Type 1 neuropeptide Y receptors and α₁-adrenoceptors in the neural control of regional renal perfusion

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Submitted 4 May 2005; accepted in final form 22 September 2005

Eppel, Gabriela A., Susan E. Luff, Kate M. Denton and Roger G. Evans. Type 1 neuropeptide Y receptors and α₁-adrenoceptors in the neural control of regional renal perfusion. Am J Physiol Regul Integr Comp Physiol 290: R331–R340, 2006. First published September 29, 2005; doi:10.1152/ajpregu.00317.2005.—The aim of this study was to determine the contribution of neuropeptide Y (NPY) Y₁ receptors in neurally mediated reductions in renal medullary perfusion. In pentobarbital sodium-anesthetized rabbits, electrical stimulation of the renal nerves (RNS, 0.5–16 Hz) decreased renal perfusion in a frequency-dependent manner. Under control conditions, 4 Hz reduced cortical and medullary perfusion by ~85 ± 3% and ~43 ± 7%, whereas 8 Hz reduced them by ~93 ± 2% and ~73 ± 4%, respectively. After Y₁ receptor antagonism with BIBO3304TF (0.1 mg/kg plus 0.2 mg kg⁻¹ h⁻¹), renal reduced perfusion less (by ~65 ± 9% and ~12 ± 8% at 4 Hz). α₁-Adrenoceptor antagonism with prazosin (0.2 mg/kg plus 0.2 mg kg⁻¹ h⁻¹) also inhibited RNS-induced reductions in renal perfusion (~80 ± 4% and ~37 ± 10% reductions in the cortex and medulla, respectively, at 8 Hz). When given after BIBO3304TF treatment, prazosin inhibited RNS-induced reductions in cortical and medullary perfusion more profoundly (~57 ± 12% and ~25 ± 9% reductions, respectively, at 8 Hz). Y₁ receptor- and α₁-adrenoceptor-blockade was confirmed by testing vascular responses to renal arterial NPY and phenylephrine boluses. NPY-positive immunolabeling was observed around interlobular arteries, afferent and efferent arterioles, and in the outer medulla. In conclusion, Y₁ receptors and α₁-adrenoceptors contribute to RNS-induced vasoconstriction in the vessels that control both cortical and medullary perfusion. Consistent with this, NPY immunostaining was associated with blood vessels that control perfusion in both regions. There also seems to be an interaction between Y₁ receptors and α₁-adrenoceptor-mediated neurotransmission in the control of renal perfusion.

renal medullary blood flow; sympathetic nervous system; BIBO3304TF

ONLY ~10% of renal blood flow (RBF) perfuses the medulla, yet renal medullary perfusion appears to play a key role in the regulation of urinary salt and water excretion, and so long-term control of arterial pressure (3). Neural and hormonal factors are known to influence medullary blood flow differently from blood flow in the bulk of the renal cortex (12). For example, a considerably greater intensity of sympathetic nerve activation is required to reduce medullary blood flow than is required to reduce total RBF or outer cortical blood flow (11).

The sympathetic cotransmitter neuropeptide Y (NPY) has been observed in close association with the renal vasculature of various species such as the guinea pig (1, 30), rat (1), monkey, and human (1, 27). It is well documented that NPY contributes to global renal vasoconstriction induced by electrical stimulation of the renal nerves (RNS) via Y₁ receptors (4, 21). However, the sympathetic neurotransmitters that contribute to the neural control of medullary perfusion remain to be determined. A role for NPY seems likely, as immunohistochemical studies have localized NPY within the juxtaglomerular and medullary regions (27, 30). Therefore, the current study was designed to test the hypothesis that NPY contributes to neurally mediated vasoconstriction in the medullary circulation via Y₁ receptors. To this end, the effects of the Y₁ antagonist BIBO3304TF on reductions in medullary laser-Doppler flux (MLDF) in response to RNS in anesthetized rabbits were determined. The distribution of NPY along the rabbit renal vasculature was also examined, because this had not previously been documented. In the kidney, activation of postjunctional NPY Y₁ receptors is known to potentiate the effects of neurally released norepinephrine on vascular tone (28). On the other hand, activation of prejunctional Y₁ receptors can inhibit norepinephrine release from renal sympathetic nerves (28). Hence, it was also hypothesized that neural NPY release would modify medullary blood flow responses to renal sympathetic nerve activation mediated by postjunctional α₁-adrenoceptors.

To test this, the impact of Y₁ receptor blockade on the effects of α₁-adrenoceptor blockade (with prazosin) on RNS-induced reductions in MLDF was determined. To compare the roles of Y₁ receptors and α₁-adrenoceptors in the neural control of medullary blood flow vs. cortical blood flow, total RBF and cortical laser-Doppler flux (CLDF) were also measured in all experiments.

METHODS

Experiments were performed on 24 male rabbits of a crossbred English strain (3.2 ± 0.1 kg). The rabbits were meal fed and were allowed water ad libitum until experimental procedures began. At the conclusion of the experiment, the animals were euthanized with an overdose of pentobarbital sodium (300 mg iv; Sigma, St. Louis, MO). All procedures were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved in advance by the Animal Ethics Committee of the Department of Physiology, Monash University.

Responses to Electrical Stimulation of the Renal Nerves

Preparative procedures. Catheters were inserted into the central ear arteries and marginal ear veins under local analgesia (1% lidocaine, (Xylocaine); Astra Pharmaceuticals, NSW, Australia). Anesthesia was induced by pentobarbital sodium (90–150 mg plus 30–50 mg/h iv) and was followed by endotracheal intubation and artificial ventilation. Throughout surgery Hartmann’s solution (compound sodium lactate;
Baxter Healthcare, Toongabbie, NSW, Australia) was infused at a rate of 0.18 ml kg\(^{-1}\) min\(^{-1}\) to maintain extracellular fluid volume. Esophageal temperature was maintained at 36–38°C.

The left kidney was exposed by a retroperitoneal incision and placed in a stabilized cup to prevent it from moving during the experiment. A catheter was placed in a side branch of the renal artery (suprarenalnurbar artery) (16). Catheter patency was maintained by a continuous infusion of 154 mM NaCl (20 µl·kg\(^{-1}\)·min\(^{-1}\)). The major renal nerve trunks were then identified, placed across a pair of hooked electrodes, and sectioned proximal to the electrode. A mixture of paraffin oil and petroleum jelly was applied to the nerves throughout the experiment to prevent dehydration.

A transit-time ultrasound flow probe (Type 2SB, Transonic Systems, Ithaca, NY) was placed around the left renal artery to measure RBF. A small hole was made in the renal capsule for insertion of a dual-fiber 26-gauge needle laser-Doppler probe (DP4s, Moor Instruments, Millwely, Devon, England), which was advanced using a micromanipulator, so that its tip lay 8–10 mm below the midregion of the lateral surface of the kidney (inner medulla). This probe provided estimates of MLDF, an estimate of medullary perfusion. A standard straight plastic probe (DP2b, Moor Instruments) was placed on the dorsal surface of the kidney, and was held in place with gauze packing. This probe provided values of CLDF, an estimate of cortical perfusion. It should be recognized that laser-Doppler flux chiefly reflects changes in erythrocyte velocity in relatively small volumes of tissue (8, 14). Once the preparative procedures were complete, the rabbits received a 10-ml bolus of a polygeline-electrolyte solution (Haemaccel; Hoechst, Melbourne, Victoria, Australia), and the maintenance infusion of Hartmann’s solution was replaced with a 1:4 parts mixture of polygeline-electrolyte solution and Hartmann’s solution.

An equilibration period of 45 min was allowed before the experiment commenced.

**Experimental protocol.** Three groups of rabbits were studied. Each rabbit was subjected to two or three sequences of RNS (see below).

One group of rabbits (Group 1, \(n = 8\)) served as a time control. Following the first stimulation sequence, they received the first vehicle treatment (distilled water; 0.25 ml/kg plus 0.5 ml·kg\(^{-1}\)·h\(^{-1}\) iv). A 15-min equilibration period was allowed before the second stimulation sequence began. A subset of these animals (\(n = 5\)) underwent a second vehicle treatment (distilled water; 0.5 ml/kg plus 0.5 ml·kg\(^{-1}\)·h\(^{-1}\) iv), followed by a 15-min equilibration period, and then a third and last sequence of RNS. The second group of rabbits (Group 2, \(n = 5\)) was subjected to a similar experimental protocol. Before the second stimulation sequence, the rabbits received the same vehicle treatment as Group 1. These rabbits then received an infusion of the α\(_1\)-adrenoceptor antagonist prazosin (0.2 mg/kg plus 0.2 mg·kg\(^{-1}\)·h\(^{-1}\) iv; Sigma) before the third sequence of nerve stimulation. A third group (Group 3, \(n = 8\)) was subjected to the same protocol but the NPY Y\(_1\) receptor antagonist BIBO3304TF was administered (0.1 mg/kg bolus plus 0.2 mg·kg\(^{-1}\)·h\(^{-1}\) iv; Boehringer Ingelheim, Germany) before the second stimulation sequence. A subset of these rabbits (\(n = 5\)) then received the α\(_1\)-adrenoceptor antagonist prazosin (as for Group 2), before the third stimulation sequence. At the end of the last stimulation sequence, all rabbits were given boluses of phentolamine (100–1,000 ng/kg; Sigma) and then NPY (50–500 ng/kg; Auspep, Parkville, Victoria, Australia) into the renal artery to determine the efficacy of the antagonist treatments. The bolus doses were given in ascending order. After each bolus, renal hemodynamic variables were allowed to recover fully, to their baseline, before the next dose was administered.

RNS was performed using purpose-written software in the LabVIEW graphical programming language (National Instruments, Austin, TX) coupled to a LabPC+ data-acquisition board (National Instruments). Initially, the nerves were stimulated at 5 Hz for short periods at different voltages to determine the voltage giving a maximal RBF response (supramaximal voltage, 5–10 V). Once determined, this voltage was used for the rest of the experiment. Stimulation was performed at six frequencies (0.5, 1, 2, 4 and 8 Hz, presented in random order, and 16 Hz always presented last). Stimulation at each frequency lasted 3 min with 2 ms pulse duration. A recovery period of 8 to 10 min was allowed between each stimulus train.

**Hemodynamic variables.** Mean arterial pressure (MAP) (mmHg) was measured by connecting the ear artery catheters to pressure transducers (Cobe, Arvada, CO) placed at the same height as the rabbit’s heart. The transit-time ultrasound flow probe was connected to a compatible flowmeter (TI08, Transonic Systems) to provide RBF (ml/min). Laser-Doppler flow probes were connected to a laser-Doppler flowmeter (DRT4, Moor Instruments) to provide values of CLDF and MLDF (units). Signals were amplified and recorded as previously described (14). Heart rate (HR, beats/min) was derived from the MAP waveform. During the 60 s after the animal was euthanized, CLDF (7 ± 1 units) and MLDF (21 ± 2 units) were measured. Before analysis, these offset values were subtracted from the values obtained throughout the experiment. The laser-Doppler flow probes were calibrated before use, so they gave a standard laser-Doppler flux of 210 units in the motility standard provided by the manufacturers (Moor Instruments). This standardized the gain of each probe.

**Data analysis.** Baseline values of hemodynamic variables were determined by averaging them over each of the 30-s control periods before each train of electrical stimulation. Reductions in RBF, CLDF, and MLDF in response to RNS were calculated from the average of each variable during the 30 s before the stimulus began (control) and the last 30 s during stimulation. In some animals, RNS at low frequencies increased MLDF during stimulation. In these cases, MLDF responses were recorded as zero. In a small number of cases, MLDF was initially reduced by RNS but then increased to a value greater than baseline before the end of the stimulus train. In these cases, the initial reduction was taken as the response to RNS. Reductions in RBF, CLDF, and MLDF in response to phenylephrine and NPY boluses were calculated from the average of each variable during the 15 s before the stimulus began and the peak reductions observed after bolus administration, for each variable.

**Statistical analysis.** Data are presented as means ± SE, and \(P\) values < 0.05 were considered to be statistically significant. Statistical analysis was performed using the computer program Systat 7.0 (SPSS, Chicago, IL). Baseline hemodynamic data and responses to RNS were subjected to ANOVA (31), the factors comprising rabbit, group (vehicle, prazosin, or BIBO3304TF plus prazosin-treated groups), and treatment period (i.e., control period, BIBO3304TF, prazosin, or the corresponding vehicle treatments). In the case of responses to RNS, the factors frequency (of stimulation), and, in some cases, region (CLDF and MLDF) were also applied. This allowed us to test whether baseline values or responses to RNS (1) differed during the first stimulation sequence between the groups (\(P_{\text{group}}\)), (2) changed during the different treatment periods (\(P_{\text{period}}\)) and 3) whether baseline values changed from one period to another in a different manner in one group compared with another (\(P_{\text{group} \times \text{period}}\)). In addition, it could be determined whether there were stimulus-dependent changes in each variable during RNS (\(P_{\text{frequency}}\)), and whether responses of RBF, CLDF, and MLDF to RNS differed (\(P_{\text{region}}\)).

Responses to the phenylephrine and NPY boluses were subjected to repeated-measures ANOVA (18) with the between-subjects factor, group, and the within subjects factor, dose. This allowed us to test (1) whether there were dose-dependent effects on each variable (\(P_{\text{dose}}\)), and (2) whether responses differed between the three groups that received vehicle, prazosin-, or BIBO3304TF plus prazosin (\(P_{\text{group}}\)).

**Neuropeptide Y Immunohistochemistry**

Three rabbits were anesthetized as described in *Preparative procedures*. MAP was recorded as described above. The abdominal aorta was cannulated in a retrograde direction just distal to the kidneys. A
bolus injection of 3 ml sodium nitrite (1% wt/vol, Sigma) plus 5,000 IU heparin (porcine mucous, MacFarca & UpJohn, Bentley, WA, Australia) was given via the ear vein to dilate the vasculature. The kidneys were initially perfused with saline and then with a fixative solution of 4% formaldehyde with 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), at a pressure equal to MAP recorded before the injection of sodium nitrite. The kidneys were removed and sliced coronally (~5 mm thick) and then sliced further into wedges containing cortex and medulla. These slices were immersed in fixative solution and left overnight at 4°C. Vibratome sections of 75- to 100-μm thickness were prepared for immunolabeling (9 sections per kidney).

Immunolabeling was performed at 4°C. Vibratome sections (five per kidney chosen at random) were washed in phosphate-buffered saline (PBS), then placed in 50% ethanol for 20 min, and then in 10% sodium borohydride in PBS for 30 min. The sections were washed again in PBS. Endogenous peroxidase activity was blocked with hydrogen peroxide (3% vol/vol) and endogenous bion was blocked using an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) together with 10% normal serum (donkey, Sigma). The sections were then incubated in a 1:5,000 dilution of neuropeptide Y (NPY) antibody raised in sheep (Auspep, Parkville, Victoria, Australia) plus 10% serum (donkey) for 36 h. The sections were washed and incubated in biotinylated donkey anti-sheep IgG (Sigma) diluted 1:200 in 10% serum (donkey) for 36 h. The sections were washed and incubated in avidin/biotin horseradish peroxidase complex using an ABC Elite Kit according to the protocol provided (Vector Laboratories). After washing, the sections were transferred to 0.5 mg/ml hydrogen peroxide (3% vol/vol) and endogenous biotin was blocked using a solution containing 1% serum for 2 h. This was followed by incubation in the avidin/biotin horseradish peroxidase complex using a 0.075% (vol/vol) and incubated until the reaction was complete and no further precipitate was formed. The reaction product was intensified with 0.1% osmium tetroxide for 30 min. The sections were then dehydrated in ethanol, infiltrated with Durecupan (Polaron Equipment, Watford, UK), mounted on glass slides in the same resin, and polymerized at 60°C for 48 h. The sections were viewed on an Olympus Provis AX70 microscope to determine the distribution of NPY in the cortical and medullary regions of the kidney. Images were taken at ×10 and ×20 magnification with an Olympus DP70 digital camera.

Control preparations were processed in an identical manner, but either without incubation of the primary antibody (negative controls, 2 sections per kidney) or with primary antibody preabsorbed with NPY (synthesized in the Biochemistry Department, Monash University) (two sections per kidney). The NPY preabsorption was performed overnight at 30°C with incubation of NPY at 10 times the concentration of antibody. All control sections lacked staining of perivascular neural plexi.

### RESULTS

#### Baseline Hemodynamics During the First and Second Stimulation Periods

Baseline values of MAP, HR, RBF, CLDF, and MLDF during the first stimulation period were similar to those previously observed in anesthetized rabbits in the same laboratory (9, 10). These variables did not differ between the three groups of rabbits, with the exception of CLDF (Table 1). There were small changes in baseline hemodynamic variables after treatment with BIBO3304TF or its vehicle. Importantly, there were no statistically significant interactions between stimulation period and group (vehicle or BIBO3304TF treatment), indicating that changes in baseline hemodynamics over the course of the two experimental periods, were similar between the two groups (Table 1, $P_{\text{period} \times \text{group}} \geq 0.5$ for all variables).

### Baseline Hemodynamics During the Second and Third Stimulation Periods

Vehicle treatment (Group 1) had little effect on hemodynamic variables, with the exception of HR, which fell by 8 ± 3% (Table 2). Similar apparent changes in HR were observed in rabbits treated with prazosin (Group 2) and in BIBO3304TF-pretreated rabbits that received prazosin (Group 3). MAP and CLDF were significantly reduced after prazosin (by 23 ± 3% and 19 ± 2%, respectively), but not its vehicle. RBF and MLDF did not change significantly after prazosin treatment, although RBF tended to fall ($P = 0.09$) and MLDF tended to increase ($P = 0.06$). The effects of prazosin were indistinguishable in BIBO3304TF-pretreated rabbits (Group 3), compared with those that received vehicle pretreatment (Group 2) ($P_{\text{group} \times \text{period}} \geq 0.6$ for all variables).

#### Responses to Graded Electrical Stimulation of the Renal Nerves Under Control Conditions

When averaged across all rabbits, RNS, during the first stimulation sequence produced small frequency-dependent increases in MAP (8 ± 1 mmHg at 16 Hz, $P_{\text{frequency}} < 0.001$) and large reductions in RBF (−93 ± 1% at 16 Hz), CLDF (−95 ± 1% at 16 Hz) and MLDF (−72 ± 5% at 16 Hz) ($P_{\text{frequency}}$ always < 0.001), but did not significantly affect HR (Fig. 1). As seen previously (9, 10, 14, 17), RBF and CLDF responses to RNS were more profound than those of MLDF ($P_{\text{region}} < 0.001$).

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Table 1. Changes in baseline hemodynamic variables from the first to the second stimulation period

<table>
<thead>
<tr>
<th>Period</th>
<th>Groups 1 and 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment 1</td>
<td>Treatment 2</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Vehicle 1</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>73 ± 2</td>
<td>+3 ± 2</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>267 ± 5</td>
<td>−16 ± 5*</td>
</tr>
<tr>
<td>RBF, ml/min</td>
<td>34 ± 2</td>
<td>−4 ± 2</td>
</tr>
<tr>
<td>CLDF, U</td>
<td>316 ± 12</td>
<td>−11 ± 8</td>
</tr>
<tr>
<td>MLDF, U</td>
<td>36 ± 5</td>
<td>−1 ± 2</td>
</tr>
</tbody>
</table>

Results are presented as means ± SE of baseline values during the first stimulation period and of changes in baseline values from the first to the second stimulation periods. MAP, mean arterial pressure; HR, heart rate; RBF, renal blood flow; CLDF, cortical laser Doppler flux; MLDF, medullary laser Doppler flux. $P$ values indicate the outcomes of ANOVA with the factors rabbit, period, and group. $P_{\text{group} \times \text{period}}$ indicates whether baseline hemodynamic variables changed from the first period to the second period in a different manner in one group compared to the other. Paired t-tests were also performed to test for significant changes, within each group, between periods 1 and 2. *$P < 0.05$, †$P < 0.005$ vs. corresponding previous period; $n = 13$ for Groups 1 and 2, $n = 8$ for Group 3.
ing responses of CLDF and MLDF to RNS by a maximum of apparent effects of BIBO3304TF treatment were small, reduc-
A sequence (Fig. 2). For example, RBF, CLDF, and MLDF
Effects of BIBO3304TF on Responses to Graded Electrical
Stimulation of the Renal Nerves
Effects of Prazosin on Responses to Graded Electrical
Stimulation of the Renal Nerves

Effects of BIBO3304TF on Responses to Graded Electrical
Stimulation of the Renal Nerves
In rabbits treated with BIBO3304TF, reductions in RBF, CLDF, and MLDF during RNS were blunted during the second stimulation sequence compared with the control stimulation sequence (Fig. 2A). For example, RBF, CLDF, and MLDF were reduced by -72 ± 5%, -79 ± 7%, and -38 ± 12%, respectively at 4 Hz during the control period. During BIBO3304TF treatment, responses to 4-Hz stimulation were -64 ± 5%, -65 ± 9%, and -12 ± 8%, respectively. The apparent effects of BIBO3304TF treatment were small, reduc-
ing responses of CLDF and MLDF to RNS by a maximum of
14 ± 3% and 26 ± 8%, respectively (at 4 Hz). The effects of BIBO3304TF were most evident at 8 Hz for RBF; 2, 4, and 8 Hz for CLDF; and 4 and 8 Hz for MLDF. RBF and CLDF responses to RNS were also significantly blunted during vehicle administration relative to the control stimulation sequence, and there was a tendency for MLDF responses to also be blunted by vehicle treatment.

To test whether the effects of BIBO3304TF administration on RBF, CLDF and MLDF responses to RNS were greater than the effects of vehicle administration, we determined the differences in the responses to RNS during the second stimulation sequence relative to the first (i.e., response to the second sequence of stimulation minus the response to the first stimulation sequence, Fig. 2B). Differences greater than zero indicate that the responses to RNS were blunted in the second stimulation sequence relative to the first. When analyzed across frequencies ≥ 4 Hz, the effect of BIBO3304TF on RBF and CLDF responses was greater than the effect of vehicle treatment (P_{group ≤ 0.04}). For example, at 8 Hz, the differences in responses between the first and second stimulation sequences were 8 ± 3% and 6 ± 4% for RBF and CLDF, respectively, in the BIBO3304TF-treated group. The corresponding values in the vehicle-treated group were only 2 ± 1% and 0 ± 2%. This analysis indicates that BIBO3304TF treatment blunted responses of RBF and CLDF over and above the effect of vehicle treatment or time. When analyzed in this manner, the effects of BIBO3304TF on MLDF responses to RNS were not statistically distinguishable from the effects of vehicle treatment.

In vehicle-treated rabbits, RBF, CLDF, and MLDF re-
sponses remained stable over the course of the second and third stimulation sequences (Fig. 3A). Thus the differences between the responses during the third stimulation sequence relative to the second stimulation sequence were close to zero throughout the frequency range (Fig. 3B). This indicates that the changes in renal hemodynamic responses to RNS in the groups of

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Table 2. Changes in baseline hemodynamic variables from the second to the third stimulation period

<table>
<thead>
<tr>
<th>Period</th>
<th>Group 1</th>
<th>P (group × period)</th>
<th>Group 2</th>
<th>P (group × period)</th>
<th>Group 3</th>
<th>P (group × period)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle 1</td>
<td>Vehicles 1 and 2</td>
<td>Vehicle 1 and Prazosin</td>
<td>Group 1 vs. Group 2</td>
<td>BIBO3304TF and Prazosin</td>
<td>Group 2 vs. Group 3</td>
</tr>
<tr>
<td></td>
<td>Baseline</td>
<td>Change</td>
<td>Baseline</td>
<td>Change</td>
<td>Baseline</td>
<td>Change</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>73 ± 4</td>
<td>+2 ± 4</td>
<td>75 ± 3</td>
<td>-14 ± 2*</td>
<td>0.009</td>
<td>76 ± 4</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>254 ± 6</td>
<td>-21 ± 7*</td>
<td>253 ± 10</td>
<td>-17 ± 7</td>
<td>0.8</td>
<td>270 ± 7</td>
</tr>
<tr>
<td>RBF, ml/min</td>
<td>30 ± 4</td>
<td>-4 ± 2</td>
<td>30 ± 2</td>
<td>-8 ± 4</td>
<td>0.5</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>CLDF, Units</td>
<td>270 ± 17</td>
<td>-3 ± 27</td>
<td>328 ± 7</td>
<td>-77 ± 10†</td>
<td>0.05</td>
<td>330 ± 16</td>
</tr>
<tr>
<td>MLDF, Units</td>
<td>21 ± 4</td>
<td>+8 ± 3</td>
<td>43 ± 13</td>
<td>+16 ± 6</td>
<td>0.7</td>
<td>39 ± 6</td>
</tr>
</tbody>
</table>

Results are presented as means ± SE of baseline values during the second stimulation period and of changes in baseline values from the second to the third stimulation periods. P_{group × period} indicates the outcome of ANOVA testing, whether baseline hemodynamic variables changed from the second period to the third period in a different manner in one group compared to the other. The effects of prazosin alone were compared to the effects of vehicle treatment (Group 1 vs. 2) and the effects of prazosin in the presence of BIBO3304TF were compared to the effects of prazosin alone (Groups 2 vs. 3). Paired t-tests were also performed to test for significant changes within each group, between periods 2 and 3. *P < 0.05, †P < 0.005, ‡P < 0.001 vs. corresponding previous period; n = 5 for Group 1; n = 5 for Group 2; and n = 5 for Group 3.
rabbits given prazosin are unlikely to be due to changes in the preparation over time or to the effects of vehicle administration. In vehicle-pretreated rabbits, renal hemodynamic responses to RNS were blunted after prazosin treatment (Fig. 3A). The differences between responses to the third and second stimulation sequences increased progressively over the frequency range from 0.5 to 4 Hz, but were less at 8 and 16 Hz compared with 4 Hz (Fig. 3B). Similarly, in BIBO3304TF-pretreated rabbits, the differences between renal hemodynamic responses to the third and second stimulation sequence also increased progressively from 0.5 to 4 Hz (0.5 to 8 Hz for MLDF). However, in contrast to rabbits treated with prazosin alone, the differences were sustained from 4 to 16 Hz (8 to 16 Hz for MLDF) in BIBO3304TF-pretreated rabbits (Fig. 3B). Thus at frequencies \( \geq 8 \) Hz, the effects of prazosin were more pronounced after BIBO3304TF pretreatment than after vehicle pretreatment. For example, in the BIBO3304TF-pretreated group, prazosin blunted RBF, CLDF, and MLDF responses to 8 Hz by 37 ± 5%, 38 ± 9%, and 55 ± 4%, respectively. In contrast, in vehicle-pretreated rabbits, prazosin reduced RBF, CLDF, and MLDF responses to 8 Hz by only 23 ± 2%, 15 ± 8%, and 28 ± 9%, respectively.

**Responses to Renal Arterial Phenytoin Boluses**

In vehicle-treated rabbits, renal arterial administration of phenylephrine produced transient and dose-dependent reductions in RBF, CLDF, and MLDF (Fig. 4) with no significant changes in MAP or HR (data not shown). At the highest dose (1 \( \mu g/kg \)), peak reductions were \(-86 \pm 12\%\), \(-74 \pm 18\%\), and \(-63 \pm 10\%\), respectively (Fig. 4). Phenylephrine-induced reductions in MLDF to those of CLDF were not significantly different (\( P_{\text{region}} = 0.2 \)). In rabbits treated with prazosin, alone or with BIBO3304TF, RBF, CLDF, and MLDF, responses to phenylephrine were blunted. Under these conditions, the highest dose of phenylephrine reduced RBF, CLDF, and MLDF by only \(-29 \pm 9\%\), \(-26 \pm 8\%\), and \(-16 \pm 5\%\), respectively (\( P_{\text{group}} \leq 0.03 \)). Hence prazosin treatment, alone, or in com-
bination with BIBO3304TF, provided significant antagonism of \(\alpha_1\)-adrenoceptors in the renal vasculature.

**Responses to Renal Arterial Neuropeptide Y Boluses**

In vehicle-treated rabbits, renal arterial administration of NPY produced transient and dose-dependent reductions in RBF, CLDF, and MLDF (Fig. 5) with no significant changes in MAP or HR (data not shown). NPY-induced reductions in MLDF were not significantly different to those of CLDF (\(P_{region} = 0.6\)). In rabbits treated with vehicle or prazosin alone, NPY at the highest dose (0.5 \(\mu g/kg\)) reduced RBF, CLDF, and MLDF by \(-79 \pm 4\%\), \(-72 \pm 8\%\), and \(-55 \pm 5\%\), respectively. In BIBO3304TF plus prazosin-treated rabbits, the responses to exogenous NPY were reduced to \(-17 \pm 3\%\), \(-12 \pm 3\%\), and \(-16 \pm 5\%\), respectively, at the highest dose (\(P_{group} \leq 0.01\)). These data indicate that there was profound antagonism of NPY Y1 receptors in the renal vasculature of rabbits treated with BIBO3304TF.

**Fig. 3.** Regional renal vascular responses to electrical stimulation of the renal nerves during the second and third stimulation periods in vehicle-, prazosin- and BIBO3304TF plus prazosin-treated rabbits (\(n = 5\) for each group). The data are presented as means \(\pm\) SE of the percentage changes in each variable at each frequency \((A)\) and percentage changes in each variable during the stimulation sequence minus responses during the second stimulation sequence \((B)\). \(P_{period}\) indicates the outcomes of ANOVA testing for significant changes in responses during the third stimulation sequence relative to the second stimulation sequence. \(P_{group \times freq}\) indicates the outcomes of ANOVA, testing whether the effects of prazosin differed in rabbits pretreated with BIBO3304TF compared with vehicle-pretreated rabbits throughout the frequency range. The \(P\) values represent the outcomes for renal blood flow, cortical laser-Doppler flux, and medullary laser-Doppler flux for each group.
Immunolabeling of Neuropeptide Y Along the Renal Vasculature

NPY-positive labeling of axon terminals was observed in the axon plexus around arterial vessels, including the interlobular, afferent, and efferent arterioles throughout the cortex, including the juxtamedullary region (Fig. 6). There was no NPY staining associated with the glomerular capillaries (Fig. 6B). In the outer medulla, dense NPY-positive staining was mainly confined to the area surrounding the vasa recta bundles with occasional lateral extensions (see Fig. 6C). NPY-positive staining did not extend into the inner medulla.

DISCUSSION

The aim of the current study was to determine the contribution of Y1 receptors, as well as any interaction between Y1 receptors and α1-adrenoceptors, in the neural control of regional kidney perfusion. The data suggest that NPY makes a small net contribution, at least to neurally mediated vasoconstriction in the cortical circulation, via Y1 receptors, because BIBO3304TF treatment reduced responses of CLDF to RNS by a maximum of 14 ± 3%. Antagonism of α1-adrenoceptors with prazosin attenuated reductions in laser-Doppler flux in response to RNS in both the cortex and medulla, demonstrating that α1-adrenoceptors mediate neurally induced vasoconstriction in the vascular elements that control both cortical and medullary blood flow. The most novel finding was that BIBO3304TF pretreatment enhanced the effect of prazosin on renal vascular responses to RNS, at least at 8 and 16 Hz. This was true for RBF, CLDF, and MLDF, indicating roles for NPY in the neural control of both cortical and medullary blood flow. One interpretation of these data is that under conditions of blockade of postjunctional α1-adrenoceptors, NPY acting at postjunctional Y1 receptors makes an enhanced contribution to neurally mediated vasoconstriction at higher frequencies of RNS. However, the fact that NPY and norepinephrine often act synergistically at postjunctional and/or extrajunctional sites argues against this explanation (15, 28). Another possibility is that blockade of prejunctional Y1 receptors by BIBO3304TF-enhanced neural norepinephrine release under the conditions of the current experiment. This hypothesis is consistent with the finding of reduced RNS-induced overflow of [3H]-norepinephrine in the rat isolated perfused kidney in response to Y1 receptor activation (15, 28). An important implication of this hypothesis is that the effects of BIBO3304TF alone observed in the current study might underestimate the contribution of postjunctional Y1 receptors in neurally mediated renal vasoconstriction.
Consistent with the observed effects of Y₁ receptor blockade on both CLDF and MLDF responses to RNS, we observed a dense plexus of NPY staining around the renal vasculature, including interlobular arteries, and afferent and efferent arteries of both cortical and juxtamedullary nephrons, but not the glomerular capillaries. The NPY-positive plexus extended from the efferent arterioles of the juxtamedullary nephrons into the outer medulla and was associated with the vasa recta. The distribution of NPY has not previously been examined in the rabbit kidney. However, the current findings are consistent with the distribution of NPY along the renal vasculature of other species, including humans (1, 30).

The effects of BIBO3304TF on renal vascular responses to RNS were greatest at higher frequencies (greater than 4 Hz). This is consistent with previous observations of the effects of Y₁ receptor blockade on RBF responses to RNS (4) and may be due to the localization of NPY within large dense cored axon vesicles that preferentially release their contents in response to high-frequency stimulation (32). It is difficult to reconcile responses to RNS with responses to changes in endogenous renal sympathetic nerve activity. The RNS protocol applied in this study activates all or most renal nerves in a square wave pattern. In contrast, physiological nerve activity consists of a bursting pattern with variations in both burst frequency and amplitude, probably reflecting selective recruitment of axons (22, 23). Therefore, future studies should test whether Y₁ receptors contribute to responses of the renal circulation to reflex maneuvers that alter sympathetic drive. Nonetheless, the fact that NPY contributes to responses to RNS at the higher frequencies suggests that NPY is unlikely to be important in the neural control of the renal vasculature under resting conditions. However, NPY may be more likely to come into play under conditions of elevated renal nerve activity, as occurs during hemorrhage (24) and during activation of diving type (nasopharyngeal) reflexes (6), where renal sympathetic nerve activity can increase three- to fourfold.

The conclusions drawn from this study depend on the efficacy and selectivity of prazosin and BIBO3304TF. We tested this directly at the conclusion of the studies by administration of bolus doses of phenylephrine and NPY. Prazosin treatment significantly attenuated RBF, CLDF, and MLDF responses to phenylephrine, indicating that there was effective antagonism of α₁-adrenoceptors. Prazosin has high affinity for all identified α₁-adrenoceptor subtypes and low affinity for...
\(\alpha_{2A}\)-adrenoceptors, but higher affinity for \(\alpha_{2B}\) and \(\alpha_{2C}\) subtypes (5, 19, 26). \(\alpha_{2}\)-Adrenoceptors in the rabbit kidney appear to be a single population with low affinity for prazosin, most likely of the \(\alpha_{2A}\)-subtype (5, 26). Consistent with this, in a previous study, we demonstrated that prazosin, at the same dose as used in the current study, did not inhibit renal vasoconstriction in response to the \(\alpha_{2}\)-adrenoceptor agonist guanabenz (10). Thus we conclude that prazosin provided effective and selective antagonism at \(\alpha_{1}\)-adrenoceptors and that \(\alpha_{2}\)-adrenoceptor mediated autoinhibition of neurotransmitter release was most likely intact under the current experimental conditions. We can also be confident that the effects of prazosin on renal vascular responses to RNS were not secondary to effects of this agent on baseline MAP or renal vascular tone. We have shown previously that modest reductions in MAP (10) or RBF (13), within the range produced by prazosin in the current study, have little impact on responses of RBF, CLDF, or MLDF to RNS. Furthermore, the effects of prazosin on baseline systemic and renal hemodynamic variables were similar in BIBO3304TF-pretreated and vehicle-pretreated rabbits, yet the effects of prazosin on responses to RNS differed considerably between these two groups of rabbits. Thus we can also be confident that the effects of prazosin on baseline hemodynamic variables did not confound our conclusions regarding interactions between \(\alpha_{1}\)-adrenoceptors and \(Y_1\) receptors in the neural control of intrarenal blood flow.

BIBO3304TF virtually abolished renal vasoconstriction in response to exogenous NPY. BIBO3304TF is highly selective for \(Y_1\) receptors and has low affinity for \(Y_2\), \(Y_4\), and \(Y_5\) receptors (7, 33). Thus we conclude that BIBO3304TF provided effective and selective antagonism at \(Y_1\) receptors, and that \(Y_2\) receptor-mediated autoinhibition of neurotransmitter release (20) was likely intact under the current experimental conditions. There is evidence that \(Y_1\) receptors may also mediate inhibition of norepinephrine release in the isolated rat kidney (28), in the pig kidney in vivo (29), and other vascular beds such as the rat mesenteric vascular bed (25). Therefore, it is possible that presynaptic \(\alpha_1\)-adrenoceptor-mediated inhibition of norepinephrine release may have been blocked by BIBO3304TF under the current experimental conditions. If this were the case, BIBO3304TF would both blunt NPY-induced vasoconstriction, and enhance norepinephrine release in response to RNS. This would increase the relative contribution of norepinephrine to RNS-induced renal vasoconstriction. Subsequent blockade of \(\alpha_1\)-adrenoceptors with prazosin could thus have a greater impact on responses to RNS than it would under conditions of intact prejunctional (and postjunctional) \(Y_1\) receptor signaling. This could explain why pretreatment with BIBO3304TF enhanced the effects of prazosin in the present study. An important corollary to this hypothesis is that blockade of prejunctional \(\alpha_1\)-adrenoceptors by BIBO3304TF would also lead to an underestimation of the role of NPY in vasoconstrictor responses to RNS, as the effect of BIBO3304TF on postjunctional \(Y_1\) receptors would be masked by enhanced norepinephrine release. This hypothesis is consistent with the current findings, and could be tested in future studies by investigating the impact of BIBO3304TF on renal norepinephrine spillover in response to RNS.

One of the initial hypotheses of this study was that differences in \(Y_1\) receptor-mediated neurotransmission, between the vessels that control blood flow in the cortex and the medulla, might contribute to the distinct responses of cortical and medullary perfusion to activation of the renal nerves. The present results allow us to confidently reject this hypothesis, since BIBO3304TF did not have a greater impact on responses of MLDF to RNS than on responses of CLDF.

Antagonism of \(\alpha_1\)-adrenoceptors with prazosin attenuated vascular responses to RNS in both the cortex and medulla, demonstrating that \(\alpha_1\)-adrenoceptors mediate neurally induced vasoconstriction in both vascular territories. This is consistent with previous observations of the effects of prazosin on regional blood flow responses to RNS using the hydrogen wash-out technique (2).

In conclusion, NPY \(Y_1\) receptors can contribute to neurally mediated vasoconstriction in vascular elements controlling cortical and medullary perfusion. Consistent with this, NPY was observed along the length of the renal vasculature from the interlobular arteries to the efferent arterioles, excluding the glomeruli. In the case of the juxtamedullary nephrons, NPY-positive neurons extend down to the outer medulla but not the inner medulla. \(\alpha_1\)-Adrenoceptors are important mediators of reductions in both cortical and medullary blood flow induced by RNS. There appear to be interactions between the two neurotransmitter pathways in the modulation of renal perfusion, which become evident in the cortical and the medullary responses to nerve stimulation when both pathways are interrupted sequentially.

ACKNOWLEDGMENTS

BIBO3304TF was a gift from Boehringer Ingelheim Pharma KG, Germany. NPY used for the immunohistochemistry was a gift from Dr. Neela Kotecha, formerly of the Department of Physiology, Monash University.

GRANTS

This work was supported by grants from the National Health and Medical Research Council of Australia (143603, 143785, 236821), the Ramaciotti Foundations (A 6370, RA159/98, RA032/01) and by a Postdoctoral Fellowship from the National Heart Foundation of Australia (PF04M 1758).

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