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Attenuated Aortic Vasodilation and Sympathetic Prejunctional Facilitation in Epinephrine-Deficient Mice: Selective Impairment of β_2-Adrenoceptor Responses

Mónica Moreira-Rodrigues, Ana L. Graça, Marlene Ferreira, Joana Afonso, Paula Serrão, Manuela Morato, Fátima Ferreirinha, Paulo Correia-de-Sá, Steven N. Ebert, and Daniel Moura

Laboratory of General Physiology (M.M.-R.) and Laboratory of Pharmacology and Neurobiology (F.F., P.C.), Unit for Multidisciplinary Investigation in Biomedicine, Institute of Biomedical Sciences Abel Salazar, University of Porto; Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Porto (A.L.G., M.F., J.A., P.S., D.M.); Neuropharmacology, Institute of Molecular and Cellular Biology, University of Porto (M.M., D.M.); Center for Drug Discovery and Innovative Medicines, University of Porto (M.M.-R., A.L.G., M.F., J.A., P.S., M.M., F.F., P.C., D.M.); Laboratory of Pharmacology, Department of Drug Sciences, Faculty of Pharmacy, University of Porto and Rede de Química e Tecnologia (REQUIMTE), Porto, Portugal (M.M.); and Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, Florida (S.N.E.).

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ABSTRACT

It has been suggested that there is a link between epinephrine synthesis and the development of β_2-adrenoceptor–mediated effects, but it remains to be determined whether this development is triggered by epinephrine. The aim of this study was to characterize β-adrenoceptor–mediated relaxation and facilitation of norepinephrine release in the aorta of phenylethanolamine-*N*-methyltransferase–knockout (Pnmt-KO) mice. Catecholamines were quantified by reverse-phase high-performance liquid chromatography–electrochemical detection. Aortic rings were mounted in a myograph to determine concentration-response curves to selective β_1- or β_2-adrenoceptor agonists in the absence or presence of selective β_1- or β_2-adrenoceptor antagonists. Aortic rings were also preincubated with [^{3}H]norepinephrine to measure tritium overflow elicited by electrical stimulation in the presence of increasing concentrations of nonselective β- or selective β_2-adrenoceptor agonists. β_2-Adrenoceptor protein density was evaluated by Western blotting and β_2-adrenoceptor localization by immunohistochemistry. Epinephrine is absent in Pnmt-KO mice. The potency and the maximal effect of the β_2-adrenoceptor agonist terbutaline were lower in Pnmt-KO than in wild-type (WT) mice. The selective β_2-adrenoceptor antagonist ICI 118,551 [(±)-*erythro*-(*S**,*S**)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride] antagonized the relaxation caused by terbutaline in WT but not in Pnmt-KO mice. Isoproterenol and terbutaline induced concentration-dependent increases in tritium overflow in WT mice only. β_2-Adrenoceptor protein density was decreased in membrane aorta homogenates of Pnmt-KO mice, and this finding was supported by immunofluorescence confocal microscopy. In conclusion, epinephrine is crucial for β_2-adrenoceptor–mediated vasodilation and facilitation of norepinephrine release. In the absence of epinephrine, β_2-adrenoceptor protein density was decreased in aorta cell membranes, thus potentially hindering its functional activity.

Introduction

In contrast to adults, human neonates have plasma concentrations of norepinephrine higher than those of epinephrine (Eliot et al., 1980). Accordingly, in the newborn canine adrenal medulla, norepinephrine is the predominant amine, whereas in adults, epinephrine predominates (Paiva et al., 1994). On the other hand, Gootman et al. (1981) showed that β-adrenoceptor–mediated vascular relaxation is immature in neonatal swine. Thies et al. (1986) also found a diminished response to the nonselective β-adrenoceptor agonist isoproterenol in human neonatal lymphocytes compared with adults. Paiva et al. (1994) also observed a parallel time course between the postnatal increase in the epinephrine content in the adrenal medulla and the development of β_2-adrenoceptor–mediated smooth muscle relaxation and facilitation of norepinephrine release by sympathetic nerve stimulation in the canine

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ABBREVIATIONS: CGP 20712 A, 1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride; CL 316243, 5-[(2*R*)-2-[[(2*R*)-2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1,3-benzodioxole-2,2-dicarboxylic acid; E_{max}, maximal effect; ICI 118,551, (±)-*erythro*-(*S**,*S**)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride; PBS, phosphate-buffered saline; pEC_{50}, negative logarithm of the molar concentration causing 50% of E_{max}; Pnmt, phenylethanolamine-*N*-methyltransferase; Pnmt-KO, phenylethanolamine-*N*-methyltransferase-knockout; RIPA, radioimmunoprecipitation assay; S1, evoked tritium overflow; TBS, Tris-buffered saline; WT, wild-type.

saphenous vein. In addition, epinephrine is the only biogenic catecholamine that has affinity for β_2-adrenoceptors at physiologically relevant concentrations, whereas both epinephrine and norepinephrine are potent β_1-adrenoceptors agonists (Lands et al., 1967a,b).

Thus, it was suggested that there is a link between epinephrine and the development of β_2-adrenoceptor–mediated effects (Paiva et al., 1994; Guimaraes and Moura, 2001). However, it remains to be proved whether the development of β_2-adrenoceptor–mediated effects is triggered by epinephrine or by other causes that might simultaneously induce epinephrine production and the functional development of the responses to β_2-adrenoceptor stimulation.

It has been difficult to decipher the role of epinephrine with the commonly used adrenal medullectomy because this procedure can damage the adrenal cortex, altering the release of corticosteroids, and it also removes the release of other adrenal amines and peptides, such as norepinephrine, chromogranin A, catestatin, and neuropeptide Y (Harrison and Seaton, 1966). An alternative approach is the use of phenylethanolamine-*N*-methyltransferase (Pnmt) inhibitors to block epinephrine synthesis in vivo (Bondinell et al., 1983), but most of them also inhibit monoamine oxidase (Mefford et al., 1981) and α-adrenoceptors (Feder et al., 1989). These drawbacks for the elucidation of the specific role of epinephrine in the development of β_2-adrenoceptor subtype are avoided by doing experiments in an epinephrine-deficient animal model generated by knocking out the Pnmt gene (Ebert et al., 2004, 2008; Sharara-Chami et al., 2010). Therefore, the aim of this study was to characterize the role of epinephrine on β-adrenoceptor–mediated aorta relaxation and facilitation of norepinephrine release from sympathetic nerve endings using Pnmt-knockout (Pnmt-KO) and wild-type (WT) mice.

Materials and Methods

Animals. All animal care and experimental protocols were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health and were approved by the Institute of Biomedical Sciences Abel Salazar, University of Porto, Portugal, ethics committee (project no. 020/2012). The Pnmt-KO mice ($Pnmt^{-/-}$) were produced by disruption of Pnmt locus by insertion of Cre-recombinase in exon 1 (Ebert et al., 2004). A couple of Pnmt-KO mice were kindly provided by S.N.E., and animals were bred in our conventional vivarium. The presence of the $Pnmt^{-/-}$ allele was verified by polymerase chain reaction of ear DNA. The animals were kept under controlled environmental conditions (12 hour light/dark cycle, room temperature 23 ± 1°C, humidity 50%, autoclaved drinking water, and mice breeding diet 4RF25/I; Ultragene, Porto, Portugal) and housed with the respective litter. Pnmt-KO (129x1/SvJ, $n = 40$) and WT (129x1/SvJ, $n = 36$) male mice (8–12 weeks old) were killed by cervical dislocation under anesthesia (isoflurane 100%, 200 μl by inhalation). Blood was collected and centrifuged (4°C, 3000*g*, 15 minutes) for plasma separation. The aorta and the left adrenal gland were rapidly removed and weighed and then placed in vials containing 0.3 ml of perchloric acid (0.2 M) and stored at –80°C until used for quantification of catecholamines. Alternatively, the aortas were dissected, removed, and used for functional or molecular studies.

Quantification of Catecholamines. Alumina extracts from plasma and tissue samples were injected in reverse-phase high-performance liquid chromatography column for separation of norepinephrine and epinephrine, which were quantified by electrochemical detection, as previously described (Paiva et al., 1994; Moreira-Rodrigues et al., 2009). The detection limit is between 350 and 1000 fmol.

Postjunctional Functional Studies. Pnmt-KO and WT mice aortas were placed in Krebs-Henseleit solution bubbled with 95% O_2 and 5% CO_2, cut in rings (1–2 mm) and mounted in a myograph (DMT, Aarhus, Denmark). Each aorta ring was allowed to stabilize for 1 hour. Afterward, optimal resting tension was settled using a standardized normalization procedure (Mulvany and Halpern, 1977). Then the arteries were precontracted with phenylephrine (α_1-adrenoceptor agonist) to about 60% of the maximum contraction, a level that has been shown to be optimal to obtain β-adrenoceptor–mediated relaxation (Guimaraes, 1975). Phenylephrine was selected for precontraction of vascular rings in our experiments because relaxation responses to β-adrenoceptor agonists in vivo occur under tonic constriction caused by α-adrenoceptor stimulation. Both in animals and in humans, changes in the balance of opposing α- and β-adrenoceptor–mediated responses result in alterations of vascular tone in vivo (Landau et al., 2002). No differences were observed between the two experimental groups concerning the degree of contraction induced by the concentration of phenylephrine used (0.3 μM; data not shown). Finally, concentration-response curves to dobutamine (selective β_1-adrenoceptor agonist) and terbutaline (selective β_2-adrenoceptor agonist) were obtained in the absence or presence of CGP 20712 A (1-[2-((3-carbamoyl-4-hydroxy) phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl) phenoxy]-2-propanol dihydrochloride; selective β_1-adrenoceptor antagonist; 40 nM) or ICI 118,551 [(±)-*erythro*-(*S**,*S**)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride; selective β_2-adrenoceptor antagonist; 14 nM], respectively. The pK_D values of terbutaline for binding to β_2- and β_1-adrenoceptors are around 5.62 and 3.82, respectively (Baker, 2005). Concentration-response curves to CL 316243 (5-[(2*R*)-2-[[(2*R*)-2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1,3-benzodioxole-2,2-dicarboxylic acid; selective β_3-adrenoceptor agonist) were not obtained (although attempt was made). The force calibration process used weight and the transducer detected 1 g [9.81 millinewtons (mN) of force]. Isometric contractions or relaxations were recorded as changes in the millinewtons of tension.

Prejunctional Functional Studies. As previously described by Trendelenburg et al. (2000), the aortas were preincubated for 30 minutes with [^{3}H]norepinephrine and then placed in superfusion chambers between platinum electrodes. Perifusion started at $t = 0$, and each preparation was challenged with seven periods of electrical stimulation (1 millisecond width, 80 mA, 120 pulses at 3 Hz). The first stimulation period (S0) was delivered at $t = 30$ minutes of perifusion and was not used for determination of tritium overflow. The subsequent stimulation periods (S1–S6) were applied at $t = 58$, 76, 94, 112, 130, and 148 minutes. Cocaine (26 μM; inhibitor of norepinephrine reuptake) and phentolamine (1 μM; nonselective α-antagonist) were present in the perifusion fluid throughout the experiment. Isoproterenol and terbutaline were added to the perifusion fluid at increasing concentrations (0.1 nM to 1 μM and 1 nM to 1 μM, respectively), 12 minutes before S2–S6. At the end of the perifusion period, tissues were placed in perchloric acid (0.2 M). Radioactivity was measured by liquid scintillation counting (liquid scintillation counter 1209 Rackbeta; LKB Wallac, Turku, Finland) in the perifusate or tissue extract after the addition of scintillation mixture (Optiphase HiSafe 3; LKB, Loughborough, Leics, UK). The spontaneous outflow of tritium was calculated as a fraction of the tritium content of the tissue at the onset of the respective collection period (fractional rate; min^{-1}). The overflow elicited by electrical stimulation was expressed as a percentage of tritium content of the tissue at the time of stimulation. Percentage changes of Sn $S1^{-1}$ ratios induced by drugs added after S1 were calculated. Finally, concentration-response curves to isoproterenol and terbutaline were obtained.

Protein Quantification. Three mice aortas in each sample were homogenized in radioimmunoprecipitation assay (RIPA) buffer (65 mM Tris-HCl, pH 7.4; 154 mM NaCl; 10 M Na_2EDTA; 1% IGEPAL; 6 mM sodium deoxycholate; 1 μM phenylmethyl sulfonyl fluoride; 1 μM NaF; 1 μM Na_3VO_4; 5 μg/ml leupeptin; 5 μg/ml aprotinin; 5 μg/ml pepstatin) and ultracentrifuged (4°C, 100,000*g*, 65 minutes). The pellets were

suspended in RIPA buffer, sonicated, and then collected for protein quantification (membrane fraction of aorta). Protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard.

Western Blot. Membrane fractions of aorta homogenates were diluted with RIPA buffer and then with 6:1 sample buffer (0.35 M Tris-HCl, pH 6.8; 4% SDS; 30% glycerol; 9.3% dithiothreitol; 0.01% bromophenol blue) and boiled at 95°C for 5 minutes. Samples (40 μg) were separated by SDS-PAGE with 10% polyacrylamide gel and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories). Blots were blocked for 1 hour with 5% nonfat dry milk in Tris-buffered saline (TBS), incubated with a rabbit polyclonal anti–β_2-adrenoceptor antibody (1:125; Santa Cruz Biotechnology, Dallas, TX) in 2.5% nonfat dry milk in TBS/Tween 20, overnight, at 4°C, then washed and incubated with an IRDye 800 goat anti-rabbit secondary antibody (1:10,000; Rockland, Gilbertsville, PA) for 1 hour at room temperature. The membranes were then washed and imaged by scanning at both 700 nm (for detection of western blot protein standard molecular weight; Precision Plus Protein Standard, Bio-Rad or NZY Color Protein Marker II; NZYtech, Lisbon, Portugal) and 800 nm (for IRDye 800 goat anti-rabbit secondary antibody detection) by fluorescence detection method, with an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Following this, the immunoblots were washed with mild stripping solution (to remove previous primary and secondary antibodies; 1% tween 20, 0.1% SDS, 200 mM glycine, pH 2.2), blocked overnight with 5% non-fat dry milk in TBS, and incubated with a mouse anti–β-actin antibody (1:10,000; Santa Cruz Biotechnology) in 2.5% nonfat dry milk in TBS/Tween 20 for 1 hour at room temperature. Finally, they were washed and incubated with an Alexa Fluor 680 goat anti-mouse secondary antibody (1:10,000; Invitrogen, Eugene, OR) for 1 hour, washed again, and imaged by scanning at 700 nm (for Western blot protein standard and Alexa Fluor 680 goat anti-mouse secondary antibody detection) (Moreira-Rodrigues et al., 2010).

Immunohistochemistry. Aortas were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 6 hours. Fixed tissue was washed and cryoprotected overnight with a solution containing 20% anhydrous glycerol (dissolved in 0.1 M phosphate buffer) and frozen. Then the tissue was sectioned (16 μm), and the tissue sections were incubated with a blocking buffer solution (10% fetal bovine serum, 1% bovine serum albumin, 0.3% Triton X-100, 0.025% NaN_3 in PBS) for 60 minutes with constant stirring. Subsequently, samples were incubated overnight (4°C) with a rabbit polyclonal anti–β_2-adrenoceptor antibody (1:50; Santa Cruz Biotechnology) diluted in dilution buffer solution (5% fetal bovine serum, 0.5% bovine serum albumin, 0.3% Triton X-100, 0.025% NaN_3 in PBS). Tissue sections were then washed and incubated for 90 minutes (at room temperature in the dark) with an anti-rabbit secondary antibody Alexa Fluor 488 (1:1500; Molecular Probes, Eugene, OR) diluted in dilution buffer solution. Negative controls were performed using the secondary antibody alone. Finally, samples were washed and mounted on optical-quality glass slides. Vectashield with DAPI (4′,6-diamidino-2-phenylindole) was used as mounting media (Vector Laboratories, Burlingame, CA). Observations and analyses were performed with a laser-scanning confocal microscope (FV1000; Olympus Fluoview, Tokyo, Japan) (Carneiro et al., 2014).

Drugs. Isoflorane 100%, Isoflo, was obtained from Abbott laboratories (Queenborough, UK). (−)-Epinephrine (+)-bitartrate salt, L-(−)-norepinephrine (+)-bitartrate salt monohydrate, isoproterenol hydrochloride, phentolamine hydrochloride, dobutamine hydrochloride, terbutaline hemisulfate salt, CGP 20712 A methanesulfonate salt, ICI 118,551 hydrochloride, *R*-(−)-phenylephrine hydrochloride, and CL 316243 were purchased from Sigma-Aldrich (St Louis, MO). Cocaine hydrochloride was obtained from Uquipa (Lisbon, Portugal), and (−)-[7-^{3}H]norepinephrine (specific activity 14.9 Ci mmol^{-1}, 1 mCi ml^{-1}) was purchased from PerkinElmer (Waltham, MA).

Statistical Analysis. Concentration-response curves were adjusted to data by nonlinear regression analysis using GraphPad Prism statistics software package (GraphPad Software Inc., La Jolla, CA). The E_{max} (maximal effect), EC_{50} , and pEC_{50} (negative logarithm of the molar concentration causing 50% of E_{max}) were estimated for each curve. Results are arithmetic means ± S.E.M. of values for the indicated number of determinations. Unless stated otherwise, statistical analysis was done by the two-tailed *t* test. Statistical analysis of the Sn S1^{-1} ratios was done by the Mann-Whitney test. $P < 0.05$ was assumed to denote a significant difference.

Results

Epinephrine and Norepinephrine Content in Adrenal Glands. Epinephrine content was baseline levels in the adrenal glands and plasma of Pnmt-KO mice when compared with WT mice, and it was below the detection level in the aorta samples of both groups (Table 1). In contrast, the norepinephrine content of the adrenal gland was higher in Pnmt-KO than in WT mice; however, norepinephrine levels in plasma and aorta were similar between the groups (Table 1).

Postjunctional β-Adrenoceptor–Mediated Responses in Aorta Rings. In aortas previously contracted with phenylephrine, terbutaline (Fig. 1A) and dobutamine (Fig. 1B) caused concentration-dependent relaxations in both Pnmt-KO and WT mice. The potency (EC_{50}, 29.00 ± 6.10 versus 5.20 ± 2.80 μM; n = 5–6) and maximal response (7.0 ± 2.4 versus 17.7 ± 3.3 mN mg^{-1}; n = 5–6) to terbutaline were lower in Pnmt-KO than in WT mice (Fig. 1A). To further confirm the results obtained, we performed the concentration-response curve of terbutaline in the presence of a β_2-adrenoceptor antagonist. ICI 118,551 antagonized the effect of terbutaline in WT (Fig. 2A) but not in Pnmt-KO mice (Fig. 2B). The potency (EC_{50}, 0.18 ± 0.05 versus 0.22 ± 0.07 μM; n = 6–8) and maximum response (13.8 ± 3.0 versus 20.0 ± 4.2 mN mg^{-1}; n = 6–8) to dobutamine were not significantly different ($P >$ 0.05) between experimental groups (Fig. 1B). CGP 20712 A antagonized the effect of dobutamine in both WT (Fig. 2C) and Pnmt-KO (Fig. 2D) mice; no differences were observed in pA_2 values between the two groups (8.1 ± 0.2 versus 8.0 ± 0.5, respectively). The β_3-adrenoceptor agonist CL 316243 (10–10,000 nM) failed to cause vasorelaxation of mice aortas (data not shown).

Prejunctional β-Adrenoceptor–Mediated Responses in Aorta Rings. No differences were found between Pnmt-KO and WT mice in basal conditions: tissue accumulation of tritium (430.50 ± 44.95 versus 404.19 ± 26.22, μmol g^{-1}; n = 7 to 8), fractional rate of loss ($4.26 \times 10^{-3} \pm 0.50 \times 10^{-3}$ versus $3.89 \times 10^{-3} \pm 0.13 \times 10^{-3}$, min^{-1}; n = 7–8) or evoked

TABLE 1
Epinephrine and norepinephrine content in the adrenal gland, aorta, and plasma in Pnmt-KO and WT mice
Values are means ± S.E.M. of 5–12 experiments per group.

	WT	Pnmt-KO
Adrenal gland		
Epinephrine (nmol mg^{-1})	2.72 ± 0.39	0.02 ± 0.01*
Norepinephrine (nmol mg^{-1})	1.66 ± 0.23	6.45 ± 0.91*
Aorta		
Epinephrine (pmol mg^{-1})	ND	ND
Norepinephrine (pmol mg^{-1})	20.06 ± 4.11	27.12 ± 5.81
Plasma		
Epinephrine (pmol ml^{-1})	4.58 ± 1.17	0.16 ± 0.03*
Norepinephrine (pmol ml^{-1})	6.00 ± 2.06	3.36 ± 1.29

ND, not detectable/below detectable limit.
*Significantly different from correspondent values in WT mice ($P < 0.05$).

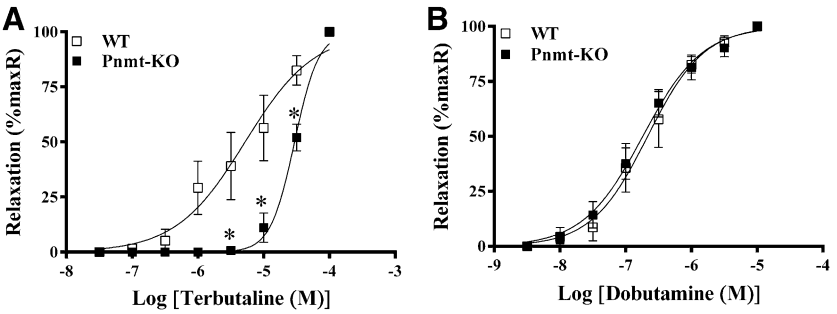

Fig. 1. (A) Terbutaline (β_2-adrenoceptor agonist) and (B) dobutamine (β_1-adrenoceptor agonist) concentration-response curves (relaxation in percentage of maximal relaxation) of phenylephrine (α_1-adrenoceptor agonist) precontracted aortas in Pnmt-KO and WT mice. maxR, maximum relaxation. Each curve point represents the mean of 5–8 experiments per group and error bars represent S.E.M. *Significantly different from correspondent values in WT mice ($P < 0.05$).

overflow of tritium induced by the control stimulation (S1, 0.43 ± 0.04 versus 0.32 ± 0.04, % of tritium content; n = 7–8). In the aorta of WT mice, both isoproterenol (Fig. 3A) and terbutaline (Fig. 3B) increased the overflow of tritium elicited by electrical stimulation in a concentration-dependent manner, with a pEC_{50} of 9.36 ± 0.55 (n = 7–8) and a maximal effect of 167.3 ± 37.5% (n = 7–8) for isoproterenol and a pEC_{50} of 7.65 ± 0.94 (n = 6) and a maximal effect of 173.3 ± 31.3% (n = 6) for terbutaline. This facilitatory effect of isoproterenol and terbutaline on norepinephrine release was absent in Pnmt-KO mice (Fig. 3). In Pnmt-KO mice, curves failed to converge to a sigmoidal equation (Fig. 3).

Quantification and Visualization of β_2-Adrenoceptors in Aorta. The protein density of β_2-adrenoceptors (~61 kDa) in membrane aorta homogenates was significantly ($P < 0.05$) lower in Pnmt-KO compared with WT mice (Fig. 4). This finding was also evidenced by immunofluorescence confocal microscopy (Fig. 5). In WT mice aorta rings, the β_2-adrenoceptor immunofluorescent labeling is concentrated mainly in the media layer (where smooth muscle cells are the most abundant cell type) but also in the endothelium. β_2-Adrenoceptor immunoreactivity was decreased in aorta rings from Pnmt-KO mice (341 ± 37 versus 190 ± 10, arbitrary fluorescence intensity units; n = 3; Fig. 5). The residual fluorescence labeling observed in the absence of the primary antibody (negative control) is due to autofluorescent elastic fibers (Fig. 5).

Discussion

Our results show that epinephrine-deficient mice do not develop aorta β_2-adrenoceptor–mediated responses both at a postjunctional level (β_2-adrenoceptor–dependent relaxation) and at a prejunctional level (facilitation of norepinephrine release from sympathetic nerve endings), strengthening the hypothesis that epinephrine is critical for the functional development of β_2-adrenoceptor–mediated responses.

The Pnmt-KO mouse is an epinephrine-deficient mouse model generated by knocking out the Pnmt gene (Ebert et al., 2004, 2008; Sharara-Chami et al., 2010). The absence of Pnmt mRNA expression alters epinephrine biosynthesis. Accordingly, we found only baseline levels of epinephrine in the adrenal medulla and plasma of Pnmt-KO mice. Our results agree with those of Sun et al. (2008) showing that epinephrine is absent from the adrenal gland and plasma of Pnmt-KO mice, whereas the norepinephrine content of the adrenal glands is significantly increased. This result might be due to an upstream accumulation of norepinephrine that would normally be methylated to epinephrine.

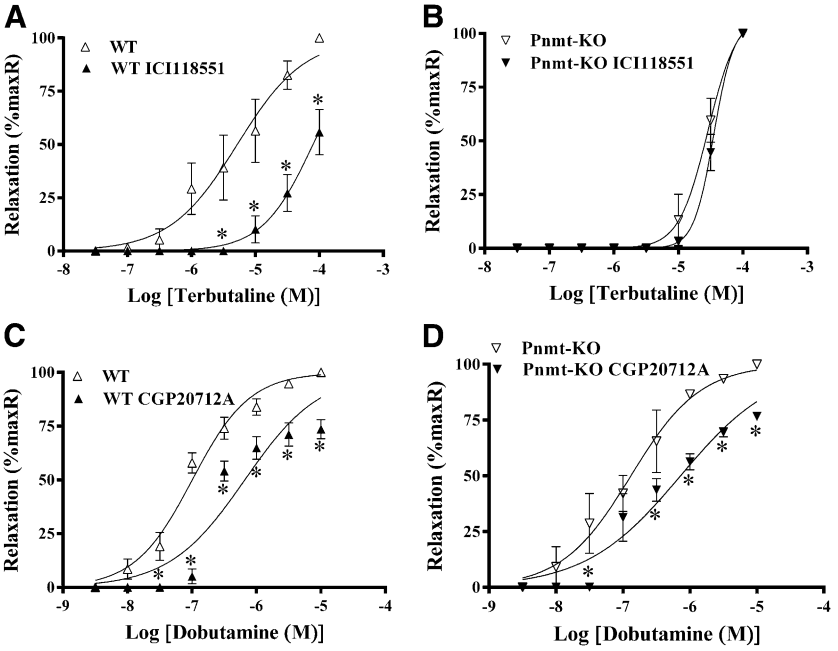

Fig. 2. (A and B) Terbutaline (β_2-adrenoceptor agonist) concentration-response curves in the absence or presence of ICI 118,551 (β_2-adrenoceptor antagonist) and (C and D) dobutamine (β_1-adrenoceptor agonist) concentration-response curves in the absence or presence of CGP 20712 A (β_1-adrenoceptor antagonist), in (A and C) WT and (B and D) Pnmt-KO mice, of phenylephrine (α_1-adrenoceptor agonist) precontracted aortas. maxR, maximum relaxation. Each curve point represents the mean of 5 experiments per group and error bars represent S.E.M. *Significantly different from correspondent values in respective mice group ($P < 0.05$).

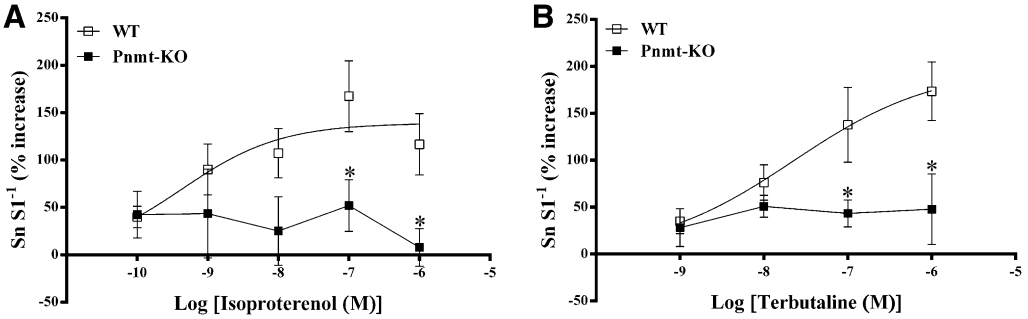

Fig. 3. (A) Isoproterenol (β-adrenoceptor agonist) and (B) terbutaline (β_2-adrenoceptor agonist) evoked tritium overflow of aortas in Pnmt-KO and WT mice. In Pnmt-KO mice, the curve failed to converge to sigmoidal equation. Each curve point represents the mean of 7 to 8 experiments per group and error bars represent S.E.M. *Significantly different from correspondent values in WT mice ($P < 0.05$).

In conduit arteries (thoracic aorta and carotid artery) of mice, both β_1- and β_2-adrenoceptors mediate smooth muscle relaxation (Chruscinski et al., 1999, 2001; Rohrer et al., 1999). We chose the mouse aorta because 1) it contains postjunctional relaxing β_2-adrenoceptors, 2) it is easy to mount in the myograph to evaluate β_2-adrenoceptor–mediated relaxation, and 3) it has enough nerve terminals to evaluate prejunctional β-adrenoceptor–mediated responses.

Our results showed that dobutamine (β_1-adrenoceptor agonist) and terbutaline (β_2-adrenoceptor agonist) caused concentration-dependent relaxation of aorta rings precontracted with phenylephrine in both WT and Pnmt-KO mice. In WT mice, terbutaline caused aorta relaxation at β_2-selective concentrations, whereas in Pnmt-KO mice, very high (nonselective) concentrations of terbutaline were required to produce relaxation. Actually, the potency and the maximal response to β_2-adrenoceptor stimulation by terbutaline were lower in Pnmt-KO mice than in WT mice. In addition, the β_2-adrenoceptor antagonist ICI 118,551 failed to modify the relaxing effect of terbutaline in Pnmt-KO mice, contrary to that observed in WT mice. Overall, these results suggest a loss of β_2-adrenoceptor–mediated relaxation in the aorta of Pnmt-KO compared with WT mice. In agreement with these results is the fact that after adrenalectomy impaired formation of high-affinity myocardial β-adrenoceptor complexes was observed (Davies et al., 1981), and after adrenal demedullation, a decrease in β-adrenoceptor cardiac density was observed in rats (Tumer et al., 1990).

On the other hand, no differences were observed in β_1-adrenoceptor–mediated aorta relaxation to dobutamine between the two groups since the potency and the maximal response to dobutamine were similar. In addition, CGP 20712 A (a β_1-adrenoceptor antagonist) antagonized the effect of dobutamine with similar potency in both groups. Thus, our results suggest that both β_1- and β_2-adrenoceptors mediate aortic relaxation in WT mice but that only β_1-adrenoceptors are operative in the absence of epinephrine, as happens in Pnmt-KO mice. This might explain why Pnmt-KO mice show normal basal blood pressure values, but blood pressure dramatically increases during treadmill exercise compared with WT mice (Bao et al., 2007). In Pnmt-KO mice at rest, β_1-adrenoceptors may be sufficient to produce vasodilation and control blood pressure. One can speculate that under stressful conditions, this mechanism might not be enough because β_2-adrenoceptor–mediated vasodilator response is blunted, and then blood pressure rises. In agreement with this hypothesis is the fact that β_2-adrenoceptor KO mice also have a normal basal resting blood pressure and become hypertensive during exercise (Chruscinski et al., 1999).

Conversely, we did not observe β_3-adrenoceptor–mediated vasorelaxation induced by CL 316243 in WT mice, which is in agreement with the fact that in aortas from $\beta_1\beta_2$-adrenoceptor double-knockout mice, the β-agonist isoproterenol does not cause any relaxation (Chruscinski et al., 2001). These findings suggest that β_3-adrenoceptors do not contribute to the adrenoceptor-mediated vasodilation in mice aorta.

In the aorta of WT mice, the facilitatory effect of isoproterenol on norepinephrine release was similar to that found by other authors in mice spleen and atria (Trendelenburg et al., 2000). To our knowledge, data from this study show for the first time that prejunctional β-adrenoceptors, in particular of the β_2 subtype, are present in the mouse aorta. In addition, we report here the absence of β_2-adrenoceptor–mediated effect on norepinephrine release in Pnmt-KO mice. In agreement with

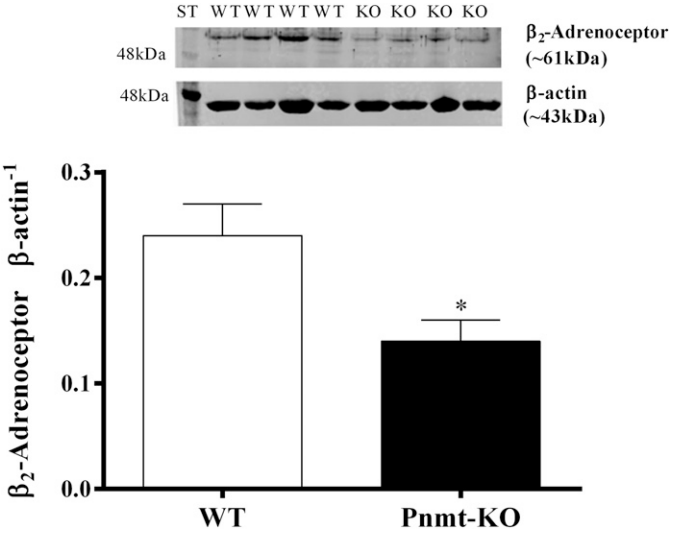

Fig. 4. Semiquantification of β_2-adrenoceptor protein (~61 kDa) in membrane aorta homogenates, in Pnmt-KO and WT mice. Immunoblot quantification is normalized with β-actin protein. Data shown are representative immunoblots; bars represent means of 7–10 experiments per group, and error bars represent S.E.M. ST, Western blot protein standard. *Significantly different from correspondent values in WT mice ($P < 0.05$).

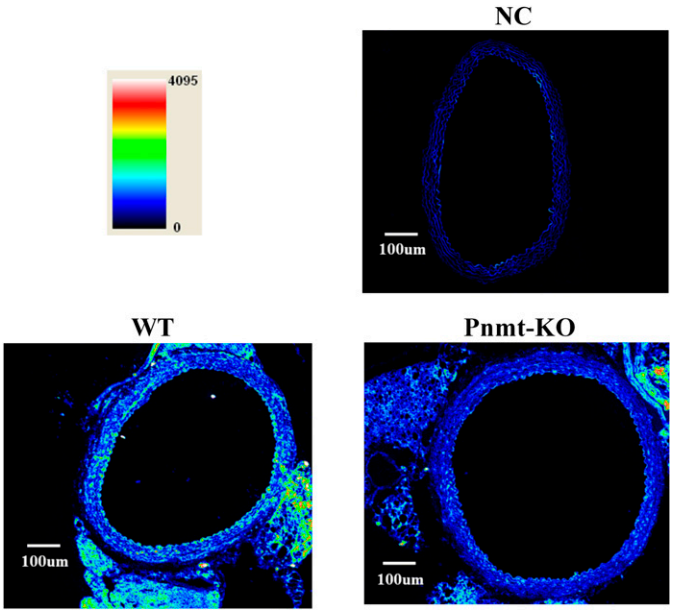

Fig. 5. Representative confocal micrographs of β_2-adrenoceptor immunoreactivity of aortas in Pnmt-KO and WT mice. The images are shown as a pseudocolor spectral display. β_2-Adrenoceptor protein immunoreactivity is represented by the signal intensity of a standardized color pallet, dark blue is low intensity, and red is high intensity. Similar results were obtained in two individual experiments. Image scale bar is 100 μm. The negative control (NC) is secondary antibody alone.

our results, it had been previously shown that carteolol (nonselective β-adrenoceptor antagonist) does not inhibit [^{3}H]norepinephrine release in a concentration-dependent manner induced by nerve stimulation from pulmonary arteries in guinea pigs subjected to adrenalectomy or adrenodemedullation, contrary to sham-operated animals (Misu et al., 1989). On the other hand, putative prejunctional β_1-adrenoceptor–mediated effects were not evaluated because in all tissues tested so far, presynaptic β-adrenoceptors are of the β_2 subtype (Kahan and Hjemdahl, 1987; Molderings et al., 1988; Trendelenburg et al., 2000; Todorov et al., 2001). Although compensatory changes may occur, the presence of presynaptic β_1- or β_3-adrenoceptors seems rather unlikely because we did not observe any β-adrenoceptor induced effect in the release of norepinephrine using isoproterenol (a nonselective β-agonist).

The lower β_2-adrenoceptor protein density observed in aorta of Pnmt-KO mice may be correlated with decreased coupling to stimulatory G protein and lower cAMP levels, which may justify impairments of vasorelaxation and of norepinephrine release facilitation on β_2-adrenoceptor activation (Guimaraes and Moura, 2001). Signaling complexes are located in specialized membrane compartments and are enriched in components of the signal transduction cascade, including G proteins and effector molecules. Thus, these complexes may act as a scaffold promoting the interaction of specific signaling proteins (Galbiati et al., 2001). In Pnmt-KO mice, one possible explanation for the presence of reduced β_2-adrenoceptor protein density (41.7 $\pm$ 8.3%) and the absence of β_2-adrenoceptor function could be a differential association of β_2-adrenoceptors to the signaling complexes in the membrane, which could alter the spatial relationship of β_2-adrenoceptors with the associated signaling proteins, thereby leading to an abrupt decrease in activation of the protein effectors.

The highest β_2-adrenoceptor immunofluorescent labeling found in aorta rings was in the media layer, where smooth muscle cells are most abundant. Immunolocalization studies also revealed the presence of β_2-adrenoceptors in the endothelial lining of mice aortic rings. Given that β_2-adrenoceptors located in vascular endothelial cells may regulate NO release (Conti et al., 2013), one cannot exclude that relaxation of mouse aortic rings produced by β_2-adrenoceptor agonists involves this mechanism.

The results from this study agree with the fact that the time course of postnatal increase in the epinephrine content of the adrenal medulla positively correlates with the development of β_2-adrenoceptor–mediated effects, as previously described in the canine saphenous vein (Paiva et al., 1994). This view is supported by the diminished response of β-adrenoceptors in lymphocytes from human newborns compared with adults (Thies et al., 1986), and lymphocytes almost exclusively express adrenoceptors of the β_2-subtype (Sanders, 2012). Interestingly, it was found that treatment of newborn rats with β_2-adrenoceptor agonists caused a surprising sensitization of β-adrenoceptors instead of evoking desensitization (Kudlacz and Slotkin, 1990). Also, a single exposure of newborn rats to terbutaline induces a strong sensitization of the β_2-adrenoceptor–mediated stimulation of adenylyl cyclase activity in the brainstem and cerebellum (Slotkin and Seidler, 2006). Epinephrine is the only biogenic catecholamine that has a good affinity for β_2-adrenoceptors (Lands et al., 1967a,b), and it is conceivable that it can act similarly, causing sensitization of β_2-adrenoceptors. Because Pnmt-KO mice were never exposed to epinephrine, β_2-adrenoceptor function does not appear to develop efficiently.

One limitation of our experimental design is the limited selectivity of terbutaline and dobutamine at β_2- and β_1-adrenoceptors, respectively (Ruffolo et al., 1984; Young et al., 2002; Baker, 2005). This prevents us from using higher nonselective concentrations of those agonists because the mouse aorta relaxes to activation of both subtypes of β-adrenoceptors.

In conclusion, epinephrine is crucial for the development of β_2-adrenoceptors and associated β_2-adrenoceptor–mediated aorta vasodilation and facilitation of norepinephrine release by sympathetic nerves. In the absence of epinephrine, β_2-adrenoceptor protein density was decreased in aorta cell membranes, thus potentially hindering its functional activity.

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Authorship Contributions

Participated in research design: Moreira-Rodrigues, Moura.

Conducted experiments: Moreira-Rodrigues, Graça, Ferreira, Afonso, Serrão, Morato, Ferreirinha, Moura.

Performed data analysis: Moreira-Rodrigues, Graça, Ferreira, Ferreirinha, Correia-de-Sá, Moura.

Wrote or contributed to the writing of the manuscript: Moreira-Rodrigues, Graça, Morato, Correia-de-Sá, Ebert, Moura.

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Address correspondence to: M. Moreira-Rodrigues, Laboratory of General Physiology, Building 2, Floor 4, Cabinet 22, ICBAS, University of Porto, R. Jorge Viterbo Ferreira, 228, 4050-313, Porto, Portugal. E-mail: mirodrigues@icbas.up.pt