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β_2-Adrenoceptor Antagonist ICI 118,551 Decreases Pulmonary Vascular Tone in Mice via a $G_{i/o}$ Protein/Nitric Oxide–Coupled Pathway

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Abstract—β_2-Adrenoceptors are important modulators of vascular tone, particularly in the pulmonary circulation. Because neurohormonal activation occurs in pulmonary arterial hypertension, we have investigated the effect of different adrenergic vasoactive substances on tone regulation in large and small pulmonary arteries, as well as in systemic vessels of mice. We found that the β_2-adrenoceptor antagonist ICI 118,551 (ICI) evoked a decrease of vascular tone in large pulmonary arteries and reduced the sensitivity of pulmonary arteries toward different contracting agents, eg, norepinephrine, serotonin, or endothelin. ICI proved to act specifically on pulmonary vessels, because it shifted the dose-response curve of norepinephrine to the right in pulmonary arteries, whereas there was no effect in the aorta. Pharmacological experiments proved that the right shift of the norepinephrine dose-response curve by ICI was mediated via a β_2-adrenoceptor/$G_{i/o}$ protein-dependent pathway enhancing NO production in the endothelium; these results were corroborated in β-adrenoceptor and endothelial NO synthase knockout mice where ICI had no effect. ICI increased vascular lumen diameter in lung sections and reduced pulmonary arterial pressure under normoxia and under hypoxia in the isolated perfused lung model. These effects were found to be physiologically relevant, because ICI specifically decreased pulmonary but not systemic blood pressure in vivo. Thus, the β_2-adrenoceptor antagonist ICI is a pulmonary arterial-specific vasorelaxant and, therefore, a potentially interesting novel therapeutic agent for the treatment of pulmonary arterial hypertension. (*Hypertension*. 2009;54:157-163.)

Key Words: basic science ■ adrenoceptors ■ blood flow regulation ■ NO ■ cell signaling ■ pulmonary hypertension

β-Adrenoceptors (β-ARs) can couple to G_s, $G_{i/o}$ and/or G_q proteins and have an important vasorelaxing function in the systemic and pulmonary circuits. All of the isoforms that are known so far (β_1-, β_2-, β_3-, and low-affinity-state β_1-AR) have been identified in pulmonary vessels,[1–3] with the β_2-AR being the most potent for vasorelaxation.[4] The pulmonary arteries (PAs) are important therapeutic targets, because pulmonary arterial hypertension often occurs after cardiac surgery in children[5] but also in adults and can result in life-threatening right heart failure. Therefore, specific and fast-acting vasodilators for PAs are urgently needed. However, most drug treatment regimens also strongly decrease vascular tone in the systemic circuit, leading to hypotension.

Other than β-AR agonists, β-blockers were also reported to show promising results in the treatment of pulmonary arterial hypertension, which was not caused by left ventricular disorders.[6] In systemic arteries, it has been demonstrated that third-generation β-blockers can directly induce vasodilation via NO release from the endothelium[7] and thereby positively affect the afterload. The aim of our study was to identify pulmonary specific vasorelaxants, and we have, therefore, investigated the impact of different β-adrenergic mediators on vascular tone in the pulmonary and systemic circulations of mouse. We have identified ICI 118,551 (ICI), a β-blocker with antagonist activity at β_2-AR that is known to be >500-fold selective over β_1- and β_3-AR,[8] as an interesting candidate. Pharmacological studies revealed β_2-AR blockade in isolated tissues, in animals, and in humans by ICI.[9,10] Moreover, inverse agonist properties of ICI were demonstrated in a transgenic mouse model of β_2-AR overexpression, where the increased baseline tension in the left atrium could be diminished by ICI binding, shifting the equilibrium of the β_2-AR to the inactive state.[11] In the present study, we have found that the specific β_2-AR blocker ICI acts as an agonist that induces vasorelaxation via a $G_{i/o}$ protein/NO-coupled pathway specifically in PA and may, therefore, be used for the therapy of pulmonary arterial hypertension.

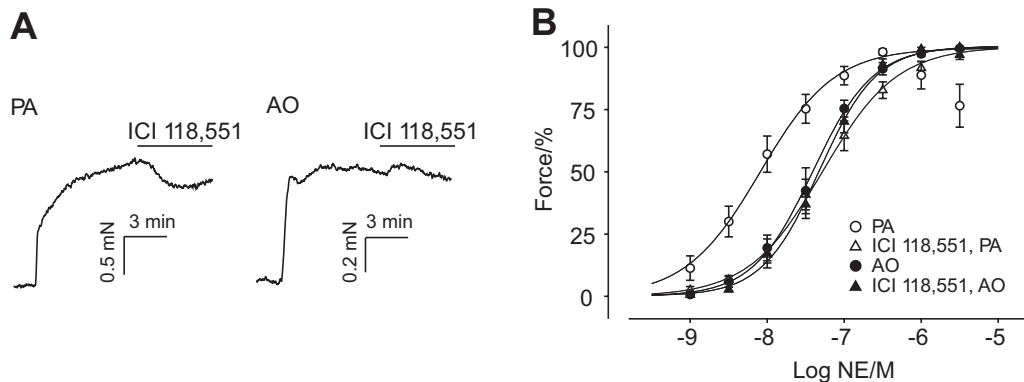**Figure 1.** The β_2-specific inverse agonist ICI reduces the sensitivity of murine PA but not AO toward different vasoconstrictors in isometric force measurements. A, ICI relaxed NE precontracted PA (left) but not AO (right). B, Dose-response curve of NE in PA and AO with and without ICI.

Methods

Please see the online data supplement available at http://hyper.ahajournals.org.

Results

ICI Reduces the Sensitivity of PAs to Different Vasoconstrictors

To identify vasoactive substances that are specific for pulmonary vessels, we have investigated the effect of adrenergic agonists and antagonists on vascular tone of murine PAs and aortas (AOs). These vessels were chosen because they are of similar size and easily accessible.

We found that the β_2-AR–specific inverse agonist ICI (10 μmol/L) induced a prominent vasorelaxation of norepinephrine (NE)-precontracted PA but not AO (Figure 1A). To investigate the pulmonary selectivity in detail, we performed dose-response curves for different vasoconstrictors in PAs and AOs after ICI treatment. Our experiments show that the PA was more sensitive to all of the vasoconstrictors tested compared with the AO (NE: pEC_{50}=8.09±0.20, n=8 [PA] versus 7.41±0.07, n=5 [AO], $P<0.05$; phenylephrine: 7.48±0.08, n=6 [PA] versus 6.51±0.09, n=6 [AO], $P<0.05$; endothelin: 9.28±0.09, n=4 [PA] versus 7.82±0.05, n=4 [AO], $P<0.05$). Similarly, there was also an apparent difference in pEC_{50} values between PA and AO for serotonin (5-HT), although it was not statistically significant (7.48±0.20, n=5 [PA] versus 6.98±0.07, n=6 [AO], $P>0.05$).

Preincubation with ICI shifted the concentration-response curves of the PA to the right, indicating a reduced sensitivity toward the vasoconstrictors (pEC_{50}: 8.09±0.20, n=8 [NE] versus 7.28±0.08, n=6 [NE+ICI], $P<0.05$ [Figure 1B]; 9.28±0.09, n=4 [endothelin] versus 8.24±0.04, n=3 [endothelin+ICI], $P<0.05$, 7.48±0.20, n=5 [5-HT] versus 6.61±0.24, n=4 [5-HT+ICI], $P<0.05$; Figure S1A through S1C, available in the online data supplement.

Vascular reactivity to NE is particularly important, because pulmonary arterial hypertension has been reported to often be associated with elevated blood levels of NE, which are also significant predictors of mortality.[5,12] Interestingly, ICI selectively decreased NE sensitivity of PAs, whereas there was no change in AOs (pEC_{50}: 7.41±0.07, n=5 [NE, AO] versus 7.32±0.05, n=6 [NE+ICI, AO], $P>0.05$; Figure 1B).

To test the potential of other β-blockers to influence NE vasoreactivity, we preincubated PAs and AOs with the β_1-AR antagonist metoprolol. This treatment was without effect on NE dose response. Similarly, the specific β_3-antagonist SR 59230A (SR; 10 μmol/L) only evoked a minor shift of NE dose response in PAs and AOs. Because the reduction of sensitivity to vasoconstrictors could be an effect of β_2-AR antagonists in general, we also examined the effect of other β_2-AR blockers. However, the β_1/β_2-blocker propranolol and the β_2-AR specific antagonist butoxamine (Butox) only slightly decreased the sensitivity toward NE in PAs (Table S1). This suggests that the reduction of vasoreactivity toward NE by ICI is not a general effect of all β_2-AR antagonists but a specific property of this compound.

ICI Selectively Reduces NE Sensitivity via β_2-Adrenoceptors

To rule out possible unspecific effects of ICI that could be independent from β-ARs, we tested the impact of ICI on NE dose response in β-AR knockout mice where β_1-, β_2-, and β_3-ARs are absent.[13] In these mice, the right shift of NE dose response in PAs by ICI was found to be abrogated (pEC_{50}: 7.70±0.20, n=4 [β-knockout {KO}+NE] versus 7.55±0.13, n=4 [β-KO+NE+ICI], $P>0.05$; Figure 2A). Thus, ICI reduced the sensitivity toward NE in PAs via β-ARs. To further investigate the specific receptor subtype involved, the vessels of wild-type mice were preincubated with the β_3-AR–selective antagonist SR or the specific β_1-AR blocker CGP 20712A (300 nmol/L). In both cases, the treatment did not affect the right shift of the NE dose-response curve by ICI (pEC_{50}: 7.62±0.05, n=4 [NE+SR, PA] versus 7.22±0.06, n=4 [NE+SR+ICI, PA], $P<0.05$; Figure 2B; pEC_{50}: 8.73±0.04, n=6 [NE+CGP 20712A, PA] versus 7.64±0.04, n=3 [NE+CGP 20712A+ICI, PA], $P<0.05$; Figure 2C), indicating that neither β_1- nor β_3-AR was involved. The mediation of the effect of ICI by β_2-AR was also corroborated by the pretreatment of vascular rings with Butox (10 μmol/L), which led to a strong attenuation of the right shift, especially at low NE concentrations (pEC_{50}: 7.70±0.06, n=4 [NE+Butox, PA] versus 7.36±0.06, n=4 [NE+Butox+ICI, PA], $P>0.05$; Figure 2D). Thus, ICI decreased the sensitivity toward NE in PA by β_2-AR.
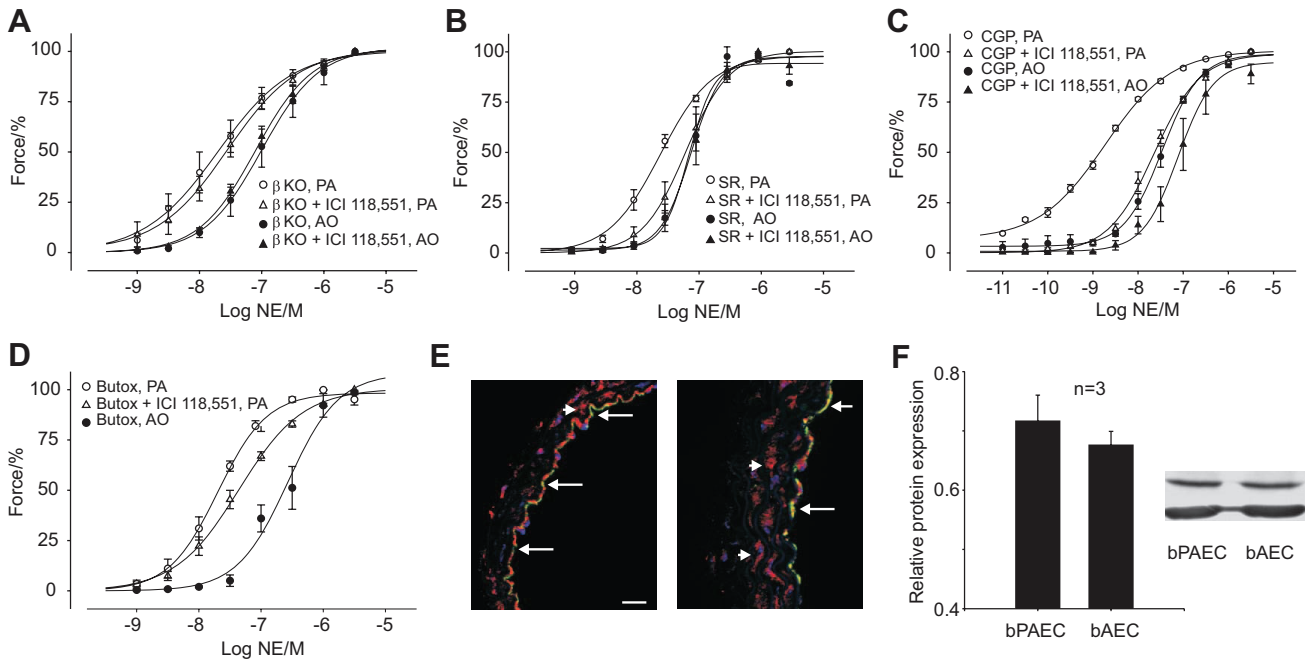
Figure 2. The effect of ICI depends on β_2-ARs. A, ICI had no effect in β_1-, β_2-, or β_3-AR KO mice (β KO). B, β_3-AR blocker SR did not prevent the reduction of sensitivity toward NE by ICI. C, β_1-AR blocker CGP 20712A (CGP) did not inhibit the effect of ICI either. D, β_2-AR blocker Butox strongly attenuated the response to ICI. E, Immunohistochemistry in murine PA (left) and AO (right) with β_2-AR (red) and CD31 (green) antibodies. In both arteries, β_2-AR could be found in the endothelium (arrow), as well as in the smooth muscle layer below (arrowheads); bar=20 μm. F, Western blot experiments revealed a similar β_2-AR expression in bPAECs and bAECs.

However, the pulmonary arterial specificity of the compound is not related to the number of β_2-ARs in PAs or AOs, because immunostainings revealed a similar distribution in both vessels (Figure 2E), and semiquantitative analysis of Western blot experiments confirmed equal amount of β_2-ARs in bovine pulmonary endothelial cells (bPAECs) and bovine aortic endothelial cells (bAECs) (Figure 2F).

Effect of ICI Is Mediated by a $G_{i/o}$/NO-Dependent Pathway

β_2-ARs are known to couple to different G proteins, and $G_{i/o}$-protein activation has been shown to be involved in the induction of vasorelaxation.[14] To examine a possible contribution of $G_{i/o}$ proteins to ICI-induced changes in vasoreactivity, we preincubated PAs and AOs with the $G_{i/o}$ protein blocker pertussis toxin (PTX; 1 μg/mL) for 12 hours. This did not change pEC$_{50}$ values for NE dose-response curves (PA: pEC$_{50}$: 8.09±0.20, n=8 [NE] versus 7.97±0.12, n=4 [NE+PTX], $P>0.05$; AO: 7.41±0.07, n=5 [NE] versus 7.16±0.05, n=4 [NE+PTX], $P>0.05$). However, PTX completely prevented the decrease of PA sensitivity by ICI (pEC$_{50}$: 8.08±0.09, n=4, [NE+PTX+ICI] $P>0.05$ versus NE+PTX; Figure 3A). Thus, ICI reduced the sensitivity of PA to NE by activation of $G_{i/o}$ proteins.

Vasorelaxation is often mediated via endothelium-derived factors like NO. Therefore, we assessed the contribution of the pulmonary vascular endothelium in ICI signaling. Removal of the endothelium alone had no effect on the cumulative NE concentration response (pEC$_{50}$: 7.88±0.08, n=5 [NE, PA without endothelium], $P>0.05$ versus NE, PA). However, in endothelium-denuded PAs, the sensitivity to-

ward NE remained unchanged after ICI application (pEC$_{50}$: 7.73±0.05, n=4 [NE+ICI, PA without endothelium], $P>0.05$ versus NE, PA without endothelium; Figure 3B), indicating that ICI evoked the release of endothelium-dependent mediators, which lowered NE sensitivity. The complete removal of the endothelium in our preparation was confirmed by immunostaining with CD31 (Figure 3C). The impact of NO was determined after incubation with the endothelial NO synthase (eNOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L). This substance strongly inhibited the right shift of the dose-response curve by ICI (pEC$_{50}$: 7.89±0.17, n=4 [NE+L-NAME+ICI], $P>0.05$ versus 8.02±0.23, n=4 [NE+L-NAME; Figure 3D]. L-NAME alone had no effect on NE dose response in PAs but increased sensitivity to NE at low concentrations in AOs (data not shown). The important role of the eNOS was further proven using vessels from eNOS KO mice where ICI had no effect on NE dose response (pEC$_{50}$: 7.51±0.07, n=4 [eNOS KO+NE+ICI] versus 7.50±0.08, n=4 [eNOS KO+NE], $P>0.05$; Figure 3E). The critical contribution of NO production to the vascular response to ICI was corroborated by fluorescence measurements with the NO indicator diamino-fluorescein–IM diacetate (DAF-FM DA), revealing that ICI induced NO production (relative NO increase: 46.9±7.8%) in bPAECs, which was significantly higher compared with the steady-state NO release under control conditions (relative NO increase: 19.5±3.7%, $P<0.05$ versus ICI; Figure 3F). The activation of the eNOS by ICI could be attributed to a time-dependent phosphorylation of the eNOS at Ser1177, which was demonstrated in Western blotting experiments after incubation of bPAECs for 2, 5, and 10 minutes (Figure 3G and 3H). From these data we conclude that ICI reduces
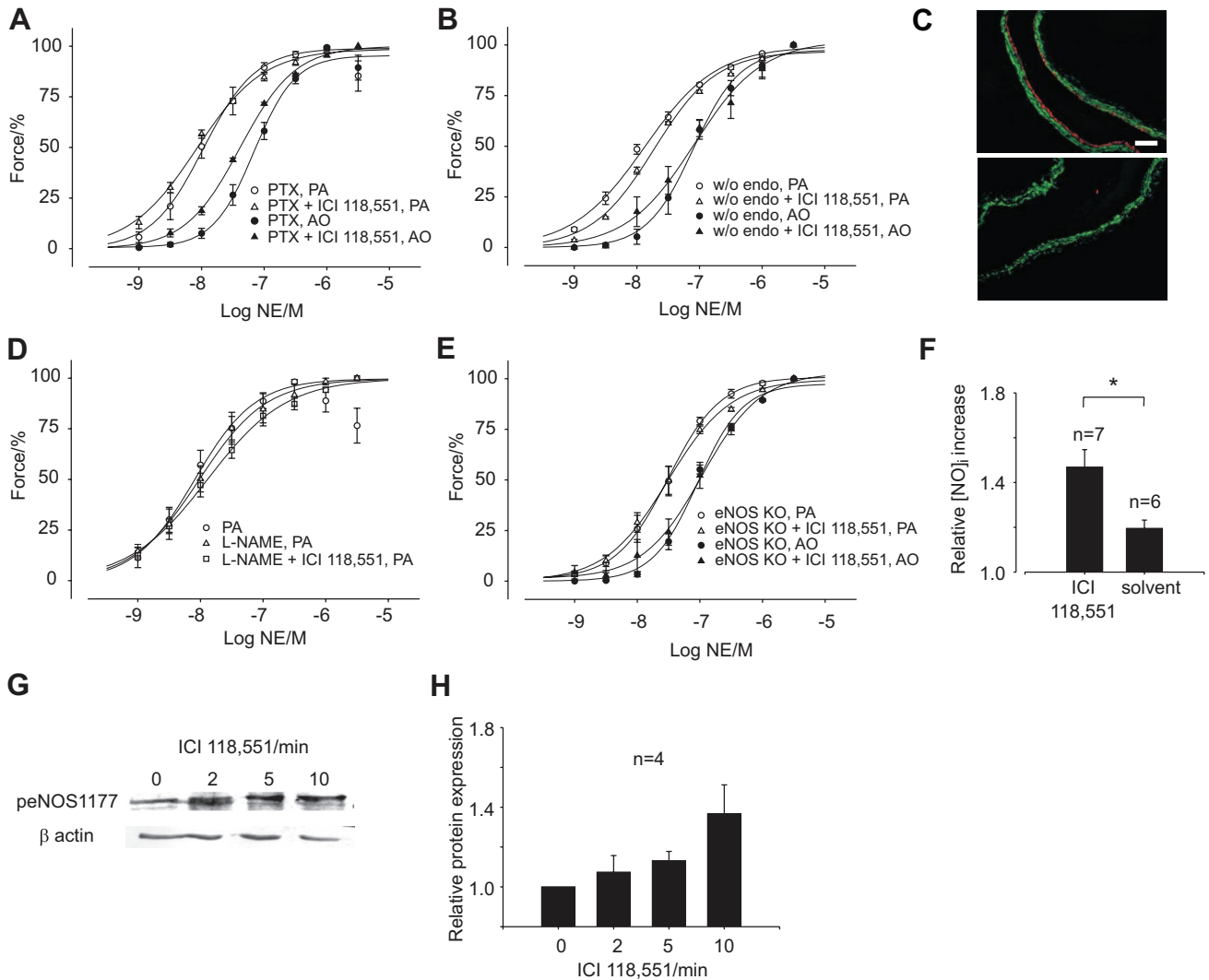
Figure 3. The right shift of NE dose-response in PA by ICI is $G_{i/o}$ and endothelin dependent and mediated by NO. A, The effect of ICI could be blocked by the $G_{i/o}$ protein blocker PTX. B, Removal of the endothelium (w/o endo) abrogated the ICI-induced right shift of the dose-response curve in the PAs of mice. C, Immunostaining with CD31 (red) and α-smooth muscle actin (green) confirmed the presence (top) or the complete removal of the endothelium (bottom) in murine PA; bar=50 μm. D, L-NAME inhibited the ICI-induced right shift. E, In eNOS KO mice, the effect of ICI was absent. F, Statistical analysis of NO measurements with the NO-sensitive dye DAF-FM DA revealed that ICI induced an increase of $[NO]_i$ in bPAECs, whereas changes in NO were significantly lower on application of the solvent. *$P<0.05$. G, Western blotting showed that ICI treatment phosphorylated eNOS at Ser1177 in bPAECs in a time-dependent manner. H, Statistical analysis of 4 independent Western blot experiments.

the sensitivity to NE in PAs via a $G_{i/o}$-dependent activation of the eNOS and the subsequent release of NO.

ICI Decreases the Tone of Small Intra-PAs

To directly assess pulmonary vascular diameter, we applied the lung section technique established by Perez and Sanderson.[15] We found that the β_2-adrenergic inverse agonist ICI (10 μmol/L) increased the lumen area of PA from $33.33\pm7.17\%$ to $78.29\pm12.74\%$ (n=4; $P<0.05$), after precontraction with 5-HT (Figure 4A through 4C), hence a prominent vasorelaxing effect ($\approx70\%$; Figure 4C). To examine whether ICI also affected the small precapillary arteries, we have used the isolated perfused lung (IPL) system. Here, ICI reduced pulmonary arterial pressure after 5-HT precontraction from 4.51 ± 1.32 mm Hg (6.15 ± 1.80 cm H_2O) to 1.94 ± 0.74 mm Hg (3.51 ± 1.12 cm H_2O; n=7; $P<0.05$); this is

equivalent to a pressure decrease of $\approx60\%$ (Figure 5A and 5B). The effect of ICI in the IPL was dose-dependent in the range of 0.01 to 10.00 μmol/L (Figure 5C and 5D). In contrast to our findings in the IPL in large PAs, only concentrations of ICI >1 μmol/L (≤10 μmol/L) evoked a dose-dependent relaxation (Figure S1D), demonstrating that these vessels are less sensitive to ICI than the small intra-PAs; responses were independent of the precontracting agent, because vasorelaxation induced by ICI (10 μmol/L) was similar after 5-HT ($31.0\pm7.4\%$) or NE ($33.4\pm10.0\%$) precontraction.

One of the main pathophysiological causes of pulmonary arterial hypertension is hypoxia. We, therefore, tested the potential of ICI to reduce the vascular pressure after hypoxic vasoconstriction. Our experiments revealed that ICI attenuated the hypoxia (0% O_2)-induced pressure increase of pulmonary vessels by 33% (Figure 5E and 5F).
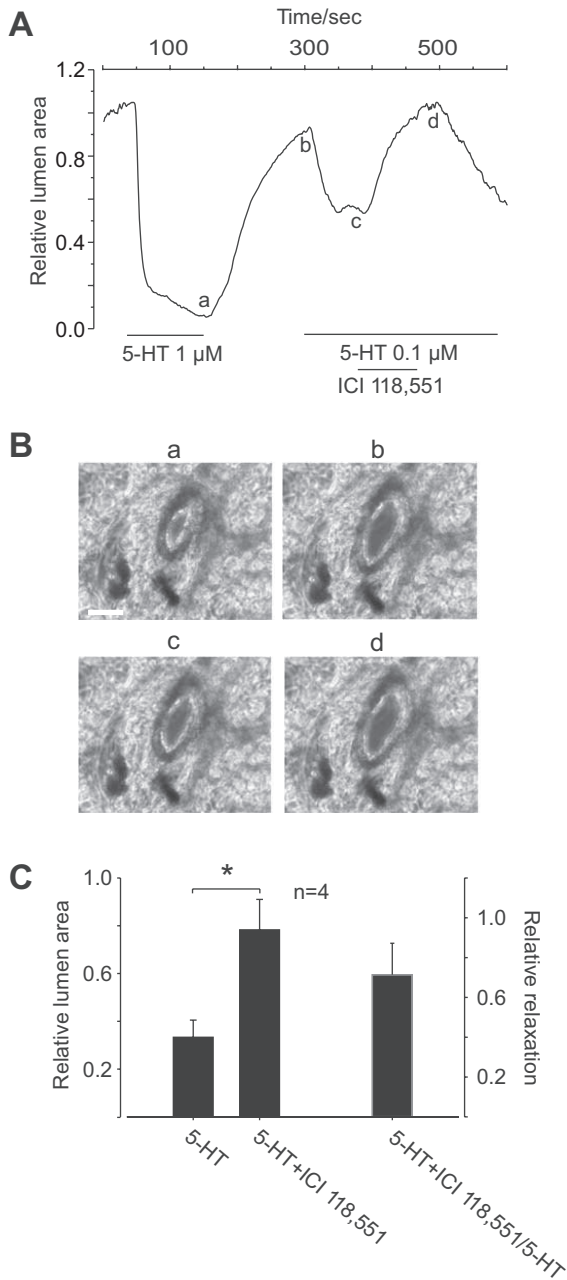
Figure 4. ICI relaxes small intra-PAs in lung sections of mice. A, Online changes of the lumen area of a small PA in response to ICI after precontraction with 5-HT. B, Series of phase-contrast images recorded at different time points indicated by the letters in A; bar=50 μm. C, Statistical analysis of 4 independent experiments. *$P<0.05$.

Thus, ICI decreases pulmonary vascular pressure by its vasodilatory action on small PAs under normoxic and hypoxic conditions.

ICI Reduces Pulmonary but not Systemic Pressure In Vivo

To prove the specificity and the physiological relevance of the ICI-induced vasorelaxation, we performed in vivo catheter measurements. Blood pressure was measured in the sedated mouse after injecting ICI (0.2 mg/kg; 6 μL over 40 seconds) into the jugular vein. Systolic pressure values

directly after the injection were compared with those obtained 2 minutes later. The analysis showed that ICI reduced systolic pressure in the pulmonary circuit from 32.3 ± 3.1 mm Hg to 23.9 ± 2.6 mm Hg (n=5; $P=0.03$), which corresponds with a pressure decrease of 25% (Figure 6A). In contrast, systolic systemic pressure values remained stable at 113.0 ± 8.1 mm Hg directly after injection and at 112.8 ± 13.8 mm Hg (n=5; $P>0.05$) 2 minutes later (Figure 6B). Thus, ICI strongly reduces pulmonary but not systemic arterial pressure in vivo.

Discussion

In the current study we show that even low concentrations of the specific β_2-AR inverse agonist ICI selectively reduced the sensitivity of PAs but not of systemic vessels to different vasoconstrictors. This finding is physiologically relevant, because it occurred in large and in small intra-PAs under normal and hypoxic conditions. The response was a specific property of ICI, because it was not mimicked by other β-blockers, regardless of whether these were either β_2 specific or not. Although in most of the experiments we used high concentrations of ICI to obtain strong effects, in the IPL there was a dose-dependent vasorelaxation already starting at a very low concentration of 0.01 μmol/L, which was in good agreement with the reported EC_{50} of 0.06 μmol/L for ICI binding to β_2-AR.[16] A direct effect on α-AR–mediated precontraction can be excluded, because ICI also affected dose-response curves of vasoconstrictors, which are devoid of adrenergic activity, eg, endothelin and 5-HT; moreover, ICI was without effect in β-AR KO mice with intact α-AR signaling. In addition, the observed differences of the response to ICI in pulmonary versus systemic vessels were not caused by the different expression or distribution of β_2-AR, because Western blots in bPAECs and bovine aortic endothelial cells, as well as immunostainings in PAs and AOs, yielded similar results. We, therefore, suggest that differences in G protein coupling most likely account for the differential effect of ICI. There is accumulating evidence that G protein–coupled receptors can couple to more than one G protein, and different agonist-induced conformations exist with different efficacies for coupling to particular G proteins. This type of ligand behavior has been termed "functional selectivity" or "stimulus trafficking"[17] and may also depend on the cell type. Hence, ICI-induced β_2-AR coupling to $G_{i/o}$ proteins could differ in pulmonary and systemic endothelial cells. Other than quantitative differences in G protein activation, differential coupling to specific $G\alpha_{i/o}$ subtypes may also occur. This important concept could be addressed in future studies by means of guanosine 5′-[γ-thio]triphosphate assays or photolabeling and immunoprecipitation of $G_{i/o}$ proteins. Furthermore, the downstream signaling of ICI-induced $G_{i/o}$ protein activation could also differ in endothelial cells from the pulmonary and the systemic circuits.

Although other studies reported ICI as an antagonist of isoprenaline-induced vasorelaxation in rat PAs,[4] we demonstrate a vascular tone decrease evoked by this specific compound in mice. These contrasting results could be explained by different basal receptor activities, which play an important role for inverse agonists like ICI. Vasorelaxation by PTX-sensitive G proteins is often transmitted via endo-
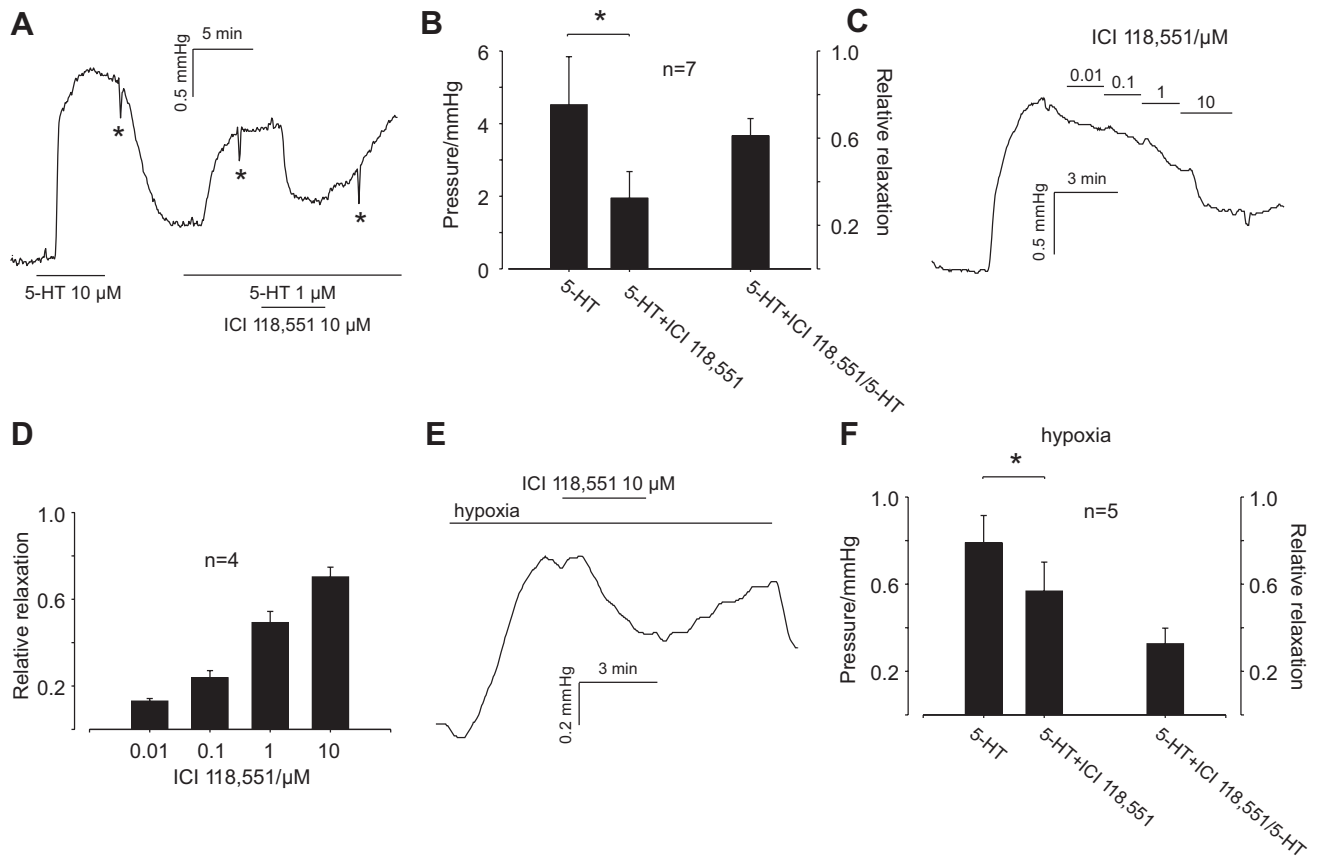
Figure 5. ICI relaxes intra-PAs in the murine IPL. A, Original trace of arterial pressure decrease by ICI (10 μmol/L) in the IPL; note regular hyperinflation every 10 minutes indicated by *. B, Statistical analysis of 7 independent experiments. *$P<0.05$. C and D, Original trace of the dose-response curve by ICI and its statistical analysis. E, Original trace showing the pulmonary arterial pressure increase during acute hypoxia. ICI (10 μmol/L) reduced arterial pressure in the IPL. F, Statistical analysis of 5 independent experiments. *$P<0.05$.

thelial NO production.[14] We provide convincing evidence for the involvement of NO in the ICI-mediated vasorelaxation of pulmonary vessels, because the right shift of NE dose-response curves was blocked by the removal of the endothelium, by treatment with the eNOS inhibitor L-NAME, and by using vessels from eNOS KO mice. Moreover, NO measurements with the fluorescence dye DAF-FM DA in bPAECs support these findings.

Figure 6. ICI reduces vascular pressure in catheter measurements in vivo. A, In vivo measurements of murine pulmonary (PAP) and (B) systemic arterial pressure (SAP) in response to ICI. Systolic pressure values immediately after injection of ICI (0.2 mg/kg; $-$ICI 118,551) and 2 minutes later ($+$ICI 118,551) were compared. *$P<0.05$.

In recent years, NO production in response to β-blockers has already been described. Many β-blockers were reported to possess intrinsic activity of their own.[18,19] For example, the third-generation β_1-blocker nebivolol was shown to have agonistic effects at endothelial β_3-AR, thereby leading to enhanced NO production and vasorelaxation.[9] However, our data clearly show that β_3-AR is not involved in the reduction of NE sensitivity by ICI. Although our in vivo experiments support the idea that the effect of ICI is mediated via NO, we cannot exclude that eicosanoids, mast cell degranulation, and/or histamine-dependent mechanisms also contribute to the observed vasodilation,[20,21] because these pathways are also influenced by β-adrenergic signaling.[22]

One of the main concerns of most pharmacological vasorelaxants in the treatment of pulmonary hypertension is their pronounced hypotensive effect on systemic vessels. However, in the case of ICI, we found that it selectively reduced pulmonary but not systemic vascular tone in vivo. In accordance with our data, 2 different studies in humans also reported that ICI did not affect systemic blood pressure.[23,24] Only Vincent et al[25] found a reduction of systemic blood pressure, however, only 1 week after the beginning of treatment and when using a much higher concentration (0.7 mg/kg for patients of 70-kg weight) compared with our study. Thus, low concentrations of ICI specifically reduce vascular tone in pulmonary vessels.
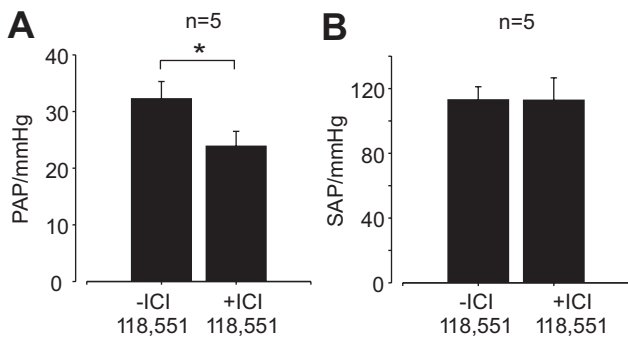
The acute reduction of pulmonary arterial pressure by ICI could be clinically beneficial in states of acute pulmonary arterial hypertension crises. Moreover, it may prevent persisting high pressure in the pulmonary circulation, which has been reported to be an important factor in the pathophysiology of chronic pulmonary arterial hypertension.[26] Whether ICI can also attenuate vascular remodeling remains to be investigated in future studies.

Perspectives

Altogether, we show that the β-blocker ICI has agonist activity at the β_2-AR, whereby it decreases vascular tone of PAs selectively via a $G_{i/o}$ protein–dependent NO increase. This finding shows that distinct substances independent from their class properties may have special features that can be exploited for therapeutic use.

Disclosures

J.B. received honoraria payment by etelion for different lectures. The remaining authors report no conflicts.

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The β2-adrenoceptor antagonist ICI 118,551 decreases pulmonary vascular tone in mouse via a $G_{i/o}$ protein/NO-coupled pathway

ICI 118,551 relaxes pulmonary arteries

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Methods

All animal experiments were performed in accordance with National Institute of Health animal protection guidelines and approved by the local authorities of the University of Bonn.

Animals

For experiments 10-12 weeks old female mice were used (CD1, Charles River Laboratories, Sulzfeld, Germany). β−AR KO mice were a kind gift of Prof. Giacobino (Geneva, Switzerland) and eNOS KO mice (C57/Bl6) were kindly provided by Prof. Gödecke (Düsseldorf, Germany).

Isometric force measurements

Isometric force measurements were performed in a wire myograph as described recently[1]. Main left and right PA and AO were dissected free of connective tissue and cut into 2-mm-long rings. In some of the experiments the endothelium of vascular rings was denuded with a mouse whisker. The complete removal of the endothelium was proven by immunostaining (see below). Arterial segments were mounted on a small vessel wire myograph (Multi Myograph 610 M, Danish Myo Technology, Aarhus, Denmark). A computer-assisted normalization protocol was performed to pre-stretch AO to $0.9 \times L_{100}$ with L_{100} = diameter mimicking a transmural pressure of 100 mmHg. Because blood pressure in the pulmonary circuit is much lower than in systemic vessels PA were pre-stretched to 5 mN, a value that was reported to allow optimal force development[2]. Rings were equilibrated for 20 min. The solution was bubbled with 100% oxygen and heated to 37°C. Prior to the experiments the arteries were maximally contracted with norepinephrine (NE) (10 µM).

Immunohistochemistry

The pharmacological agents acetylcholine or bradykinin which are usually applied to test the functional integrity of the endothelium showed only inconsistent responses in PA. We therefore examined the presence of the endothelium after the experiments with immunostaining. Vascular rings were carefully removed from the wire myograph, embedded in tissue-tek solution, cryopreserved and sections of 10 µm thickness were cut with a cryotome (CM3050S, Leica, Wetzlar, Germany). Immunostaining was performed using primary antibodies against CD31 (1:800, BD Biosciences), α-smooth muscle actin (1:800, Sigma-Aldrich) or $β_2$-adrenoceptor (Santa Cruz, dilution 1:100). Primary antibodies were visualized with secondary antibodies conjugated with Cy3 and Cy5 (1:400, Dianova, Hamburg, Germany) and nuclei were stained with Hoechst 3342 (1:1000, Sigma-Aldrich, Taufkirchen, Germany).

Western blotting

Bovine pulmonary arterial endothelial cells (bPAEC) were incubated with ICI 118,551 (10 µM) for 0, 2, 5 and 10 minutes. Then, proteins were isolated using RIPA buffer containing Protease Inhibitor C (Boehringer Ingelheim, Germany). Protein concentrations were determined by a Bradford Assay (Bio Rad, Munich, Germany). SDS-PAGE was performed according to Laemmli[3]. After denaturation of the proteins by boiling in 0.8% SDS and 0.5% β-mercaptoethanol (v/v) electrophoresis was performed in a 7.5% polyacrylamide gel. Proteins were blotted with a semidry system (Transblot SD, Bio Rad, Germany) on a nitrocellulose-membrane (0.45 µM, Whatman, Dassel, Germany) for 45 minutes at 15 V. The membrane was then blocked with 5% BSA in TBST buffer (20 mM Tris/HCl, 137 mM NaCl, 0.1% Tween 20, pH 7.6) for 1 hour at RT. Incubation with β-actin (Sigma-Aldrich, dilution of 1:20000), peNOS 1177 (Upstate, dilution of 1:1000) or $β_2$-

adrenoceptor antibody (Santa Cruz, dilution 1:100) was performed over night at 4°C. The secondary antibody, goat-anti-rabbit conjugated HRP (Sigma-Aldrich) was used at a dilution of 1:10000. For detection an ECL solution (chemiluminescence, GE Healthcare, Munich, Germany) was employed. Four series of peNOS Western blots (see also Fig. 3H) were performed. For the quantitative evaluation peNOS/β-actin values after ICI 118,551 treatment were normalized to the respective peNOS/β-actin values without ICI treatment.

Single-cell NO measurements
NO measurements were performed as described before[1]. Briefly, bPAEC were plated on glass coverslips and loaded with DAF-FM DA (10 µM for 10 min at room temperature). After washout, ICI 118,551 (10 µM) or the solvent were applied to the bath and fluorescence measurements were performed. As excitation wavelength 480nm was chosen and emission was recorded through a 535/50 nm bandpass filter. For analysis the fluorescence increase after 20 min was normalized to fluorescence intensity immediately prior to application of the test substance. Cells that displayed a change of shape were excluded from the analysis.

Lung slice technique
Lung slices were prepared according to the protocol of Perez and Sanderson[4]. Briefly, the trachea was cannulated and the lungs were filled with a warm solution of agarose (2%, low gelling temperature). Then, the agarose was flushed into the alveoli by injecting a small volume of air. Subsequently, a gelatin solution (6%, type A, porcine skin) was perfused through the pulmonary vasculature via the right ventricle. Agarose and gelatin were gelled with ice-cold PBS. The lung lobes were removed and cut into serial slices of 200 µm thickness with a vibratome (Microm HM 650 V, Microm, Walldorf, Germany). The slices were cultivated in growth media at 37°C and 5% CO_2. Experiments were performed on the next day when the gelatin in the vessel lumen was dissolved.
For measurements the slices were placed in a custom-made perfusion chamber and exposed to different agonists. The slices were monitored by phase-contrast microscopy with an inverted microscope and a 10x objective. Digital images were recorded with a CCD camera in the time-lapse mode at 1 Hz. For analysis the lumen area of the vessels was calculated with a custom-written software by pixel summing.

The isolated perfused lung (IPL)
Experiments were performed with the IPL setup of Hugo Sachs Elektronik (March-Hugstetten, Germany). Mice were sacrificed, the trachea was cannulated and the lungs were ventilated with positive pressure at a ventilation rate of 80 breaths/min and a tidal volume of 200 µl. After laparotomy and thoracotomy the diaphragm was removed and heparin was injected into the right ventricle. Mice were exsanguinated by cutting the aorta, then the PA was cannulated and secured with a pre-looped thread. Next, the cannula for the venous limb was inserted through a ventricular incision into the left ventricle and perfusion was started with physiological salt solution (PSS: NaCl 118 mM, KCl 5 mM, Na_2HPO_4 1.2 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 1.6 mM, Hepes 24 mM, glucose 10 mM, pH 7.4, 340 mosm/kg) at a flow rate of 1.0 ml/min with a roller pump. After the lid of the chamber was closed negative pressure ventilation was applied (-2 to -10 cmH_2O). Every 10 min a hyperinflation (-20 cmH_2O) was performed. Artificial thorax chamber pressure was measured with a differential pressure transducer and the air flow velocity with a pneumotachograph tube connected to a differential pressure transducer. For hypoxia experiments, mice were ventilated with positive

pressure and 100% N_2. The arterial pressure was continuously monitored by means of a pressure transducer connected to the cannula in the PA. Pharmacological agents were added to the perfusion solution.

In vivo measurement of pulmonary and systemic pressure
For in vivo measurements of pulmonary and systemic pressure, CD 1 mice were mildly sedated (isoflurane 1.5%, O_2 0.6 l/min, N_2O 0.4 l/min) and pressure recorded with a pressure catheter using the Millar ARIA 1 system (Millar). The catheter was inserted into the right jugular vein or carotid artery, respectively. The pressure of the pulmonary circuit was recorded in the right ventricle while the systemic pressure was determined in the left ventricle or the carotid artery. During the measurements ICI 118,551 (0.2 mg/kg) was injected into the left jugular vein. Because of the very small (6μl) volume no acute changes of pressure were induced by the injection. For analysis, systolic pressures immediately after the injection of ICI 118,551 (control) were compared to the pressure values 2 min later.

Chemicals
All chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany) with the exception of ICI 118,551 (Tocris Bioscience, Bristol, U.K). CGP 20712A was kindly provided by Novartis (Basel, Switzerland). All drugs were prepared as stock solutions in deionized water with the exception of indomethacin which was dissolved in ethanol (100 mM).

Data analysis
Contractions are expressed as force development relative to the maximal NE-induced contraction. Dose-response curves were fitted with the Hill equation and EC50 values were determined. Dose-response curves with NE in PA showed a decrease of vascular tone for very high NE doses which was reported to occur via β-AR[5]. As we were only interested in α-AR-dependent vasoconstriction, NE concentrations ≥ 1 μM were not used for the fitting routine; this did not alter EC50 values. In lung slice and IPL experiments the relaxation was normalized to the submaximal pre-contraction. Data are indicated as mean ± standard error of the mean (SEM). Differences were determined by ANOVA and Bonferroni *post hoc* analysis for multiple comparisons or Student`s t-test. $P < 0.05$ was considered significant. Raw data for original trace figures were smoothed using 5 point adjacent averaging (AA) except for Fig.6 where 9 point AA was used.

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	Pulmonary artery			Aorta			
Condition	pEC50	SEM	n	pEC50	SEM	n	
ET	9.28	0.09	4	7.82	0.05	4	*
ET + ICI	8.24	0.04	4	7.74	0.05	4	+
5-HT	7.48	0.20	5	6.98	0.07	6	
5-HT + ICI	6.61	0.24	4	6.21	0.01	4	+
NE	8.09	0.20	8	7.41	0.07	5	*
NE β KO	7.70	0.20	4	7.00	0.11	4	
NE β KO + ICI	7.55	0.13	4	7.10	0.04	4	
NE + Butox	7.70	0.06	4	6.57	0.11	4	
NE + Butox + ICI	7.36	0.06	4	7.12	0.03	4	
NE + CGP	8.73	0.04	6	7.46	0.04	4	
NE + CGP + ICI	7.64	0.04	3	7.11	0.10	4	+
NE eNOS KO	7.50	0.07	4	7.03	0.04	4	
NE eNOS KO + ICI	7.51	0.08	4	6.97	0.09	4	
NE + ICI	7.28	0.08	6	7.32	0.05	6	+
NE + L-NAME	8.02	0.23	4	7.65	0.20	4	
NE + L-NAME + ICI	7.89	0.17	4	7.45	0.05	4	
NE + Met	8.04	0.18	4	7.23	0.05	3	
NE + Prop	7.77	0.10	4	7.36	0.03	4	
NE + PTX	7.97	0.11	4	7.16	0.05	4	+
NE + PTX + ICI	8.08	0.09	4	7.38	0.02	4	
NE + SR	7.62	0.05	4	7.17	0.06	4	
NE + SR + ICI	7.22	0.06	4	7.13	0.03	4	+
NE w/o endo	7.88	0.08	5	7.10	0.06	5	
NE w/o endo + ICI	7.73	0.05	4	7.08	0.12	4	
Phe	7.48	0.08	6	6.51	0.09	6	*

Table S1. pEC50 values of dose-response curves in PA and AO. Values are pEC50 ± SEM and listed in alphabetical order. * indicates significant difference between PA and AO ($p<0.05$); + indicates

significant difference between PA with and without ICI (p<0.05). ET = endothelin, ICI = ICI 118,551, 5-HT = serotonin, NE = norepinephrine, β KO = β−AR KO mouse, Butox = butoxamine, CGP = CGP 20712A, eNOS KO = eNOS KO mouse, Met = metoprolol, Prop = propranolol, PTX = pertussis toxin, SR = SR 59230A, w/o endo = endothelium denuded, Phe = phenylephrine.
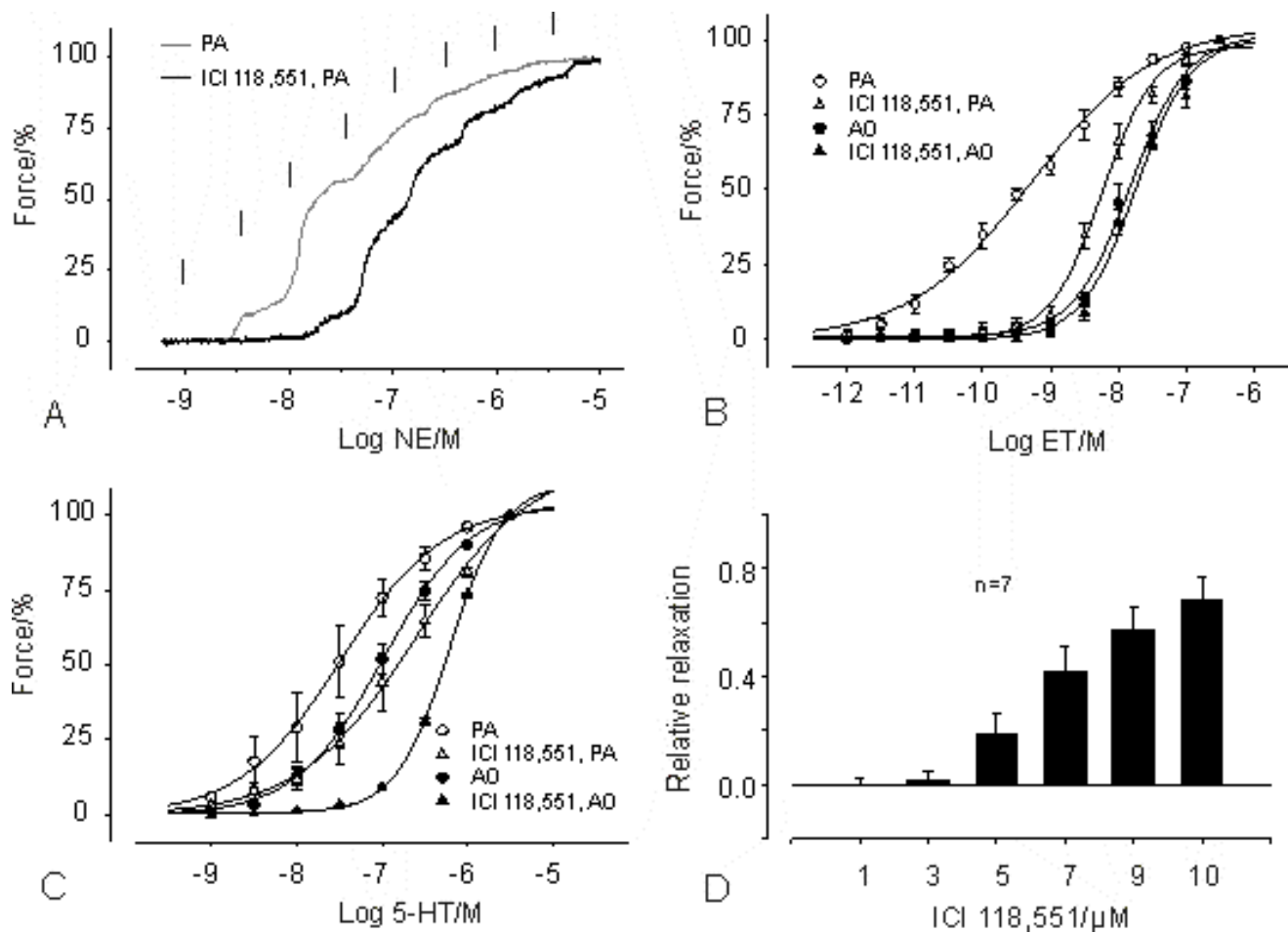
Figure S1. Analysis of the effect of ICI 118,551 in a wire-myograph. A, Original trace of a dose-response curve with norepinephrine (NE) in PA with and without ICI 118,551, NE addition is marked by dashes. B, Dose-response curve of endothelin (ET) in PA and AO with and without ICI 118,551. C, Dose-response curve of serotonin (5-HT) in PA and AO with and without ICI 118,551. D, Statistical analysis of ICI 118,551 dose-response curve in PA in a wire-myograph. After NE pre-contraction different concentrations of ICI 118,551 were applied to a PA in a cumulative manner. Relaxations relative to pre-contraction with NE are displayed.