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Neuropeptide Y_2 receptors are involved in enhanced neurogenic vasoconstriction in spontaneously hypertensive rats

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1 The present study addressed the role of neuropeptide (NPY) Y_2 receptors in neurogenic contraction of mesenteric resistance arteries from female spontaneously hypertensive rats (SHR). Arteries were suspended in microvascular myographs, electrical field stimulation (EFS) was performed, and protein evaluated by Western blotting and immunohistochemistry.

2 In vasopressin-activated endothelium-intact arteries, NPY and fragments with selectivity for Y_1 receptors, [Leu^{31},Pro^{34}]NPY, Y_2 receptors, NPY(13–36), and rat pancreatic polypeptide evoked more pronounced contractions in segments from SHR than in Wistar Kyoto (WKY) arteries, even in the presence of the Y_1 receptor antagonist, BIBP3226 (0.3 μM, (*R*)-*N*(2)-(diphenacetyl)-*N*-[(4-hydroxyphenyl)methyl]D-arginineamide).

3 In the presence of prazosin and during vasopressin activation, EFS-evoked contractions were larger in arteries from SHR compared to WKY. EFS contractions were enhanced by the Y_2 receptor selective antagonist BIIE0246TF (0.5 μM, (*S*)-*N*2-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6h)-oxodibenz[b,e]azepin-11-y1]-1-piperazinyl]-2-oxoethyl]cyclo-pentyl-*N*-[2-[1,2-dihydro-3,5 (4*H*)-dioxo-1,2-diphenyl-3*H*-1,2,4-triazol-4-yl]ethyl]-argininamide), reduced by BIBP3226, and abolished by the combination of BIBP3226 and BIIE0246TF.

4 Immunoblotting showed NPY Y_1 and Y_2 receptor expression to be similar in arteries from WKY and SHR, although a specific Y_2 receptor band at 80 kDa was detected only in arteries from WKY.

5 Immunoreaction for NPY was enhanced in arteries from SHR. In contrast to arteries from WKY, BIIE0246TF increased NPY immunoreactivity in EFS-stimulated arteries from SHR.

6 The present results suggest that postjunctional neuropeptide Y_1 and Y_2 receptors contribute to neurogenic contraction of mesenteric small arteries. Moreover, both enhanced NPY content and altered neuropeptide Y_1 and Y_2 receptor activation apparently contribute to the enhanced neurogenic contraction of arteries from SHR.
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Abbreviations: BIIE0246TF, (*S*)-*N*2-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6h)-oxodibenz[b,e]azepin-11-y1]-1-piperazinyl]-2-oxoethyl]-cyclo-pentyl-*N*-[2-[1,2-dihydro-3,5(4*H*)-dioxo-1,2-diphenyl-3-*H*-1,2,4-triazol-4-yl]ethyl]-argininamide; BIBP3226, (*R*)-*N*2-(diphenacetyl)-*N*-(4-hydroxyphenyl) methyl)-D-arginineamide; EFS, electrical field stimulation; KPSS, potassium-rich physiological salt solution; NPY, neuropeptide Y; NTS, nucleus tractus solitarius; rPP, rat pancreatic polypeptide; SHR, spontaneously hypertensive rats; WKY, Wistar Kyoto rats

Introduction

Neuropeptide Y (NPY) is a 36-amino-acid peptide that activates a class of G-protein-coupled NPY receptors, where five classes have been cloned and classified as Y_1, Y_2, Y_4, Y_5, and y_6 on the basis of their molecular and pharmacological profile (Gehlert, 1998; Michel *et al*., 1998). The Y_1, Y_2, and Y_5 receptors preferentially interact with the endogenous ligands NPY and peptide YY, whereas the NPY Y_4 receptor is characterized by its high affinity for rat pancreatic polypeptide (rPP) of the same species (Michel *et al*., 1998). However, characterization of NPY receptors is no longer solely based on use of analogues and fragments of NPY, as highly selective non-peptide NPY receptor antagonists have been developed (Doods *et al*., 1996; 1999; Daniels *et al*., 2002).

NPY is widely distributed in the central and peripheral nervous system and is a potent vasoconstrictor producing contraction of vascular smooth muscle cells either directly or indirectly by potentiating the effects of other vasoconstrictors through the activation of NPY Y_1 receptors (Abounader *et al*., 1995; Prieto *et al*., 1995; 1998; Malmstrom, 1997). In mice lacking the NPY Y_1 receptor, blood pressure and heart rate were similar to those in wild-type animals (Pedrazzini *et al*., 1998). However, these observations do not exclude that NPY plays a role in the regulation of blood pressure. Recently, we found NPY vasoconstriction to be enhanced in isolated arteries from female spontaneously hypertensive rats (SHR) (Gradin *et al*., 2003). This effect can probably be ascribed to an

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upregulation of NPY content in the sympathetic nerves, enhanced inhibition of cyclic AMP-mediated relaxation, and impaired endothelium-dependent inhibition of NPY vasoconstriction in arteries from SHR.

It has been postulated that prejunctional inhibitory effects of NPY are mediated by activation of the NPY Y_2 receptor subtype (Malmstrom *et al*., 2002), whereas NPY released from sympathetic nerves is thought to cause vasoconstriction through activation of postjunctional neuropeptide Y_1 receptors. However, postjunctional Y_2 receptors exist in a variety of tissues and Y_2 mRNA is found in peripheral tissue (Michel *et al*., 1998). Postjunctional neuropeptide Y_2 receptors have been suggested to be involved in angiogenesis (Zukowska-Grojec *et al*., 1998), vasoconstriction (McAuley & Westfall, 1992; Tessel *et al*., 1993; Malmstrom, 2001; Chu *et al*., 2003), and vasorelaxation resistant to the neuropeptide Y_1 receptor antagonist, BIBP3226, in cerebral and penile arteries (You *et al*., 2001; Prieto *et al*., 2004).

Recently, we have observed that the enhanced neurogenic response to electrical field stimulation (EFS) in rat mesenteric arteries from female SHR is not blocked by the NPY Y_1 receptor antagonist, BIBP3226 (Gradin *et al*., 2003). Therefore, in the present study, we hypothesized that NPY Y_2 receptors are involved in the enhanced vasoconstriction of mesenteric small arteries from SHR rats. The receptors involved in vasoconstriction were characterized by use of selective agonists and antagonists for the NPY receptors. Western blots were performed for the Y_1 and Y_2 receptors, and alterations in the prejunctional receptors were addressed by use of EFS and immunohistochemistry performed in the absence and presence of the selective NPY Y_2 receptor antagonist, BIIE0246TF.

Methods

Mechanical recordings

All the procedures performed on animals in the present study were in accordance with Swedish and Danish animal law and regulations. Female SHR and WKY rats (200–250 g) were obtained from Møllegaard Breeding Center, Skensved, Denmark. The rats were anaesthetized with sodium pentobarbital (60 mg kg, i.p.) and the arteries supplying the proximal jejunum were carefully freed from the surrounding tissue. Segments of 2 mm length were threaded onto two stainless-steel wires suspended in microvascular myographs for measurement of isometric tension as previously described (Gradin *et al*., 2003). The myographs were temperature controlled (37°C) and the segments were kept in physiological salt solution (for composition see Gradin *et al*., 2003). Solutions were equilibrated with 5% CO_2 in O_2 to maintain a pH of 7.4. Potassium-rich physiological salt solution (KPSS) was similar to physiological salt solution except that NaCl was exchanged with KCl on equimolar basis. The preparations were stimulated with 124 mM KPSS until reproducible responses were obtained, that is, when the active developed contraction was within 10% of the previous contraction to KPSS. In almost all experiments, this was reached with the third KPSS stimulation.

Experiments were performed in preparations with and without endothelium. The endothelial cells were removed by insertion of a wire into the lumen of the artery and rubbing back and forth for 5 min. Endothelial cell function was assessed by adding acetylcholine (10 μM) in arteries contracted with noradrenaline (10 μM).

The preparations were incubated with prazosin (1 μM) and propranolol (1 μM) to inhibit α- and β-adrenoceptors, and contraction was evoked by addition of vasopression (0.5–0.8 $U\,l^{-1}$). Once the contraction to vasopressin was stable, concentration–response curves were constructed for NPY receptor agonists in the absence and presence of the neuropeptide Y_1 receptor antagonist, BIBP3226 (0.3 μM). Moreover, concentration–response curves for NPY and the NPY Y_2 receptor agonist NPY(13–36) were obtained in the presence of the NPY Y_2 receptor antagonist, BIIE0246TF (0.03–0.5 μM).

Electrical field stimulation

EFS was performed with platinum electrodes (Danish Myo Technology, Aarhus, Denmark), measuring 2×2 mm, secured in plastic mounting heads on either side of the artery, approximately 1 mm from the vessel wall. The electrodes were connected to an electrical stimulator with constant current output adjusted to 35 mA. To investigate the role of neuropeptide receptors, the preparations were treated with prazosin (1 μM) and propranolol (10 μM) to antagonize the adrenoceptors, and contracted with vasopressin, before responses to EFS (90 s trains, 0.3 ms square pulses, 16 Hz) were obtained in the absence and presence of the Y_1 receptor antagonist, BIBP3226, the Y_2 receptor antagonist, BII0246TF, and the combination of the two antagonists.

Western blot

From each rat, a pool of mesenteric arteries with a total length of 25 mm were collected, stimulated twice with KPSS to remove blood, quick-frozen, and stored at −80°C. The arteries were homogenized and after centrifugation, the amount of protein was measured in each sample by a modified Lowry (Bio-Rad, Hercules, CA, U.S.A.). The samples were applied to a 10% SDS gel and then the proteins were transferred to PVDF membranes (Immobilon-P, Millipore, MA, U.S.A.). The membranes were washed in TBS-T (10 mM Tris, pH 7.5, 0.1 mM NaCl, 1 mM EDTA, 0.1% Tween 20) and incubated in TBS-T, 5% fat-free dry-milk, and antibodies against the neuropeptide Y_1 receptor (1 : 1280) and the neuropeptide Y_2 receptor (1 : 640, generous gift from Astra Zeneca, Göteborg, Sweden). Coincubations were also performed with the peptide sequences (Ross-Petersen, Holte, Denmark) used to create the antibodies. For the Y_1 receptor the peptide sequence was SLAAFKDKYVCFDKFPSDSH, and for the Y_2 receptor VACTEKWPGEEKSIYGTVYS. The membranes were incubated with secondary antibodies with conjugated horseradish peroxidase (1 : 5000; Zymed, San Francisco, CA, U.S.A.). Enhanced chemiluminescence and a Storm 860 phosphor imager were used for visualization and quantification. Biotinylated SDS–PAGE standards were used for estimation of molecular weight.

Immunohistochemistry

The advantage of using immunofluorescence for quantification of NPY content is that it allows measurements in small arterial

segments in which the functional studies are performed. The fixation procedure in the present study differed from previous studies, where preparations were fixed 10 min after stimulation with 124 mM KPSS (Gradin *et al*., 2003). Thus, in the present study, isolated mesenteric arteries were treated with 0.5 μM BIIE0246TF or vehicle for 30 min and then either kept in resting conditions or activated with EFS (0.3 ms, 16 Hz, 90 s trains), and immediately immersion fixed in 4% paraformaldehyde for 10 min and then rinsed in PBS. Indirect immunofluorescence incubations were carried out using a rabbit anti-NPY, produced against synthetic NPY (Sigma, St Louis, MO, U.S.A.), dilution 1 : 4000, as previously described (Gradin *et al*., 2003). To avoid variations due to processing, vehicle- and BIIE0246TF-treated preparations from WKY and SHR were processed together in pairs. Briefly, normal donkey serum was used for preincubation, followed by rabbit anti-NPY. After rinsing, the vessel samples were incubated with secondary antibodies (donkey anti-rabbit conjugated with FITC) (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.). Control sections, included in every incubation series, were incubated with normal rabbit or donkey sera, instead of the primary antisera, followed by all subsequent incubations as described above. These control sections both in the absence and presence of BIIE0246TF were always negative for immunofluorescence (results not shown).

The sections were examined in a confocal laser scanning microscope with a krypton/argon laser (Bio-Rad, MRC 1024). Control and drug-stimulated samples were scanned in the same parameter setting throughout each series of immunoincubation, including same size of pin hole, gain level, black level, and laser power.

The quantification analysis of NPY immunoreactivity was carried out on single confocal image using 'Pixel Anatomy' software, as described previously (Gradin *et al*., 2003). Briefly, as a first step, background fluorescence was estimated by analysing the distribution of the pixel intensities in the image areas that did not contain any immunoreactive objects (the background threshold). The background was subtracted by setting the baseline of pixel intensities to the background value. In the next step, an arbitrarily outlined polygon, which covered the vessel-occupied and imaged area, was chosen, for quantification of NPY-IR. In the polygon area, the relative area of immunolabelled pixels with an intensity value above the background was calculated.

Substances

NPY, vasopressin, forskolin, prazosin, propranolol, guanethidine, (Leu^{31},Pro^{34})-NPY, NPY (13–36) were from Sigma (U.S.A.), rPP was from ICN Biomedicals (Costa Mesa, CA, U.S.A.), and BIBP3226 (R-*N*2-(diphenylacetyl)-*N*-(4-hydroxyphenyl)-methyl)-arginiamidel) and BIIE246TF ((*S*)-*N*2-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6h)-oxodibenz[b,e]azepin-11-y1]-1-piperazinyl]-2-oxoethyl]cyclo-pentyl-*N*-[2-[1,2-dihydro-3,5(4*H*)-dioxo-1,2-diphenyl-3-*H*-1,2,4-triazol-4-yl]ethyl]-argininamide) were gifts from Dr Stephan Mueller and Dr Karl Thomae (GMBH, Germany). Drugs were dissolved in distilled water, except forskolin, BIBP3226, and BII246TF, which were diluted in dimethylsulphoxide (DMSO) and kept as stock solution at -20°C and further diluted in distilled water. The resulting concentration of DMSO (0.03%) had no effect on the preparations.

Calculations and statistical analyses

For quantification, nerves immunoreactive for NPY were statistically processed with the Statview software. The nerve density is different in arteries from WKY and SHR as previously published (Gradin *et al*., 2003; Luff *et al*., 2005), and therefore preparations were compared in the absence and presence of the Y_2 receptor antagonist, and the change expressed relative to control tissue from the same animal. Images were processed using Adobe Photoshop.

Mechanical responses of vessels were measured as force and expressed as increase in tension (ΔT), which is the increase in force above baseline (ΔF), divided by the segment length. Sensitivity to the agonists is given as pD_2 values, where $pD_2 = -\log EC_{50}$ (M). The results are expressed as mean $\pm$ s.e.m. and n represents the number of animals. Statistical differences between groups were tested by use of analysis of variance (ANOVA) and Student's unpaired t-test when appropriate. $P < 0.05$ was considered as statistically significant.

Results

The systolic tail blood pressure in female SHR was significantly increased (160–180 mmHg, $n = 6$) compared to WKY rats (120–130 mmHg, $n = 6$) and lumen diameter significantly smaller in third-order mesenteric arteries from SHR (150–185 μm) *versus* WKY (220–270 μm), whereas the response to KPSS was not different in mesenteric arteries from WKY and SHR, as previously described (Gradin *et al*., 2003).

Functional receptor studies

In mesenteric arteries from WKY rats incubated with prazosin (10 μM) and contracted with vasopressin (0.5–0.8 U l^{-1}), NPY and NPY receptor agonists induced concentration-dependent contractions with the following order of potency (pD_2; Table 1): NPY $\geqslant$ [Leu^{31}, Pro^{34}]NPY > rPP > NPY(13–36) (Figure 1a). In mesenteric arteries from SHR rats, the maximal contractions induced by NPY and NPY receptor agonists were markedly enhanced (Figure 1b and Table 1), and the curves were leftward shifted: NPY = [Leu^{31}, Pro^{34}]NPY > rPP > NPY(13–36). In the presence of the Y_1 receptor antagonist, BIBP3226 (0.3 μM), contractions evoked by NPY and NPY receptor agonists with the exception of the Y_2 receptor agonist were markedly reduced in arteries from both WKY and SHR. However, contractions evoked by the agonists in arteries from SHR were still markedly enhanced compared to contractions evoked in arteries from WKY rats (Figure 1c and d).

In preparations without endothelium, concentration–response curves for NPY were enhanced in segments from SHR *versus* WKY rats (Figures 2 and 3). Incubation with BIBP3226, the NPY Y_2 receptor antagonist, BIIE0246TF, and the combination of the two antagonists did not change the vasopressin contraction (Table 1). However, BIBP3226 (0.3 μM) inhibited NPY contraction completely in arteries from WKY rats and caused rightward shifts in the concentration–response curves for NPY in arteries from SHR rats (Figure 2). In contrast, the selective NPY Y_2 receptor antagonist, BIIE0246TF (0.5 μM), did not change concentration–response curves for NPY in arteries from WKY and leftward shifted concentration–response curves for NPY in arteries from SHR (Figure 2a and

Table 1 Contractile responses to high-potassium solution (124 mM KPSS), vasopressin (0.5–0.8 U l^{-1}) in the absence and presence of the NPY Y_1 receptor antagonist, BIBP3226 (0.3 μM), or the NPY Y_2 receptor antagonist, BIIE0246 (0.5 μM); parameters for concentration–response curves for NPY, [Leu^{31}, Pro^{34}]NPY, rPP, and NPY(13–36) constructed in vasopressin-contracted arteries are also shown

	WKY		*SHR*	
Parameter	pD_2 ($-log\,EC_{50}$)	ΔT (N m^{-1})	pD_2 ($-log\,EC_{50}$)	ΔT (N m^{-1})
KPSS	—	2.0±0.10 (12)	—	2.0±0.30 (12)
Vasopressin	—	1.20±0.18 (12)	—	1.45±0.30 (12)
BIBP3226 + vasopressin	—	1.12±0.10 (12)	—	1.42±0.10 (12)
BIIE0246TF + vasopressin	—	1.24±0.20 (12)	—	1.58±0.30 (12)
BIBP3226, BIIE0246TF				
Vasopressin	—	1.10±0.19 (12)	—	1.59±0.30 (12)
NPY	8.16±0.04 (5)	0.79±0.07 (5)	8.60±0.03 (5)*	1.19±0.20 (5)*
[Leu^{31}, Pro^{34}]NPY	8.15±0.03 (5)	0.76±0.02 (5)	8.60±0.04 (5)*	1.12±0.30 (5)*
RPP	7.80±0.09 (5)	0.76±0.05 (5)	8.46±0.08 (5)*	0.96±0.24 (5)*
NPY(13–36)	6.90±0.09 (5)	0.46±0.04 (5)	7.00±0.04 (5)	0.83±0.20 (5)*

Values are mean±s.e.m.. ΔT is the maximal increase in tension (N m^{-1}), whereas $pD_2 = -\log(EC_{50})$, where EC_{50} is the concentration of agonist required to induce half-maximal contraction. Numbers in parentheses represent number of animals examined.
*$P<0.05$, statistically significant difference *versus* WKY.

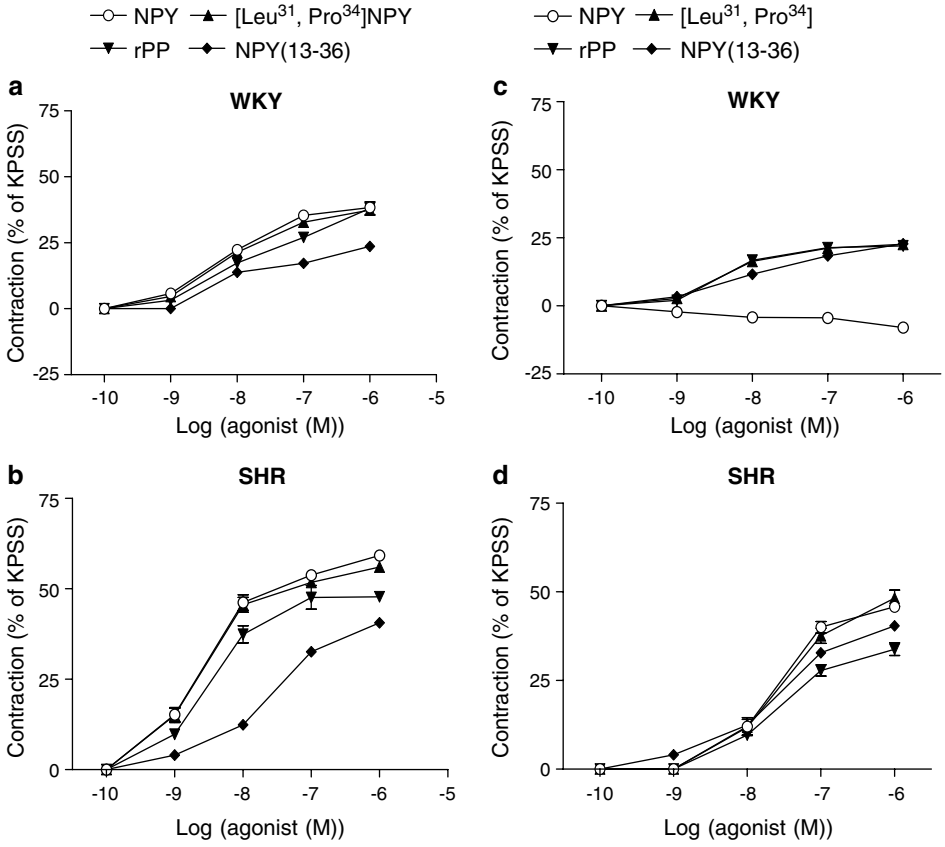

Figure 1 Contractions induced by NPY receptor agonists are more pronounced in arteries from SHR, even in the presence of the neuropeptide Y_1 receptor agonist, BIBP3226. Mesenteric endothelium-intact small arteries from (a, c) WKY and (b, d) SHR were constricted with vasopressin 0.5–0.8 U l^{-1} and concentration–response curves were constructed for NPY receptor agonists in the absence (a, c) and the presence (b, d) of the neuropeptide Y_1 receptor antagonist, BIBP3226 (0.3 μM). Responses are expressed as percentage of the contraction induced by 124 mM KPSS and represent mean±s.e.m. of arteries from five animals in each group.

b). However, in the presence of the combination of BIBP3226 and BIIE0246TF, NPY contraction was abolished in arteries without endothelium (Figure 2a and b).

In preparations with endothelium and in the presence of BIBP3226, NPY induced contractions in arteries from SHR, whereas NPY evoked relaxations in arteries from WKY (Figure 3). In the presence of the combination of BIBP3226 and BIIE0246TF, NPY relaxation in arteries from WKY was abolished, whereas NPY contraction was enhanced in arteries from SHR (Figure 3).

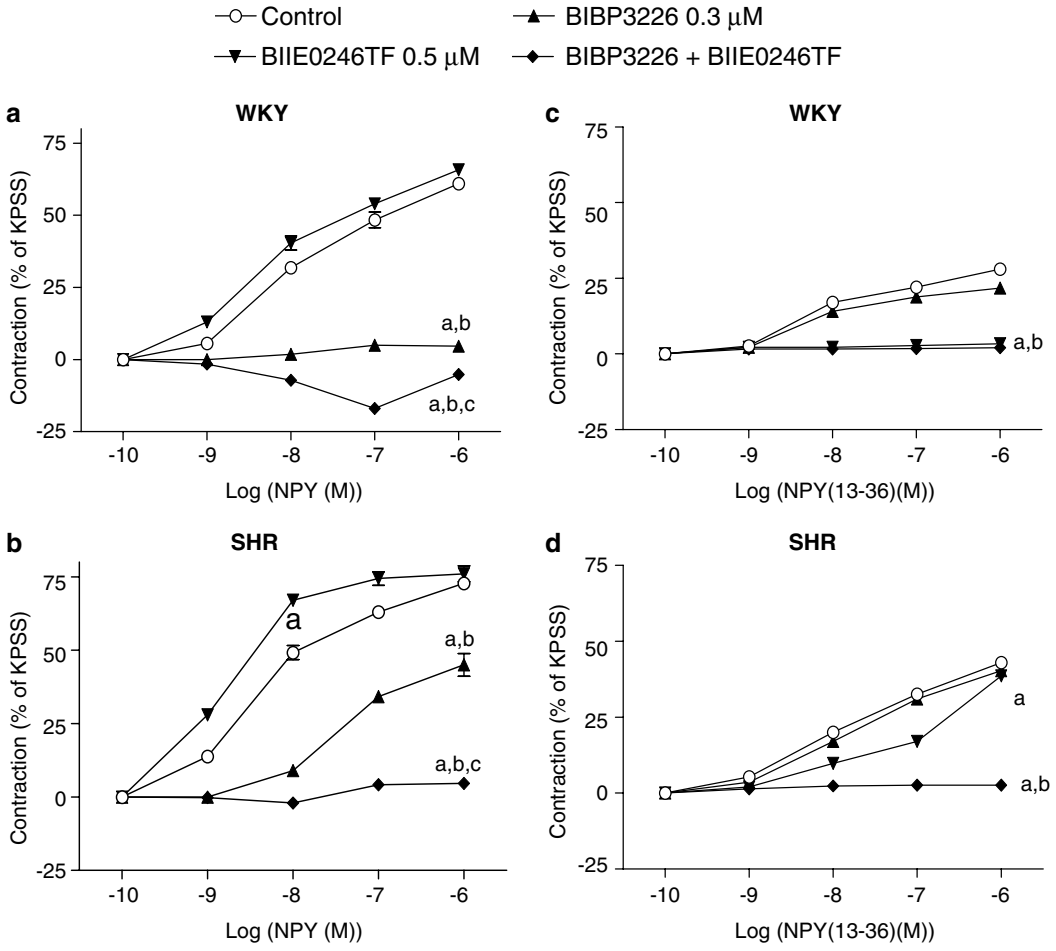

Figure 2 Combination of the neuropeptide Y_1 and Y_2 selective receptor antagonists, BIBP3226 and BIIE0246TF, abolishes NPY-evoked contractions in arteries from SHR and WKY. Average contractions for (a, b) NPY and (c, d) the selective Y_2 receptor agonist NPY(13–36) in prazosin-treated and vasopressin-activated endothelium-denuded arteries from SHR and WKY. The responses were obtained in the absence and presence of either BIIE0246TF (0.5 μM), BIBP3226 (0.3 μM), or the combination of BIIE0246TF and BIBP3226. Significantly different responses evaluated by two-way ANOVA followed by *t*-test: $^*P<0.05$ *versus* WKY; (a) $P<0.05$ *versus* control; (b) $P<0.05$ *versus* the response with BIIE0246TF; (c) $P<0.05$ *versus* the response with BIBP3226. The columns are means$\pm$s.e.m. of arteries from six animals.

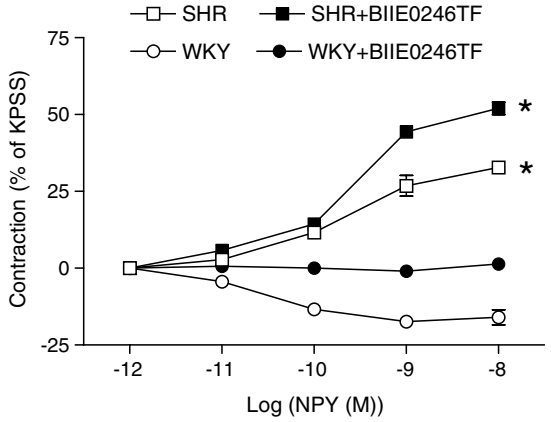

Figure 3 Average concentration–response curves for NPY in vasopressin-contracted arteries with endothelium. The concentration–response curves were constructed in the presence of the NPY Y_1 receptor antagonist, BIBP3226 (0.3 μM), or the combination of BIBP3226 and BIIE0246TF (0.5 μM). Responses are expressed as percentage of the contraction induced by 124 mM KPSS and represent mean$\pm$s.e.m. of arteries from five animals in each group. Significantly different responses evaluated by two-way ANOVA followed by *t*-test: $^*P<0.05$ *versus* WKY.

Also in arterial preparations without endothelium, concentration–response curves for NPY(13–36) were enhanced in segments from SHR *versus* WKY rats (Figure 4). The NPY Y_2 selective receptor agonist, NPY(13–36), contraction did not differ in the presence of BIBP3226, but was significantly inhibited in the presence of the Y_2 selective antagonist, BIIE0246TF (Figure 2c and d). In preparations without endothelium, BIIE0246TF caused rightward shifts in the concentration–response curves for NPY(13–36), and also depressed the maximum response for the agonist in arteries from WKY (Figure 4a and b).

Reversal of vasodilator responses by NPY

In mesenteric arteries contracted with vasopressin, an activator of adenylyl cyclase, forskolin (3 μM), evoked sustained relaxations of a similar magnitude in arteries from WKY and SHR. When the preparation was fully relaxed by forskolin, the addition of NPY reversed the relaxations (Figure 5). As in previous studies, the concentration–response curves for NPY were shifted leftward and the maximal reversal effect was enhanced in arteries from SHR compared to WKY (Figure 5a and b). In

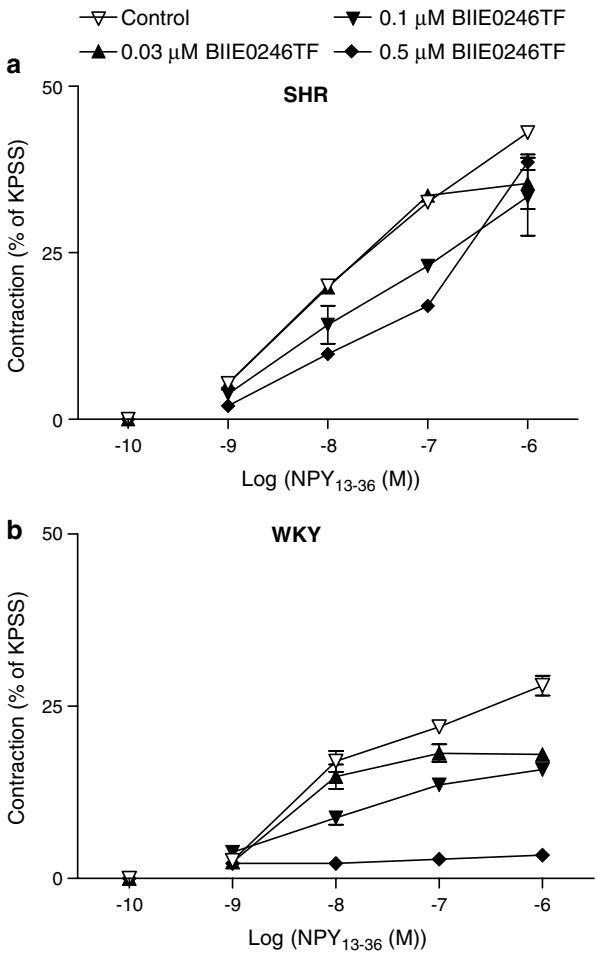

Figure 4 Endothelium-denuded small arteries from (a) SHR and (b) WKY were constricted with vasopressin and concentration–response curves were constructed for the NPY Y_2 receptor agonist, NPY(13–36), in the absence or presence of increasing concentrations of the NPY Y_2 receptor antagonist, BIIE0246TF. Responses are expressed as percentage of the contraction induced by 124 mM KPSS and represent mean ± s.e.m. of arteries from 5 to 6 animals in each group.

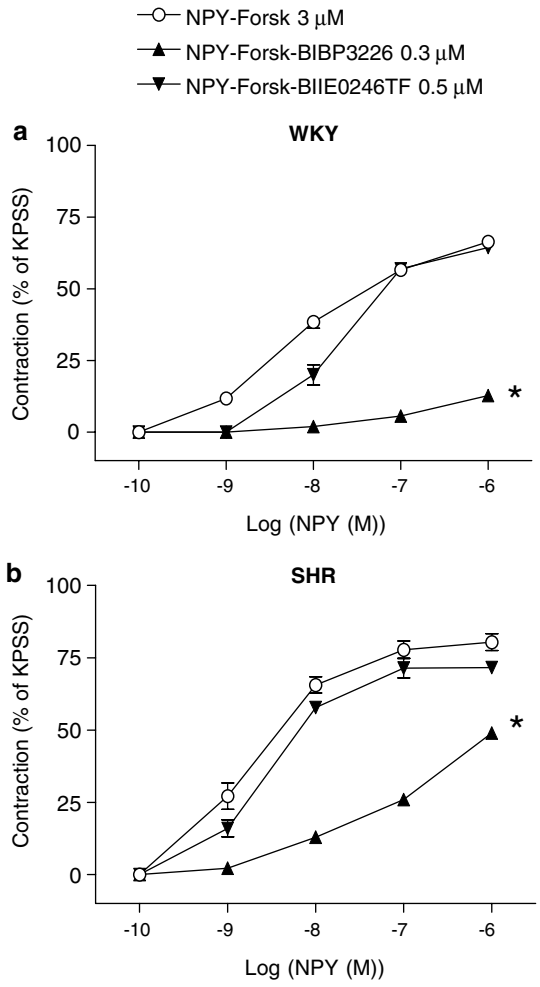

Figure 5 Inhibition of forskolin-evoked vasodilatation by NPY in arteries from SHR is mediated by Y_1 receptors. Mesenteric small arteries from (a) WKY and (b) SHR were constricted with vasopressin and relaxed with the adenylyl cyclase activator, forskolin (3 μM), and when full vasodilatation was attained, increasing concentrations of NPY were added in the presence of vehicle, BIBP3226, or BIIE0246TF. Responses are expressed as percentage of the contraction induced by 124 mM KPSS and represent mean ± s.e.m. of arteries from five animals in each group. Significantly different responses evaluated by *t*-test: $^*P<0.05$ *versus* control.

contrast, NPY(13–36) did not reverse forskolin relaxation in arteries from WKY and SHR (results not shown, $n=5$ animals from each group). The reversal effect of forskolin by NPY was largely unaltered in the presence of BIIE0246TF, but markedly inhibited in the presence of the NPY Y_1 receptor antagonist BIBP3226 (Figure 5).

Contractions to electrical field stimulation

In endothelium-denuded preparations, EFS caused contractions, which were significantly enhanced in arteries from SHR rats compared to WKY rats (Figure 6). The contractions induced by EFS were enhanced in the presence of BIIE0246TF in both SHR and WKY rats, and were markedly inhibited in the presence of BIBP3226 (Figure 6). However, only in the presence of the combination of the selective Y_1 and Y_2 receptor antagonists, BIBP3226 and BIIE0246TF, contractions were abolished (Figure 6).

Western blot for neuropeptide Y_1 and Y_2 receptors

Western blots of crude cell proteins from rat mesenteric small arteries demonstrated expression of both neuropeptide Y_1 and Y_2 receptors. Thus, by using a polyclonal antibody raised against a peptide sequence of the NPY Y_1 receptor, a single band of 52 kDa was detected (Figure 7a). This band was not detected when coincubated with the peptide to which the antibody was raised (Figure 7b). The bands for the Y_1 receptor in arterial samples from WKY and SHR did not differ. In samples from both WKY and SHR, two bands with molecular weights of 40 and 24 kDa, and two faint and less consistent bands at 39 and 45 kDa were detected with the antibody to the Y_2 receptor, whereas a band at around 80 kDa was seen only in samples from WKY rat arteries (Figure 7c). These bands were not detected when coincubated with the peptide to which the antibody was raised (Figure 7c). Moreover, a blast

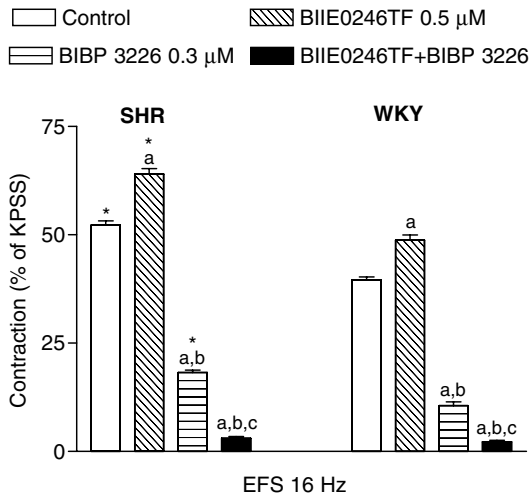

Figure 6 Enhanced neurogenic contractions in arteries from SHR are only abolished by the combination of the neuropeptide Y_1 and Y_2 selective receptor antagonists, BIBP3226 and BIIE0246TF. Average responses to EFS (16 Hz, 0.3 ms, 90 s trains) in prazosin-treated and vasopressin-activated endothelium-intact arteries from SHR and WKY. The responses were obtained in the absence and presence of either BIIE0246TF (0.5 μM), BIBP3226 (0.3 μM), or the combination of BIIE0246TF and BIBP3226. Significantly different responses evaluated by two-way ANOVA followed by *t*-test: $^*P<0.05$ *versus* WKY; (a) $P<0.05$ *versus* control; (b) $P<0.05$ *versus* response with BIIE0246TF present; (c) $P<0.05$ *versus* the response with BIBP3226. The columns are means ± s.e.m. of arteries from six animals.

for the binding of the peptide sequence used to block the Y_2 receptor antibody did not reveal binding to other known or predicted proteins based on the rat genome. Quantification of the 24 and 40 kDa bands for the Y_2 receptor showed no difference between arteries from WKY and SHR (Figure 7d).

Immunoreactivity for neuropeptide Y

NPY immunoreactive fibers were localized in the adventitia of mesenteric small arteries. The distribution pattern was homogenous and similar in whole-mounted arterial preparations isolated from WKY and SHR (Figure 8). The fluorescence intensity of NPY immunoreactivity was significantly higher both in unstimulated and EFS-stimulated arteries from SHR compared to the WKY strain (Figure 8). In unstimulated preparations, incubation with the Y_2 receptor antagonist, BIIE0246TF (0.5 μM), did not cause significant changes in the immunofluorescence compared to the control tissue. However, in the EFS-stimulated arteries, BIIE0246TF (0.5 μM) lowered NPY immunoreactivity in arteries from WKY, but enhanced NPY immunoreactivity in arteries from SHR (Figure 8c–f).

Discussion

The present study demonstrates the presence of NPY Y_2 receptors in mesenteric small arteries, and we found that enhanced vasoconstriction is abolished only in the presence of the NPY Y_2 receptor antagonist, BIIE0246TF. Moreover, the different modulation of the NPY content in nerves suggests profound alterations of the NPY neurotransmission both at pre- and postjunctional level in the vasculature of female SHR rats.

Role of NPY Y_2 receptors in vasoconstriction

The NPY Y_1 receptor is considered the predominant receptor subtype in the vasculature (Malmstrom, 1997; Prieto *et al*., 1998; 2000), but both prejunctional inhibitory and postjunctional vasoconstrictor receptors belonging to the Y_2 receptor subtype have been described in rat femoral artery (Tessel *et al*., 1993) and porcine spleen (Malmstrom 2001; Malmstrom *et al*., 2002). Immunostaining also showed the presence of Y_2 receptors in nerve cell bodies of the vasculature (Uddman *et al*., 2002). In the present study, both immunoblotting and the contractile effect of the Y_2 receptor agonist NPY(13–36) provide evidence for expression of functional Y_2 receptors in mesenteric arteries from WKY and SHR rats. Activation of postjunctional Y_2 receptors is confirmed by the results obtained with BIIE0246TF, as this NPY Y_2 receptor antagonist inhibited NPY(13–36) contraction and abolished neurogenic contractions to EFS obtained in the presence of the NPY Y_1 receptor antagonist, BIBP3226. Moreover, in the absence of blockade of NPY Y_1 receptors, BIIE0246TF enhanced neurogenic contractions. BIIE0246TF possesses high affinity for the NPY Y_2 receptor and is devoid of affinity for NPY Y_1, Y_4, and Y_5 receptors (Doods *et al*., 1999; Malmstrom, 2001). Moreover, in the present study, BIIE0246TF did not affect vasopressin contraction. Therefore, the present results demonstrate that in addition to NPY Y_1 receptors, both prejunctional inhibitory and postjunctional vasoconstrictory NPY Y_2 receptors are present in mesenteric small arteries.

In vivo studies of the hindlimb (Jackson *et al*., 2005) and kidney (Dubinion *et al*., 2006) have recently suggested that postjunctional Y_2 receptors do not appear to contribute to vasoconstriction in these vascular beds in normotensive anaesthetized rats without sympathetic nerve activation. Although the contractions to EFS in the presence of the NPY Y_1 receptor antagonist, BIBP3226, was only one-fifth of the response to EFS in the presence of the NPY Y_2 receptor antagonist, BIIE0246TF, in mesenteric arteries from female WKY (Figure 6), the present study suggests that both NPY Y_1 and Y_2 receptors appear to play a role in the neurogenic contractions of rat mesenteric small arteries.

Role of NPY Y_2 receptors in hypertension

There are several lines of evidence supporting a role of neuropeptide Y_2 receptors in hypertension. Thus, there is an increased Y_2 receptor binding in the nucleus tractus solitarius (NTS) of SHR rats (Aguirre *et al*., 1995), and Y_2 receptor mRNA expression is increased in NTS after 2 h of induction of coarctation hypertension in rats (Coelho *et al*., 2004). Taken together with the observation that Y_2 receptor activation in NTS induces hypertension, this suggests that Y_2 receptors are implicated in blood pressure regulation. In previous studies, we observed that the enhanced neurogenic response to EFS in rat mesenteric small arteries is increased even in the presence of the neuropeptide Y_1 receptor antagonist, BIBP3226 (Gradin *et al*., 2003), an observation confirmed in the present study, and the persisting neurogenic response was blocked by the

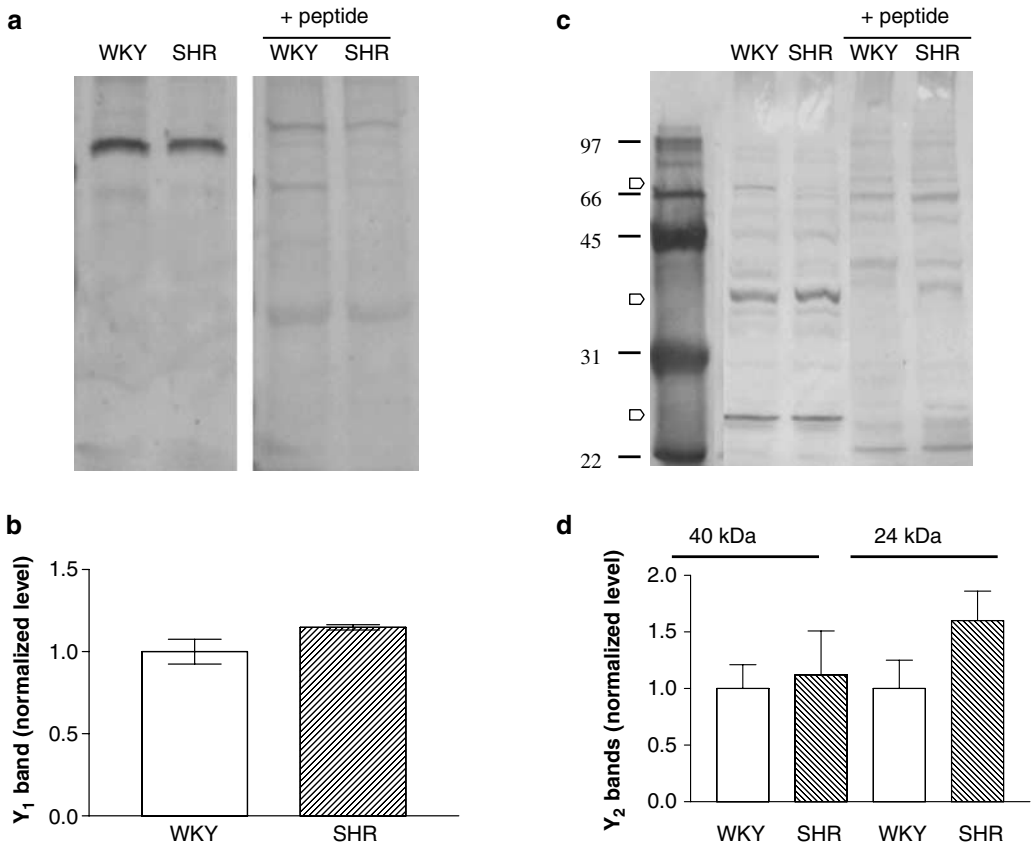

Figure 7 Expression of neuropeptide Y_1 and Y_2 receptors in rat mesenteric small arteries. Representative Western blots and quantitative analysis for (a, b) neuropeptide Y_1 receptor in arteries from WKY and SHR. Blots were performed in the (a) absence and (b) presence of blocking peptide. (c, d) Representative Western blots for neuropeptide Y_1 receptor in arteries from WKY and SHR. (c) Notice three bands in control conditions in arteries from WKY, but only two bands in arteries from SHR. (d) Incubation with blocking peptide for the neuropeptide Y_2 receptor. Results are means ± s.e.m. of arteries from five animals in each group.

Y_2 receptor antagonist, BIIE0246TF (see Figure 6). These findings suggest that in addition to neuropeptide Y_2 receptors in NTS in hypertensive rats, altered activation of peripheral Y_2 receptors plays a role in arteries from female SHR rats.

The contraction evoked by the Y_2 agonist, NPY(13–36), appears low compared to contractions evoked by the Y_1 receptor agonist in arteries both from WKY and SHR. However, NPY(13–36) contraction was also low in comparison with NPY contraction in the presence of the Y_1 receptor antagonist BIBP3226. Therefore, we cannot exclude that other contractile receptors apart from Y_1 and Y_2 receptors are activated by NPY in rat mesenteric arteries. This is also supported by the observation that NPY contraction in arteries with endothelium is enhanced in SHR *versus* WKY. However, neurogenic responses appear to be mediated by Y_1 and Y_2 receptors only. Therefore, despite an enhanced response to NPY in the presence of both BIBP3226 and BIIE0246TF to block Y_1 and Y_2 receptors, it is unlikely this receptor subtype contributes to the enhanced neurogenic contractions observed in arteries from SHR *versus* WKY.

The increased maximal contraction in mesenteric small arteries from SHR rats evoked by activation of Y_2 receptors by either the NPY Y_2 receptor agonist or nerve stimulation suggests altered expression or signal transduction pathway linked to the Y_2 receptors. In the present study, immunoblotting of mesenteric arteries from WKY rats for the neuropeptide Y_1 receptor showed specific bands at 52 kDa, whereas immunoreactivity for the neuropeptide Y_2 receptor was found at 80, 40, and 24 kDa. Although this was also the case for two additional faint bands, the bands at 80, 40, and 24 kDa disappeared in the presence of the peptides against which the antibodies were raised, hence suggesting these are specific for the Y_2 receptor. Moreover, the molecular sizes of the receptors in the range of 40–52 kDa agree with previous studies and nucleotide sequences of neuropeptide Y_1 and Y_2 receptors (Pedrazzini *et al*., 1998; Naveilhan *et al*., 1999; Goumain *et al*., 2001). Based on these findings, the expression of the Y_1 and Y_2 receptors does not appear to be changed, although further studies are needed to address the consequences of the lack of the Y_2 receptor band at 80 kDa in arteries from the SHR rats.

The unaltered Y_2 receptor expression found by immunoblotting contrasts with the enhanced maximal contraction induced by the Y_2 receptor agonist NPY(13–36) and the neurogenic contractions sensitive to the Y_2 receptor antagonist, BIIE0246TF. In a previous study, we found that acetylcholine relaxation was decreased and that responses to NPY were increased in arteries from SHR rats, indicating that endothelial dysfunction could play a role in the increased response (Gradin *et al*., 2003). In the present study, NPY induces relaxation in arteries from WKY in the presence of BIBP3226 that could be abolished by the combination of BIBP3226 plus BIIE0246TF. These findings suggest that NPY

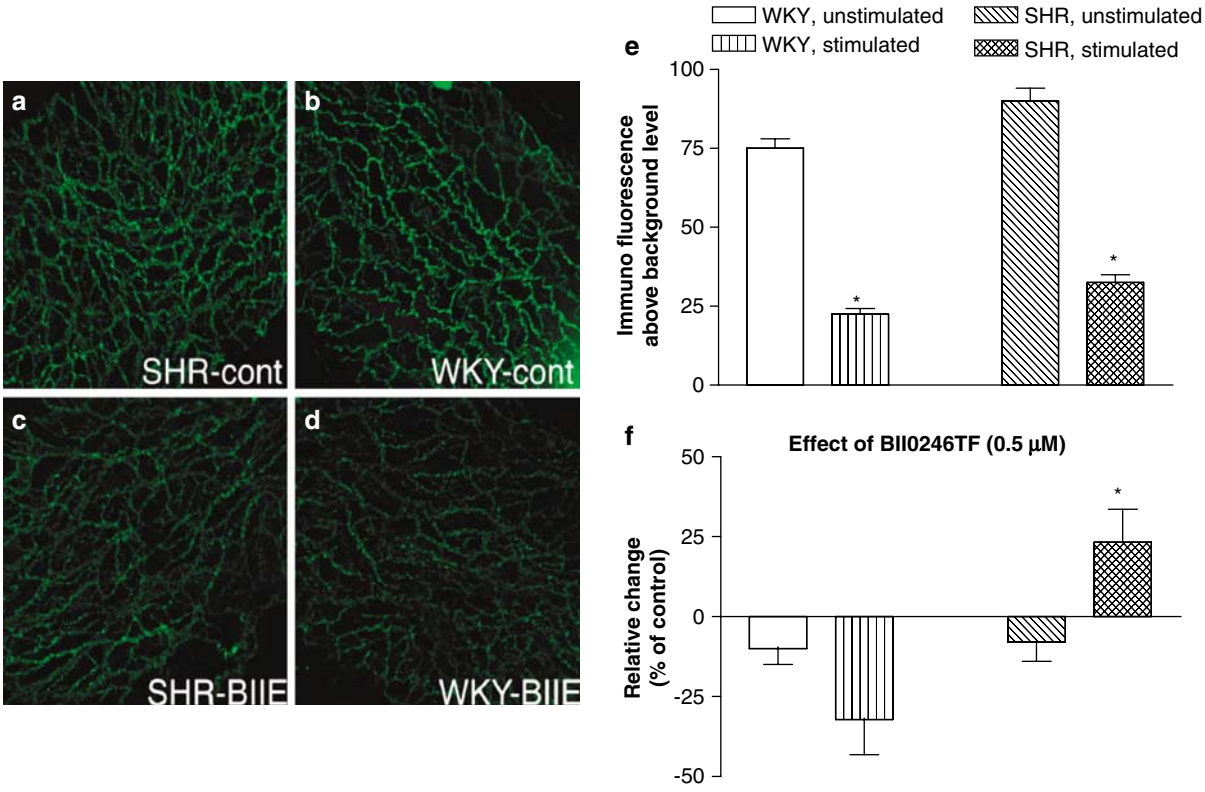

Figure 8 NPY immunoreactive (NPY-IR) nerves in EFS stimulated mesenteric small arteries from SHR (a, c) and WKY (b, d) rats in the absence (a, b) and presence (c, d) of a neuropeptide Y_2 antagonist, BIIE0246TF (0.5 μM). (e) Quantitative analyses showing that immunointensity of NPY-IR nerves was higher both in unstimulated and stimulated arteries from SHR rats compared to WKY rats. (f) Quantitative analyses showing immunofluorescence in the presence of BIIE0246TF expressed relative to a parallel control. Results are means$\pm$s.e.m. of arteries from 7 to 26 animals in each group. *$P<0.05$ *versus* control.

activates Y_2 receptors in the endothelial cell layer. However, in arteries from SHR, incubation with BIIE0246TF also induced a leftward shift in the concentration–response curves for NPY obtained in the presence of BIBP3226, suggesting that activation of endothelial NPY Y_2 receptors is intact. Moreover, the response to the Y_2 receptor agonist was also increased in endothelium-denuded arteries in the present study. Therefore, another explanation for the increased response is that the Y_2 agonist NPY(13–36) activates inhibitory receptors in the smooth muscle layer or adventitia. Indeed, in horse penile arteries, NPY(13–36) was described to activate both contractile Y_2 receptors and in the presence of BIIE0246TF an atypical receptor population different from Y_1 and Y_2 receptors and mediating relaxation (Prieto *et al*., 2004). The observation that increasing concentrations of BIIE0246TF decrease maximal contraction induced by NPY(13–36) supports that NPY(13–36) activates an inhibitory receptor population in arteries from WKY, but this is not the case in arteries from SHR. Therefore, these findings suggest that the increased contractions to high concentrations of NPY(13–36) can be ascribed to decreased activation of these relaxant non-Y_1 non-Y_2 receptors. In previous studies, media thickness of arteries from female SHR rats of the same age as in the present study was increased (Gradin *et al*., 2003), and therefore it cannot be excluded that structural remodelling of the arteries from hypertensive animals contributes to the increased maximal response to the Y_2 receptor agonist and neurogenic contractions.

An alternative explanation for the enhanced Y_2 receptor-mediated vasoconstriction induced by EFS in mesenteric arteries from SHR could be alterations in the release of vasodilatory neurotransmitters, as activation of NPY receptors was suggested to inhibit vasodilatation mediated by increases in cyclic AMP (Nilsson *et al*., 1996; Prieto *et al*., 1997). However, the present study shows that inhibition by NPY of the vasodilatation evoked by an activator of adenylate cyclase, forskolin, is mainly mediated by Y_1 receptors. Although it cannot be excluded that unchanged or decreased vasodilatation and release of vasodilatory neurotransmitters play a role in the vascular bed from SHR (Kawasaki *et al*., 1990), it does not appear a likely explanation for the enhanced neurogenic Y_2 receptor-mediated contraction in mesenteric arteries from SHR compared to WKY.

Altered prejunctional Y_2 receptor modulation of NPY neurotransmission?

Sympathetic nerve hyper-reactivity is thought to play a role in the increased vascular resistance in essential hypertension (Mancia *et al*., 1999), and SHR have been suggested to be in a state of sympathetic hyper-reactivity compared to normotensive rats (Norman & Dzielak, 1986). We have previously shown that immunoreactivity for noradrenergic and NPYergic fibres is increased in mesenteric small arteries from SHR in line with increased NPY content (Gradin *et al*., 2003). In the present study, we also found an increased immunoreactivity

for NPY in arteries from SHR, and it appears that NPY immunoreactivity can be modulated by the Y_2 receptor antagonist BIIE0246TF.

Activation of prejunctional Y_2 receptors in peripheral nerves is associated with decreased acetylcholine release in the heart (Smith-White *et al*., 2001), and reduced release of noradrenalin and NPY from postganglionic sympathetic nerves (Michel *et al*., 1998; Smith-White *et al*., 2001). In the present study, the Y_2 receptor antagonist, BIIE0246TF, increased responses to EFS and decreased NPY immunoreactivity in arteries from WKY rats, suggesting that inhibition of prejunctional receptors increases release of NPY. In contrast, in arteries from SHR, immunoreactive content seems unaltered or even tended to increase, whereas the increase observed in EFS contraction in the presence of BIIE0246TF was similar in arteries from SHR compared to WKY. Taken into account the enhanced postjunctional NPY-induced contraction and larger NPY content detected by immunoreactivity in arteries from SHR, a larger increase in EFS contraction would be expected by blocking the prejunctional Y_2 receptors in arteries from SHR. NPY Y_2 receptors are mainly expressed in adventitia of small arteries (Uddman *et al*., 2002), and therefore the lack of alterations of NPY content may suggest a downregulation of adventitial Y_2 receptors. However, it cannot be excluded that other mechanisms such as increased synthesis or decreased degradation play a role in the increased and unaltered NPY immunoreactivity in the presence of Y_2 receptor antagonists in mesenteric small arteries from SHR.

An alternative explanation for the altered regulation showing higher NPY content in SHR *versus* WKY could be alterations in prejunctional α_2-adrenoceptor or dopamine receptor regulation of neurotransmitter release. In superior mesenteric arteries, differences have been postulated between SHR and WKY rats in the population of postjunctional but not prejunctional α_2-adrenoceptors (Feres *et al*., 1998), and similar studies in rat anococcygeus muscle also found that regulation of prejunctional α_2-adrenoceptor receptors did not explain the enhanced sympathetic neurotransmission in SHR (Jimenez-Altayo *et al*., 2003).

In summary, the present results suggest that in addition to neurogenic Y_1 receptors, postjunctional Y_2 receptors contribute to neurogenic contraction of mesenteric small arteries. Moreover, both enhanced NPY content and altered neuropeptide Y_2 receptor activation apparently contribute to the enhanced neurogenic contraction of arteries from SHR.

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