the present study are consistent with those of De Vriese and colleagues, who demonstrated in STZ diabetic rats treated with insulin, that the global RBF response to
a 100 ng intra renal bolus dose of ACh was attenuated compared with control animals (4). Evidence from *in vitro* studies of arteries from a number of different vascular beds indicates that insulin treatment and improved metabolic control can improve endothelium-dependent vasodilation in diabetes (12, 17, 31). The extent of improvement does vary with the vascular bed and how well the hyperglycaemia is controlled.

Our current observations indicate that NO dominates control of basal endothelium-mediated vasodilator tone, and the integrated hyperemic responses to endothelial stimulation by both ACh and BK, in the renal circulation of the rat *in vivo*. EDHF does contribute to the initial phase of ACh- and BK-induced renal vasodilation, but whether EDHF is under “blockade” by NO has yet to be resolved. Prostanoids appear to make little net contribution. Integrated renal hyperemic responses to endothelial stimulation are blunted in diabetes, due chiefly to diminished NO function. Thus, NO appears to be the dominant player in renal endothelial vasodilator function in the healthy kidney, and reduced NO function appears to be the dominant factor in dysfunction of endothelium-dependent vasodilation in type 1 diabetes. Whether this reflects reduced NO release, bioavailability, and/or reduced sensitivity of the vasculature to NO, remains to be determined.

**PERSPECTIVES AND SIGNIFICANCE**

The vascular endothelium is a major regulator of organ blood flow. Vascular beds in different parts of the body rely to differing extents on one or more endothelial vasodilators for regulation of tissue perfusion. The relative contributions of these factors to endothelium-dependent vasodilation in a tissue may also vary depending on species. Vasodilation in larger vessels is generally mediated by a combination of NO and
prostacyclin, with the role of EDHF becoming more prominent in smaller arteries and arterioles. In the rat renal circulation NO and EDHF are the dominant players. In diabetes, oxidative stress and reactive oxygen species will directly target NO, reducing its bioavailability and role in vasodilation. Whether or not EDHF is also vulnerable to this insult depends on the nature of EDHF, which varies across the circulation. In diabetes EDHF may serve as mechanism by which to sustain to some extent, organ perfusion in the presence of a reduced NO influence. However, significant loss of both endothelial vasodilator pathways may ultimately contribute to end organ damage and failure.
Acknowledgements

This work was supported by grants from the National Health and Medical Research Council of Australia (143603, 143785, 384101 and 236872) and the Diabetes Australia Research Trust. The authors would like to thank Dr Harry Coleman for his help in preparing this manuscript.
REFERENCES


22. **Mieyal P, Fulton D, McGiff JC, and Quilley J.** NO-independent vasodilation to acetylcholine in the rat isolated kidney utilizes a charybdotoxin-sensitive, intermediate-conductance 


42. **Zygmunt PM, and Hogestatt ED.** Role of potassium channels in endothelium-dependent relaxation resistant to nitroarginine in the rat hepatic artery. *Br J Pharmacol* 117: 1600-1606, 1996.
Figure Legends

Figure 1: Responses of mean arterial pressure and renal blood flow to intravenous injection of \( N^o\)-nitro- \( L\)-arginine methylester (L-NAME) and indomethacin. L-NAME was administered as a bolus followed by an infusion, whereas indomethacin was administered as a bolus only. Data represent the mean ± SE of averages over 60 s periods in control (\( n = 7 \)) and diabetic (\( n = 8 \)) rats. * \( P < 0.01 \) for the interaction term (group×time) from repeated measures ANOVA.

Figure 2: Basal systemic and renal hemodynamic variables in control and diabetic rats. Responses were recorded under basal conditions, in the presence of L-NAME and indomethacin only and finally in the additional presence of charybdotoxin (ChTx) and apamin. \( n = 8 \) each for control and diabetic groups. MAP = mean arterial pressure, HR = heart rate, RBF = renal blood flow, RVC = renal vascular conductance.

Figure 3: Typical responses evoked by renal arterial injection of acetylcholine (ACh) in a control rat. Responses to ACh (25 pmol/kg) were evoked under basal conditions, after administration of L-NAME + indomethacin, and then in the presence of ChTx + apamin. Mean arterial pressure (upper panels), heart rate (middle panels), and renal blood flow (lower panels) are shown as 2 s averages.

Figure 4: Typical responses evoked by renal arterial injection of bradykinin (BK) in a control rat. Responses to BK (25 pmol/kg) were tested under basal conditions, after administration of L-NAME + indomethacin, and then in the presence of ChTx + apamin.
Figure 5: Maximum (peak) increases and decreases in renal blood flow (RBF) in response to ACh (15-25 pmol/kg, left panels) and BK (15-25 pmol/kg, right panel) in control ($n = 8$) and diabetic ($n = 9$) rats. Shown are RBF responses evoked by ACh and BK under basal conditions, in the presence of L-NAME plus indomethacin and after subsequent administration of ChTx plus apamin. * $P < 0.05$ control versus diabetic rats prior to L-NAME plus indomethacin (basal responses). † $P < 0.05$, †† $P < 0.01$, for comparison with basal responses within the same treatment group. ‡ $P < 0.05$ for comparison of responses during infusion of ChTx plus apamin versus responses after treatment with L-NAME plus indomethacin.

Figure 6: Integrated (area under the curve) RBF responses evoked by ACh and BK. Shown are RBF responses recorded in control ($n = 8$) and diabetic ($n = 9$) rats under basal conditions, following exposure to L-NAME plus indomethacin and after subsequent administration of ChTx plus apamin. * $p<0.05$ basal responses in control vs diabetic rats. †† $p<0.01$, ††† $p<0.001$ for comparison of RBF responses after inhibitor treatment with those under basal conditions within the same treatment group.

Figure 7: Effects of progressive administration of indomethacin and L-NAME on RBF responses evoked by ACh and BK in control rats. Peak changes (top panels) and integrated (area under the curve) responses (bottom panels) are shown. Shown are responses recorded under basal conditions, after indomethacin administration, and after subsequent exposure to L-NAME (L+I). † $P < 0.05$ for comparison with responses under basal conditions within the same treatment group.
**Figure 8:** Effects of charybdotoxin and apamin on RBF responses evoked by ACh and BK in control animals \((n = 5)\). Peak changes (top panels) and integrated (area under the curve) responses (bottom panels) are shown. Shown are RBF responses under basal conditions, in the presence of ChTx plus apamin (Toxins), and after recovery from ChTx plus apamin infusion (Recovery).

**Figure 9:** Analysis of integrated hyperemic and ischemic responses to ACh and BK in control \((n = 8)\) and diabetic \((n = 9)\) rats. The integrated response of RBF in diabetic rats is represented as a % of the mean response in control rats (designated as 100%). Shown are the proportions of the response attributable to NO, EDHF and the residual (including the ischemic) portion of the response not attributable to NO, prostanoids, or EDHF.
**Table 1 Legend**

Data are presented as the mean ± SE of variables averaged over the 3 min before responses to bradykinin and acetylcholine were tested. Data are shown for basal conditions, and after administration of various combinations of L-NAME, indomethacin (Indo), and ChTx plus apamin. MAP, mean arterial pressure; HR, heart rate; RBF, renal blood flow; RVC, renal vascular conductance. † $P \leq 0.05$, ††† $P \leq 0.001$ for comparison with basal within the same group of rats (Tukey test). ‡ $P \leq 0.05$ for comparison of the third treatment period with the second treatment period within the same group of rats (Tukey test).
Table 1  Basal systemic and renal hemodynamic variables in protocols 1, 2 and 3.

<table>
<thead>
<tr>
<th>Protocol 1</th>
<th>n</th>
<th>MAP (mmHg)</th>
<th>HR (bpm)</th>
<th>RBF (ml min⁻¹ g dry kidney⁻¹)</th>
<th>RVC (µl min⁻¹ mmHg⁻¹ g dry kidney⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>88±4</td>
<td>353±14</td>
<td>16.9±1.6</td>
<td>193±17</td>
</tr>
<tr>
<td>L-NAME + Indo</td>
<td>8</td>
<td>140±5†††</td>
<td>330±11</td>
<td>6.7±1.0†††</td>
<td>48±6†††</td>
</tr>
<tr>
<td>ChTx + apamin</td>
<td>8</td>
<td>127±7†††</td>
<td>313±12</td>
<td>5.3±0.6†††</td>
<td>41±4†††</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protocol 2</th>
<th>n</th>
<th>MAP (mmHg)</th>
<th>HR (bpm)</th>
<th>RBF (ml min⁻¹ g dry kidney⁻¹)</th>
<th>RVC (µl min⁻¹ mmHg⁻¹ g dry kidney⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>112±4</td>
<td>378±24</td>
<td>18.1±1.8</td>
<td>160±12</td>
</tr>
<tr>
<td>Indo</td>
<td>4</td>
<td>106±5</td>
<td>385±20</td>
<td>17.9±1.5</td>
<td>170±19</td>
</tr>
<tr>
<td>Indo + L-NAME</td>
<td>4</td>
<td>119±13</td>
<td>307±12‡</td>
<td>6.7±0.6†‡</td>
<td>59±11†‡</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protocol 3</th>
<th>n</th>
<th>MAP (mmHg)</th>
<th>HR (bpm)</th>
<th>RBF (ml min⁻¹ g dry kidney⁻¹)</th>
<th>RVC (µl min⁻¹ mmHg⁻¹ g dry kidney⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>88±5</td>
<td>378±23</td>
<td>17.1±1.2</td>
<td>197±18</td>
</tr>
<tr>
<td>ChTx + Apamin</td>
<td>5</td>
<td>108±5</td>
<td>359±19</td>
<td>17.8±1.3</td>
<td>166±14</td>
</tr>
<tr>
<td>Recovery</td>
<td>5</td>
<td>106±7</td>
<td>377±18</td>
<td>17.2±1.7</td>
<td>161±10</td>
</tr>
</tbody>
</table>
Figure 2

MAP

HR

RBF

RVC

Control

Diabetic

mmHg

bpm

ml min\(^{-1}\) g dry kidney\(^{-1}\)

μl min\(^{-1}\) mmHg\(^{-1}\) g dry kidney\(^{-1}\)

Basal

N&I

ChTx & apamin
Figure 3

Acetylcholine

<table>
<thead>
<tr>
<th>Basal</th>
<th>L-NAME + Indomethacin</th>
<th>ChTx + Apamin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Arterial Pressure (mmHg)</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Renal Blood Flow (ml/min)</td>
<td>12</td>
<td>9</td>
</tr>
</tbody>
</table>

Time from intra-renal bolus (secs)
Figure 4

Bradykinin

Basal

Mean Arterial Pressure (mmHg)

Heart Rate (bpm)

Renal Blood Flow (ml/min)

Time from intrarenal bolus (secs)

L-NAME + Indomethacin

ChTx + Apamin

Time from intrarenal bolus (secs)
FIGURE 5

Peak decrease in Renal Blood Flow (ml.min\(^{-1}\).g dry kid wt\(^{-1}\))

-1.5
-1.0
-0.5
0.0
0.5
1.0

Peak increase in Renal Blood Flow (ml.min\(^{-1}\).g dry kid wt\(^{-1}\))

0.0
2.0
4.0
6.0
8.0

Basal
L-NAME + Indo
+ ChTx + Apamin

Control Diabetic
Control Diabetic
Control Diabetic

Acetylcholine
Bradykinin
FIGURE 6

Renal Blood Flow Area under the curve (ml/ g dry ktg wt)

Acetylcholine

- Basal
- L-NAME + Indo
- + ChTx + Apamin

Bradykinin

- Basal
- L-NAME + Indo
- + ChTx + Apamin

Control  Diabetic  Control  Diabetic
FIGURE 7

[Graph showing the peak change and area under the curve for renal blood flow with respect to Acetylcholine and Bradykinin under Basal, Indo, and L+ I conditions.]
FIGURE 8

Peak change renal blood flow (ml.min\(^{-1}\).g dry kid wt\(^{-1}\))

Acetylcholine

Bradykinin

Area under curve renal blood flow (ml. g dry kid wt\(^{-1}\))

Basal Toxins Recovery

Basal Toxins Recovery
FIGURE 9

[Graph showing blood flow area under curve (% total of basal) for Acetylcholine and Bradykinin in Control and Diabetic groups.]

- **Acetylcholine**
  - Control
  - Diabetic

- **Bradykinin**
  - Control
  - Diabetic

Legend:
- NO
- EDHF
- Residual