European Journal of Pharmacology 654 (2011) 86–91

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Pulmonary, Gastrointestinal and Urogenital Pharmacology

Lack of evidence that nebivolol is a β_3-adrenoceptor agonist

Elfaridah P. Frazier [a], Martina B. Michel-Reher [a], Pieter van Loenen [a], Carsten Sand [b], Tim Schneider [c], Stephan L.M. Peters [a], Martin C. Michel [a,*]

[a] Department of Pharmacology and Pharmacotherapy, University of Amsterdam, The Netherlands
[b] Department of Pharmacology, University of Duisburg-Essen, Germany
[c] Department of Urology, University of Duisburg-Essen, Germany

ARTICLE INFO

Article history:
Received 7 October 2010
Received in revised form 9 November 2010
Accepted 12 November 2010
Available online 21 December 2010

Keywords:
Nebivolol
Urinary bladder
Chinese hamster ovary cell
β_3-Adrenoceptor

ABSTRACT

Nebivolol is a selective β_1-adrenoceptor antagonist which, in addition, displays endothelium-dependent vasodilating properties in humans and other species. β_3-Adrenoceptors have been proposed to be a molecular target of nebivolol-induced vasodilatation. Therefore, we have investigated possible β_3-adrenoceptor agonism by nebivolol for relaxation of the human and rat urinary bladder (prototypical β_3-adrenoceptor-mediated responses) as well as for cAMP accumulation in Chinese hamster ovary cells stably transfected with the human β-adrenoceptor subtypes. Nebivolol concentration-dependently relaxed both human and rat isolated urinary bladder strips but with low potency, similar to that reported for vasodilatation. However, nebivolol-induced bladder relaxation in either species was not inhibited by the β_3-adrenoceptor antagonist SR 59,230A (10 μM), although this compound inhibited the isoprenaline-induced relaxation with the expected potency. In radioligand binding studies nebivolol had lower affinity for human β_3-adrenoceptors than the other two β-adrenoceptor subtypes, but this low affinity was in line with its potency to relax the bladder or isolated blood vessels. In functional studies nebivolol even in high concentrations did not stimulate cAMP formation via any of the three cloned human β-adrenoceptors or in rat bladder smooth muscle cells. Taken together these data demonstrate that nebivolol can relax not only vascular but also urinary bladder smooth muscle. However, they do not support the hypothesis that nebivolol is an agonist at cloned human β_3-adrenoceptors or in rat or human urinary bladder.

1. Introduction

Nebivolol is a β-adrenoceptor antagonist which is selective for β_1- as compared to β_2-adrenoceptors (Pauwels et al., 1988; Bundkirchen et al., 2003; Baker, 2010). In contrast to many other β-adrenoceptor antagonists, nebivolol has direct dilating effects in both animal and human isolated blood vessels, which typically are endothelium-dependent and in most cases involve the generation of NO (Ignarro, 2008; Tran Quang et al., 2009; Kamp et al., 2010). As the nebivolol concentrations required for vasodilatation are much higher than those needed to occupy β_1-adrenoceptors, it is generally assumed that this vasodilatation occurs via a molecular target distinct from the β_1-adrenoceptor. Further support for this conclusion may come from the clinical finding that the β_1-adrenoceptor antagonist effects primarily reside in the D-enantiomer of nebivolol, whereas the vasodilating effects of nebivolol are mainly associated with its L-enantiomer in some studies (van Nueten and De Cree, 1998); however, others found nebivolol-induced vasodilatation *in vitro* not to be stereo-selective (Chlopicki et al., 2002; Tran Quang et al., 2009).

There is considerable controversy what the molecular target for nebivolol-induced endothelium-dependent vasodilatation might be. Proposed nebivolol targets include effects on NO bioavailability (Evangelista et al., 2007), oestrogen receptors (Garban et al., 2004), purinergic receptors (Kalinowski et al., 2003), α_1-adrenoceptors (Rozec et al., 2006), β_2-adrenoceptors (Broeders et al., 2000; Tran Quang et al., 2009), and β_3-adrenoceptors (de Groot et al., 2003; Tran Quang et al., 2009; Rozec et al., 2009). Evidence for the latter possibility comes from several sources including the use of antagonists such as cyanopindolol (de Groot et al., 2003), SR 59,230A (Evangelista et al., 2007) and L-748,337 (Rozec et al., 2006, 2009; Tran Quang et al., 2009). However, many of the antagonists which have previously been used have ancillary properties. For example, cyanopindolol is also a ligand at serotonin receptors (Schlicker et al., 1985), and SR 59,230A can act on muscarinic receptors, does not discriminate human β-adrenoceptor subtypes and can be a β_3-adrenoceptor partial agonist in some cases (Vrydag and Michel, 2007). While L-748,337 has one of the highest β_3-selectivities among generally available antagonists (Candelore et al., 1999; Baker, 2010), the concentrations used in the above mentioned studies may also block β_1- and β_2-adrenoceptors. Therefore, these antagonist data can only be seen as circumstantial evidence for an

* Corresponding author. Academic Medical Center, Department Pharmacology and Pharmacotherapy, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. Tel.: +31 20 566 6762; fax: +31 20 696 5976.
E-mail address: m.c.michel@amc.nl (M.C. Michel).

0014-2999/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2010.11.036

involvement of β_3-adrenoceptors. More conclusive evidence comes from β_3-adrenoceptor knock-out mice, in which nebivolol-induced vasodilatation was abolished (Dessy et al., 2005).

While it is possible that the various proposed molecular targets of nebivolol are not mutually exclusively, i.e., that nebivolol causes vasodilatation via different molecular mechanisms in different vascular preparations, β_3-adrenoceptor agonism is a strong candidate to explain vasodilating nebivolol effects. The present study was designed to investigate a possible β_3-adrenoceptor agonism by nebivolol for a prototypical β_3-adrenoceptor-mediated response, i.e. relaxation of the rat and human urinary bladder (Michel and Vrydag, 2006; Yamaguchi and Chapple, 2007), as well as cAMP accumulation in Chinese hamster ovary (CHO) cells stably transfected with the human β-adrenoceptor subtypes (Niclauß et al., 2006).

2. Material and methods

2.1. Relaxation studies

Human bladder tissue was obtained from patients undergoing cystectomy due to bladder cancer from macroscopically tumour-free parts of the bladder, and transported to the laboratory within 30 min after surgical removal. Human tissues were obtained with informed patient consent based upon a protocol approved by the ethical committee of the University of Duisburg-Essen. Bladder strips of approximately 1 mm diameter, 18 ± 1 mm length and 30 ± 1 mg weight ($n = 30$ muscle strips) were prepared.

Male Wistar rats (260–280 g) were obtained from Charles River (Maastricht, The Netherlands). Animals were sacrificed by injection of pentobarbital (100 mg/kg). Thereafter, the bladder was removed and placed into Krebs–Henseleit buffer. After removing surrounding connective tissue, adipose tissue and serosa, the bladder was cut longitudinally into four strips (1 mm in diameter, 17 ± 0.03 mm in length and 8.6 ± 0.2 mg, $n = 79$ muscle strips). All experimental procedures had been approved by the institutional animal welfare committee according to the Dutch legislation for the protection of experimental animals.

The relaxation studies were performed as previously described (Frazier et al., 2006). Briefly, bladder strips were mounted under a resting tension of 10 mN in organ baths containing 7 ml (10 ml for human tissues) Krebs–Henseleit buffer of the following composition (mM): NaCl 118.5, KCl 4.7, $MgSO_4$ 1.2, Na_4EDTA 0.025, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25, Hepes 10 (not in rat experiments), and glucose 5.5 at 37 °C, yielding a total potassium concentration of 5.9 mM. The organ baths were continually gassed with 95% O_2/5% CO_2 to maintain a pH of 7.4. The bladder strips were equilibrated for approximately 75 min during which the buffer solution was refreshed every 15 min. Following the equilibration, the tissues were challenged with 50 mM KCl for 6 min and washed again with fresh buffer. They were again equilibrated and readjusted to a baseline tension of 10 mN every 10 min until stabilization had occurred, usually within 45 min. Thereafter, SR 59,230A or its vehicle, dimethylsulfoxide, was added. After 10 min strips were pre-contracted with 50 mM KCl. After 30 min (20 min after KCl), when tension in KCl-pre-contracted strips had reached a plateau, concentration–response curves for nebivolol or isoprenaline (0.1 nM–0.3 mM) were generated. To avoid desensitization, only a single relaxation curve was generated in each bladder strip. Based upon present (Fig. 1) and our previous data (Frazier et al., 2005), the contractile response to KCl declined by less than 5% with time, and hence no corrections for spontaneous tension run-down were made.

Force of contraction immediately prior to the addition of the first agonist concentration within a given experiment was defined as 0% relaxation, and a force of contraction of 0 mN was defined as 100% relaxation. The mean of up to 4 bladder strips from a given patient or animal was considered as one experiment for KCl-induced contraction, whereas only a single strip was tested per animal for the relaxation with isoprenaline or nebivolol.

2.2. Radioligand binding

CHO cells expressing the β-adrenoceptor subtypes at densities of 118, 202 and 199 fmol/mg for β_1-, β_2- and β_3-adrenoceptors, respectively (Niclauß et al., 2006), were obtained from Dr. Carsten Hoffmann (University of Würzburg, Germany) and cultured in Dulbecco's Modified Eagle's Medium with nutrient mixture F12 in the presence of 10% foetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.6 g/l $NaHCO_3$ and 0.2 mg/ml geneticin (Invitrogen, Breda, The Netherlands) until sub-confluent. CHO cells were harvested and membranes were prepared as described (Niclauß et al., 2006). They were washed twice by centrifugation at 200 *g* and then homogenized in ice-cold buffer (50 mM Tris, 0.5 mM EDTA, pH 7.5) with an Ultra-Turrax (Janke & Kinkel, Staufen, Germany) for 10 s at full speed and then twice for 20 s at 2/3 speed. The homogenates were centrifuged for 20 min at 50,000 *g* at 4 °C. The pellets were re-suspended in buffer and stored at −80 °C. Protein content was measured by the method of Bradford (Bradford, 1976) using bovine immunoglobulin G as the standard.

The radioligand binding experiments were performed as previously described (Niclauß et al., 2006). Briefly, the frozen samples were thawed and re-homogenized (10 s at full speed) in buffer. Saturation binding studies were performed using [^{125}I]-cyanopindolol ([^{125}I]-CYP) as the radioligand. The concentrations of [^{125}I]-CYP were adapted to the subtypes under investigation ($\beta_1 \approx 45$ pM, $\beta_2 \approx 37$ pM, and $\beta_3 \approx 339$ pM). Non-specific binding was defined as binding in the presence of 100 µM isoprenaline. All experiments were performed in duplicate in 96 well plates, and incubations were terminated by rapid vacuum filtration over Whatman GF/C using a Filtermate harvester (Perkin Elmer, Zaventem, Belgium). Each filter was washed with approximately 10 ml of buffer. Radioactivity adherent to the filters was quantified in a Topcount NXT (Perkin Elmer) using Microsint O scintillator (Perkin Elmer).

2.3. Cyclic AMP accumulation

Rat bladder smooth muscle cell (RBSMC) isolation was performed as described previously (Ma et al., 2002). RBSMCs were cultured in RPMI-1640 in the presence of 10% foetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin until confluent. The RBSMCs were plated at 20,000 cells/well in 96-well plates two days before the measurement. On the day of the experiment, following overnight serum starvation, the RBSMCs were washed with stimulation buffer (HBSS containing 0.05% BSA (fatty-acid free) and 5 mM HEPES) and subsequently stimulated for 15 min with various concentrations of nebivolol or isoprenaline in stimulation buffer with 0.5 mM IBMX at room temperature. After removal of the ligand the cells were lysed in 50 µl 0.5% Triton X-100 in stimulation buffer with 0.5 mM IBMX, and 10 µl of the lysate was added to 384-well optiplates in triplicate.

CHO cells were cultured as described above to confluency and serum-starved overnight. On the day of the experiment, cells were detached with enzyme-free dissociation buffer and added to 384 well plates at 1250 cells/well in a final volume of 10 µl. They were incubated with the indicated agonist concentrations for 30 min in stimulation buffer with 0.5 mM IBMX at room temperature. Reactions were stopped by addition of 5 µl detection mix and 5 µl cAMP antibody.

Detection of the cAMP formed during stimulation in both cell types was performed with the LANCE cAMP 384 kit according to the manufacturer's protocol. Measurements were carried out using a Victor 2 plate reader (Wallac, Perkin Elmer, Zaventem, Belgium) 3 h after adding the detection buffer and antibody mixture.

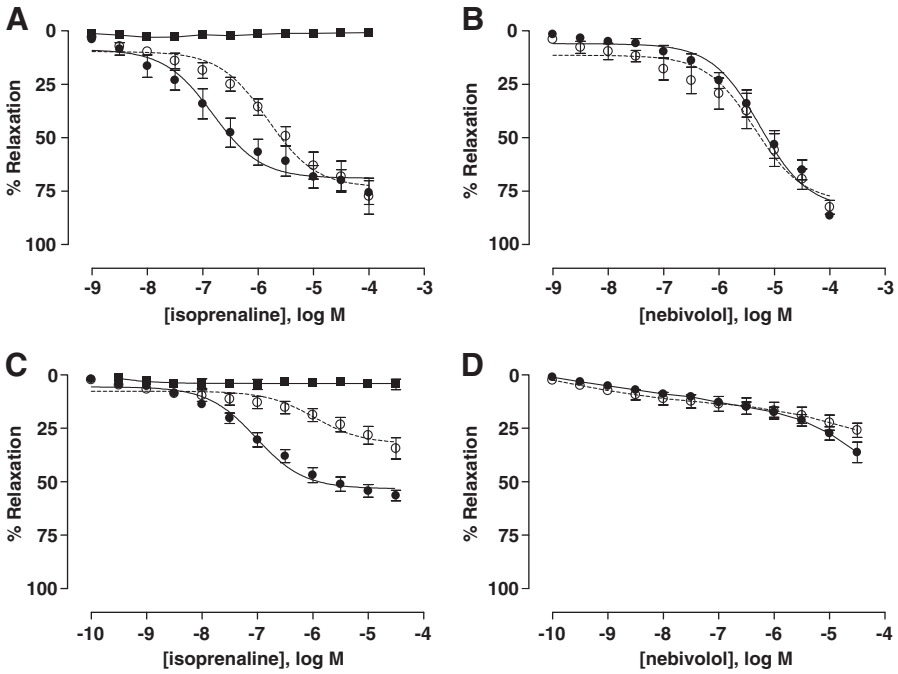

Fig. 1. Isoprenaline- and nebivolol-induced relaxation in human (A,B) and rat (C,D) urinary bladder strips in the absence (filled circles) and presence of 10 μM SR 59,230A (open circles). Data are means of S.E.M. of 6–8 experiments. A quantitative analysis of the data is shown in Table 1. Data from time control experiments in the absence of agonist are shown in filled squares ($n = 11$ and 6 for human and rat bladder, respectively).

After completion of the experiments, we found that our stocks of β_1- and β_2-adrenoceptor expressing CHO cells were mycoplasma positive. While this may have affected the overall magnitude of the response, it is unlikely to affect the analysis of nebivolol effects relative to those of isoprenaline in the same experiments.

2.4. Chemicals

Pentobarbital was obtained from OPG^{FARMA} Groothandel B.V. (Utrecht, Netherlands). Racemic nebivolol was a kind gift from the Johnson & Johnson (Beerse, Belgium). [^{125}I]-CYP (specific activity 2200 Ci/mmol) was obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). All other compounds were obtained from Sigma (Deisenhofen, Germany). Nebivolol and SR 59,230A (3-(2-Ethylphenoxy)-1-[[(1S)-1,2,3,4,-tetrahydronaphth-1-yl]amino]-(2S)-2-propanol oxalate salt) were dissolved in dimethylsulfoxide. Isoprenaline hemisulfate salt was dissolved in deionized water. All stocks are in 10 mM solutions of their respective solvents.

2.5. Data analysis

The functional and radioligand competition binding data were analyzed using non-linear sigmoidal curve fitting to calculate the pEC_{50} and E_{max} or IC_{50} values, respectively; however, in some cases curve fitting was not possible due to strong inhibition of the response. The IC_{50} values were converted to K_i values based upon the Cheng–Prusoff equation (Cheng and Prusoff, 1973) and previously obtained K_d values at the three subtypes (Niclauß et al., 2006). The statistical significance of group differences was assessed by two-tailed *t*-tests or one way ANOVA followed by Dunnett's post-test. A $P<0.05$ was considered significant. All curve fitting and statistical calculations were performed using Prism (Graphpad Software, San Diego, CA, USA). All data shown are mean values ± S.E.M. of *n* experiments.

3. Results

3.1. Relaxation studies

The KCl-induced tension in human bladder strips was 2.6 ± 0.3 vs. 1.9 ± 0.2 mN/mm ($P<0.05$) or 1.6 ± 0.2 vs. 1.3 ± 0.2 mN/mg ($n = 15$ muscle strips) in the absence and presence of 10 μM SR 59,230A, respectively. This tone was stable over time (Fig. 1). Isoprenaline caused a concentration-dependent relaxation (Fig. 1, Table 1). SR 59,230A (10 μM) did not markedly affect the maximum response but shifted the isoprenaline concentration–response curve to the right by an average of 1.1 log units (Fig. 1A); the corresponding apparent pA_2 value of 6.1 is in line with its reported affinity at human β_3-adrenoceptor (Hoffmann et al., 2004; Niclauß et al., 2006) and in the human bladder (Yamanishi et al., 2006). Nebivolol caused a similar concentration-dependent relaxation of the human bladder strips. Its maximum effects were similar to those of isoprenaline but its potency was somewhat lower; in contrast to the isoprenaline

Table 1
Isoprenaline- and nebivolol-induced relaxation in rat and human urinary bladder strips in the absence (control) and presence of 10 μM SR 59,230A. Data are means of ± S.E.M. of 6–8 experiments and are expressed as percent relaxation and as pEC_{50} values. The nebivolol-induced relaxation in the rat did not follow a sigmoidal concentration–response curve and hence did not permit curve fitting; therefore, E_{max} represents relaxation in response to 30 μM nebivolol and no pEC_{50} was calculated. a: $P<0.05$ vs. data in absence of SR 59,230A; n.d.: not determined.

Compounds	E_{max}		pEC_{50}	
	Control	+ SR 59,230A	Control	+ SR 59,230A
Human bladder				
Nebivolol	87 ± 2	83 ± 3	5.5 ± 0.5	5.9 ± 0.7
Isoprenaline	76 ± 6	77 ± 8	7.1 ± 0.1	6.2 ± 0.2[a]
Rat bladder				
Nebivolol	36 ± 5	26 ± 3	n.d.	n.d.
Isoprenaline	57 ± 2	34 ± 5[a]	7.0 ± 0.2	6.2 ± 0.3[a]

response, relaxation by nebivolol was not significantly antagonized by SR 59,230A (Fig. 1, Table 1).

In the rat bladder the KCl-induced tension was 1.9 ± 0.1 vs. 1.4 ± 0.1 mN/mm or 4.4 ± 0.2 vs. 3.1 ± 0.1 mN/mg ($P<0.05$ each, $n = 18–23$ animals) in the absence and presence of 10 μM SR 59,230A, respectively, and remained stable with time (Fig. 1). Isoprenaline caused a concentration-dependent relaxation (Fig. 1, Table 1). SR 59,230A (10 μM) caused a similar right-shift of this curve as in the human bladder, but in the rat additionally reduced the maximum relaxation (Fig. 1, Table 1). In contrast to the human bladder, nebivolol caused a much smaller relaxation than isoprenaline in the rat bladder (Fig. 1). However, its concentration–response curve was very shallow and lacked a sigmoidal shape not allowing reliable potency calculations. In line with the human bladder data, rat bladder relaxation by nebivolol was not affected by the presence of SR 59,230A (Fig. 1).

3.2. Radioligand binding

Nebivolol competed for β-adrenoceptor subtype binding with an order of potency of $\beta_1 > \beta_2 > \beta_3$, and the calculated pK_i values were 9.17 ± 0.23, 7.96 ± 0.07 and 5.66 ± 0.06, respectively ($n = 4–8$), demonstrating a much lower affinity for β_3- than for β_1- or β_2-adrenoceptors.

3.3. Cyclic AMP accumulation

Basal cAMP levels were 17.7 ± 1.0, 18.5 ± 6.3, 16.2 ± 1.0, and 80.2 ± 28.9 fmol/well in CHO cells expressing β_1-, β_2- or β_3-adrenoceptors and in RBSMCs, respectively ($n = 8–9$). Isoprenaline concentration-dependently stimulated cAMP accumulation in all four cell types (Fig. 2, Table 2). In contrast, over a concentration range from 0.1 nM to 30 μM nebivolol did not stimulate cAMP accumulation in either cell type (Fig. 2). At a concentration of 10 nM, which occupies >95% of β_1-adrenoceptors, nebivolol markedly inhibited isoprenaline-induced cAMP accumulation via β_1-adrenoceptors, but did not significantly affect that via β_2- or β_3-adrenoceptors (Fig. 2, Table 2). Interestingly, inhibition of the β_1-adrenoceptor apparently involved a reduced maximum response.

Table 2
Cyclic AMP elevation by isoprenaline in the absence (control) or presence of 10 nM nebivolol in CHO cells stably transfected with human β-adrenoceptor subtypes or rat bladder smooth muscle cells (RBSMCs). Data are means of S.E.M. of 3–4 experiments and are expressed as nM increase above basal cAMP levels. a: $P<0.05$ vs. data in absence of nebivolol; b: these values are only estimates because isoprenaline did not exhibit a saturating sigmoidal concentration–response curve in the presence of nebivolol (see Fig. 2A).

	Control		+ Nebivolol	
	E_{max} (fmol/well)	pEC_{50}	E_{max} (fmol/well)	pEC_{50}
CHO-β_1	271 ± 27	7.8 ± 0.2	102 ± 21[a,b]	6.9 ± 0.2[a,b]
CHO-β_2	387 ± 31	9.1 ± 0.3	369 ± 15[a]	8.8 ± 0.2
CHO-β_3	372 ± 27	7.5 ± 0.2	317 ± 20	7.4 ± 0.3
RBSMCs	1436 ± 468	8.0 ± 0.1	–	–

4. Discussion

Nebivolol is a β_1-adrenoceptor antagonist with vasodilating properties (Ignarro, 2008). The molecular target mediating such vasodilatation has not been defined unequivocally, but several studies have suggested it to be a β_3-adrenoceptor (de Groot et al., 2003; Tran Quang et al., 2009; Rozec et al., 2009; Dessy et al., 2005). On the other hand, some studies have specifically excluded an involvement of β_3-adrenoceptors in nebivolol-induced vasodilatation (Chlopicki et al., 2002). The present study was designed to investigate possible β_3-adrenoceptor agonism of nebivolol in non-vascular preparations which are considered prototypical for this receptor subtype.

In clinical studies the β_1-adrenoceptor antagonist and vasodilating properties of nebivolol may reside in distinct stereo-isomers (van Nueten and De Cree, 1998), whereas experimental *in vitro* studies found nebivolol-induced vasodilatation not be stereo-selective (Chlopicki et al., 2002; Tran Quang et al., 2009). The use of racemic nebivolol in the present study should avoid false negative findings in this regard. Another methodological consideration is the use of SR 59,230A. While this compound is not selective for β_3-adrenoceptors, in contrast to many other β-adrenoceptor antagonists it clearly blocks them at a

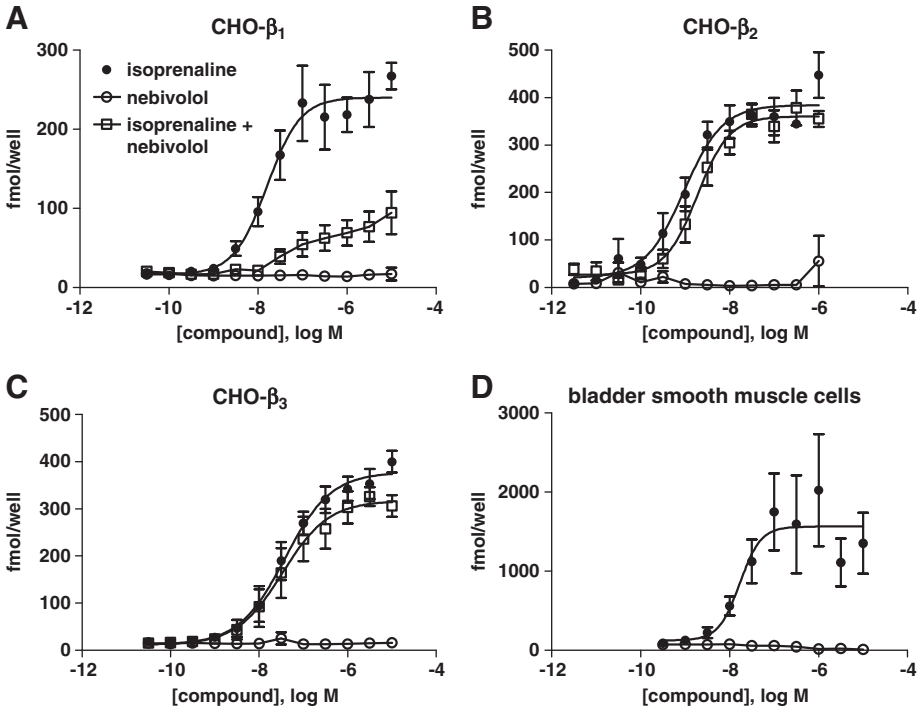

Fig. 2. cAMP accumulation induced by isoprenaline in the presence or absence of 10 nM nebivolol or by nebivolol alone in human β_1-adrenoceptor (A), β_2-adrenoceptor (B), β_3-adrenoceptor (C) and RBSMC (D). Data are means of S.E.M. of 3–4 experiments. A quantitative analysis of the data is shown in Table 2.

concentration of 10 μM (Hoffmann et al., 2004; Niclauß et al., 2006). Moreover, SR 59,230A can be a partial agonist at β_3-adrenoceptors in some preparations (Vrydag and Michel, 2007), as reflected in our data by a lower KCl-induced rat and human bladder tone in the presence of SR 59,230A. On the other hand, we have previously shown that the degree of pre-contraction does not affect the ability of a β-adrenoceptor agonist to cause relaxation in the rat bladder (Michel and Sand, 2009).

The overall role of β_3-adrenoceptors in human blood vessels is not fully clear, and no single human vascular preparation has been confirmed to exhibit vasodilatation primarily via a β_3-adrenoceptor (Rozec and Gauthier, 2006). Therefore, we have used relaxation of the human urinary bladder smooth muscle, a prototypical β_3-adrenoceptor response (Michel and Vrydag, 2006; Yamaguchi and Chapple, 2007), to initially explore possible β_3-adrenoceptor effects of nebivolol. Indeed nebivolol caused human bladder relaxation to a similar extent as isoprenaline, albeit with lower potency which is in line with its low potency to cause vasodilatation (Ignarro, 2008). However, the antagonist SR 59,230A did not significantly affect the relaxation by nebivolol, despite being used in a concentration occupying >90% of human β_3-adrenoceptors (Hoffmann et al., 2004; Niclauß et al., 2006) and despite causing a shift of the isoprenaline concentration–response curve in line with its reported antagonist potency against isoprenaline in human bladder (Yamanishi et al., 2006). These findings do not support the hypothesis of nebivolol acting as a β_3-adrenoceptor agonist in the human bladder.

We have attempted to further explore possible β_3-adrenoceptor-mediated smooth muscle relaxation by nebivolol in a second preparation, the rat urinary bladder. While the overall evidence suggests that this is also a mainly β_3-adrenoceptor preparation, in contrast to the human bladder additional involvement of other β-adrenoceptor subtypes, particularly β_2-adrenoceptors, has been proposed in rat bladder (Michel and Vrydag, 2006). In rat bladder isoprenaline-induced relaxation was similarly inhibited by SR 59,230A as in human bladder. However, nebivolol caused much less relaxation in rat bladder than isoprenaline and its concentration–response curve was very shallow and did not follow the expected simple sigmoidal function. Moreover, as in the human bladder, the nebivolol-induced relaxation of rat bladder was insensitive to SR 59,230A in a concentration where it should occupy more than 90% of β_3-adrenoceptors. Taken together the findings in rat and human bladder extend previous observations of low-potency smooth muscle relaxation by nebivolol to a non-vascular tissue of two mammalian species, but cast doubt on the involvement of a β_3-adrenoceptor.

As the bladder data did not yield a conclusive answer to the question whether nebivolol is a β_3-adrenoceptor agonist, we turned to cloned human β-adrenoceptor subtypes stably transfected into CHO cells at physiological densities (Hoffmann et al., 2004; Niclauß et al., 2006). Our binding data confirm selectivity of nebivolol for β_1- over β_2-adrenoceptors but show only low affinity for β_3-adrenoceptor, which is more than 1000-fold different from that at β_1-adrenoceptors. Nevertheless the affinity estimate for nebivolol at cloned human β_3-adrenoceptors is in line with its potency to induce vasodilatation (Ignarro, 2008) and to cause human bladder relaxation.

Finally, we have tested whether nebivolol indeed is an agonist by investigating whether it activates the prototypical signalling response of β-adrenoceptors, i.e., enhances cAMP formation. Our data do not support agonism of nebivolol at any of the three cloned β-adrenoceptor subtypes over a wide concentration range, which spans its affinities at all three subtypes. Similarly, nebivolol failed to induce cAMP formation in RBSMCs under conditions where isoprenaline was effective. In a concentration selected to imitate therapeutic blockade of β_1-adrenoceptors, nebivolol inhibited the cAMP formation via the β_1-adrenoceptor subtype but not via β_2- or β_3-adrenoceptors, a finding in line with the lower potency at the latter two as compared to β_1-adrenoceptors. After completion of our experiments, other investigators have also reported a lack of agonism of nebivolol for cAMP formation in CHO cells transfected with human β_1-, β_2- or β_3-adrenoceptors (Baker, 2010). These findings demonstrate a low affinity of nebivolol at human β_3-adrenoceptors and do not support the hypothesis that it acts as a low-potency agonist at these receptors.

In conclusion, nebivolol can cause low-potency relaxation of rat and human urinary bladder smooth muscle which, in contrast to the effects of isoprenaline, is not sensitive to inhibition by the β_3-adrenoceptor antagonist SR 59,230A. Nebivolol has low affinity for cloned human β_3-adrenoceptors, which is in line with its low potency to induce vascular and bladder smooth muscle relaxation. This questions the idea that therapeutic nebivolol doses may act on β_3-adrenoceptors. Nebivolol also failed to induce cAMP formation in RBSMCs or at any cloned β-adrenoceptor in the present or a previous study (Baker, 2010). Therefore, our data do not support the hypothesis that nebivolol causes smooth muscle relaxation by β_3-adrenoceptor agonism, certainly not for non-vascular preparations. However, the possibility exists that nebivolol is a β_3-adrenoceptor agonist inducing ligand-directed signalling also known as biased agonism (Michel and Alewijnse, 2007), i.e., nebivolol fails to promote cAMP formation but may activate other β_3-adrenoceptor-coupled signalling pathways. Specific examples of ligand-directed signalling at β_3-adrenoceptor have been reported (Sato et al., 2007). Such alternative signalling pathways of β_3-adrenoceptors include activation of BK_{Ca} or other potassium channels, which are important in smooth muscle tone (Ferro, 2006; Scherer et al., 2007). β-Adrenoceptor-mediated bladder relaxation largely occurs cAMP independently potentially via activation of BK_{Ca} potassium channels (Frazier et al., 2008). Thus, nebivolol not only failed to promote cAMP formation but also to induce prototypical potassium channel-mediated β_3-adrenoceptor-mediated responses. This makes ligand-direct signalling an unlikely hypothesis to support β_3-adrenoceptor agonism by nebivolol. Finally, it remains possible that β_3-adrenoceptor agonist properties of nebivolol *in vivo* are at least partly mediated by active metabolites of the drug (Ignarro, 2008) but in this case the question arises why the parent compound would have caused vasodilatation but not bladder relaxation via β_3-adrenoceptors *in vitro*. With regard to the bladder, only clinical studies will allow to determine whether the low-potency relaxation observed here *in vitro* has a relevant correlate *in vivo*.

Acknowledgement

We thank Dr. Henk Sipma (Johnson & Johnson) for providing us with nebivolol.

References

Baker, J.G., 2010. The selectivity of β-adrenoceptor agonists at human β_1-, β_2- and β_3-adrenoceptors. Br. J. Pharmacol. 160, 1048–1061.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Broeders, M.A., Doevendans, P.A., Bekkers, B.C., Bronsaer, R., van Gorsel, E., Heemskerk, J.W., Egbrink, M.G., van Breda, E., Reneman, R.S., van Der Zee, R., 2000. Nebivolol: a third-generation beta-blocker that augments vascular nitric oxide release: endothelial β_2-adrenergic receptor-mediated nitric oxide production. Circulation 102, 677–684.

Bundkirchen, A., Brixius, K., Bölck, B., Nguyen, Q., Schwinger, R.H.G., 2003. β_1-adrenoceptor selectivity of nebivolol and bisoprolol. A comparison of [^{3}H]CGP 12.177 and [^{125}I]iodocyanopindolol binding studies. Eur. J. Pharmacol. 460, 19–26.

Candelore, M.R., Deng, L., Tota, L., Guan, X.-M., Amend, A., Liu, Y., Newbold, R., Cascieri, M.A., Weber, A.E., 1999. Potent and selective human β_3-adrenergic receptor antagonists. J. Pharmacol. Exp. Ther. 290, 649–655.

Cheng, Y.-C., Prusoff, W.H., 1973. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Chlopicki, S., Kozlovski, V.I., Gryglewski, R.J., 2002. NO-dependent vasodilation induced by nebivolol in coronary circulation is not mediated by β-adrenoceptors or by 5 HT_{1A}-receptors. J. Physiol. Pharmacol. 55, 615–624.

de Groot, A.A., Mathy, M.-J., van Zwieten, P.A., Peters, S.L.M., 2003. Involvement of the β_3 adrenoceptor in nebivolol-induced vasorelaxation in the rat aorta. J. Cardiovasc. Pharmacol. 42, 232–235.

Dessy, C., Saliez, J., Ghisdal, P., Daneau, G., Lobysheva, I.I., Frerart, F., Belge, C., Jnaoui, K., Noirhomme, P., Feron, O., Balligand, J.L., 2005. Endothelial β_3-adrenoreceptors mediate nitric oxide-dependent vasorelaxation of coronary microvessels in response to the third-generation beta-blocker nebivolol. Circulation 112, 1198–1205.

Evangelista, S., Garbin, U., Pasini, A.F., Stranieri, C., Boccioletti, V., Cominacini, L., 2007. Effect of DL-nebivolol, its enantiomers and metabolites on the intracellular production of superoxide and nitric oxide in human endothelial cells. Pharmacol. Res. 55, 303–309.

Ferro, A., 2006. β-Adrenoceptors and potassium channels. Naunyn-Schmiedeberg's Arch. Pharmacol. 373, 183–185.

Frazier, E.P., Mathy, M.-J., Peters, S.L.M., Michel, M.C., 2005. Does cyclic AMP mediate rat urinary bladder relaxation by isoproterenol? J. Pharmacol. Exp. Ther. 313, 260–267.

Frazier, E.P., Schneider, T., Michel, M.C., 2006. Effects of gender, age and hypertension on β-adrenergic receptor function in rat urinary bladder. Naunyn-Schmiedeberg's Arch. Pharmacol. 373, 300–309.

Frazier, E.P., Peters, S.L.M., Braverman, A.S., Ruggieri Sr., M.R., Michel, M.C., 2008. Signal transduction underlying control of urinary bladder smooth muscle tone by muscarinic receptors and β-adrenoceptors. Naunyn-Schmiedeberg's Arch. Pharmacol. 377, 449–462.

Garban, H.J., Buga, G.M., Ignarro, L.J., 2004. Estrogen receptor-mediated vascular responsiveness to nebivolol: a novel endothelium-related mechanism of therapeutic vasorelaxation. J. Cardiovasc. Pharmacol. 43, 638–644.

Hoffmann, C., Leitz, M.R., Oberdorf-Maass, S., Lohse, M.J., Klotz, K.-N., 2004. Comparative pharmacology of human β-adrenergic receptor subtypes — characterization of stably transfected receptors in CHO cells. Naunyn-Schmiedeberg's Arch. Pharmacol. 369, 151–159.

Ignarro, L.J., 2008. Different pharmacological properties of two enantiomers in a unique β-blocker, nebivolol. Cardiovasc. Ther. 26, 115–134.

Kalinowski, L., Dobrucki, L.W., Szczepanska-Konkel, M., Jankowski, M., Martyniec, L., Angielski, S., Malinski, T., 2003. Third-generation β-blockers stimulate nitric oxide release from endothelial cells through ATP efflux: a novel mechanism for antihypertensive action. Circulation 107, 2747–2752.

Kamp, O., Metra, M., Bugatti, S., Bettari, L., Dei Cas, A., Petrini, N., Dei Cas, L., 2010. Nebivolol. Haemodynamic effects clinical significance combined β blockade nitric oxide release. Drugs 70, 41–56.

Ma, F.H., Higashira-Hoshi, H., Itoh, Y., 2002. Functional muscarinic M_2 and M_3 receptors and β-adrenoceptors in cultured rat bladder smooth muscle. Life Sci. 70, 1159–1172.

Michel, M.C., Alewijnse, A.E., 2007. Ligand-directed signaling: 50 ways to find a lover. Mol. Pharmacol. 72, 1097–1099.

Michel, M.C., Sand, C., 2009. Effect of pre-contraction on β-adrenoceptor-mediated relaxation of rat urinary bladder. World J. Urol. 27, 711–715.

Michel, M.C., Vrydag, W., 2006. α_1-, α_2- and β-adrenoceptors in the urinary bladder, urethra and prostate. Br. J. Pharmacol. 147, S88–S119.

Niclauß, N., Michel-Reher, M.B., Alewijnse, A.E., Michel, M.C., 2006. Comparison of three radioligands for the labelling of human β-adrenoceptor subtypes. Naunyn-Schmiedeberg's Arch. Pharmacol. 374, 79–85.

Pauwels, P.J., Gommeren, W., van Lommen, G., Janssen, P.A.J., Leysen, J.E., 1988. The receptor binding profile of the new antihypertensive agent nebivolol and its stereoisomers compared with various β-adrenergic blockers. Mol. Pharmacol. 34, 843–851.

Rozec, B., Gauthier, C., 2006. β_3-Adrenoceptors in the cardiovascular system: putative roles in human pathologies. Pharmacol. Ther. 111, 652–673.

Rozec, B., Quang, T.T., Noireaud, J., Gauthier, C., 2006. Mixed β_3-adrenoceptor agonist and α_1-adrenoceptor antagonist properties of nebivolol in rat thoracic aorta. Br. J. Pharmacol. 147, 699–706.

Rozec, B., Erfanian, M., Laurent, K., Trochu, J.-N., Gauthier, C., 2009. Nebivolol, a vasodilating selective β_1-blocker is a β_3-adrenoceptor agonist in the nonfailing transplanted heart. J. Am. Coll. Cardiol. 53, 1532–1538.

Sato, M., Horinouchi, T., Hutchinson, D.S., Evans, B.A., Summers, R.J., 2007. Ligand-directed signaling at the β_3-adrenoceptor produced by SR59230A relative to receptor agonists. Mol. Pharmacol. 74, 1359–1368.

Scherer, D., Kiesecker, C., Kulzer, M., Günth, M., Scholz, E.P., Kathöfer, S., Thomas, D., Maurer, M., Kreuzer, J., Bauer, A., Katus, H.A., Karle, C.A., Zitron, E., 2007. Activation of inwardly rectifying Kir2.x potassium channels by β_3-adrenoceptors is mediated via different signaling pathways with a predominant role of PKC for Kir2.1 and of PKA for Kir2.2. Naunyn-Schmiedeberg's Arch. Pharmacol. 375, 311–322.

Schlicker, E., Gothert, M., Hillenbrand, K., 1985. Cyanopindolol is a highly potent and selective antagonist at the presynaptic serotonin autoreceptor in the rat brain cortex. Naunyn-Schmiedeberg's Arch. Pharmacol. 311, 398–401.

Tran Quang, T., Rozec, B., Audigane, L., Gauthier, C., 2009. Investigation of the different adrenoceptor targets of nebivolol enantiomers in rat thoracic aorta. Br. J. Pharmacol. 156, 601–608.

van Nueten, L., De Cree, J., 1998. Nebivolol: comparison of the effects of dl-nebivolol, D-nebivolol, L-nebivolol, atenolol, and placebo on exercise-induced increases in heart rate and systolic blood pressure. Cardiovasc. Drugs Ther. 12, 339–344.

Vrydag, W., Michel, M.C., 2007. Tools to study β_3-adrenoceptors. Naunyn-Schmiedeberg's Arch. Pharmacol. 374, 385–398.

Yamaguchi, O., Chapple, C.R., 2007. β_3-Adrenoceptors in urinary bladder. Neurourol. Urodyn. 26, 752–756.

Yamanishi, T., Yasuda, K., Kitahara, S., Nakai, H., Yoshida, K., Iizuka, H., 2006. Effects of 138-355, a β_3-adrenoceptor selective agonist, on relaxation of the human detrusor muscle in vitro. Neurourol. Urodyn. 25, 815–819.