View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by CiteSeerX

The Journal of Pharmacology and Experimental Therapeutics
JPET 321:409–419, 2007
Vol. 321, No. 1
114298/3193585
Printed in U.S.A.
Copyright © 2007 by The American Society for Pharmacology and Experimental Therapeutics

Hypoxic Induction of Receptor Activity-Modifying Protein 2 Alters Regulation of Pulmonary Endothelin-1 by Adrenomedullin: Induction under Normoxia Versus Inhibition under Hypoxia

Thomas Dschietzig, Christoph Richter, Louay Asswad, Gert Baumann, and Karl Stangl

Department of Cardiology and Angiology, University Medical Center Charité, Campus Mitte, Berlin, Germany

Received September 21, 2006; accepted January 22, 2007

ABSTRACT

The vasodilator adrenomedullin (AM) is up-regulated in pulmonary hypertension, and inhaled AM is beneficial in patients. Therefore, we investigated the effects of AM on pulmonary endothelin-1 (ET-1). In normoxic isolated rat lungs (IRL) and rat pulmonary artery endothelial cells (RPAEC), the calcitonin gene-related peptide type-1 receptor (CGRP1R) antagonist human (h)CGRP(8-37) decreased ET-1 secretion, and the AM receptor antagonist hAM(22-52) had no effect. Exogenous AM (1 and 10 pM) increased ET-1 levels, which was abolished by hCGRP(8-37) and protein kinase A (PKA) inhibition. At 50 and 100 pM, AM decreased ET-1, an effect sensitive to hAM(22-52), NO inhibition, and protein kinase G (PKG) inhibition. In RPAEC, these results were attributed to altered ET-1 gene expression; low exogenous AM also promoted activity of endothelin-converting enzyme, and high AM increased the number of endothelin type-B (ETB) receptor sites. Hypoxia significantly elevated AM and ET-1 levels in IRL and RPAEC, and hAM(22-52), NO inhibition, or PKG inhibitors caused a further ET-1 rise. These interventions also prevented the hypoxia-related increase in ETB sites in RPAEC. In RPAEC, both high AM and hypoxia down-regulated receptor activity-modifying protein (RAMP)1, but they up-regulated RAMP2 protein and AM receptor sites, and RAMP2 silencing by small interference RNA proved its pivotal role for signal switching. In conclusion, endogenous pulmonary AM up-regulates ET-1 and endothelin-converting enzyme activity under physiological conditions, via CGRP1R and PKA. In contrast, hypoxia-induced high AM levels, via AM1 receptor and NO/PKG, down-regulate ET-1 gene expression and promote expression of ETB receptors. This hypoxia-related switch of AM signaling can be attributed to up-regulation of the RAMP2/AM1 receptor system.

Adrenomedullin (AM), first discovered by Kitamura et al. (1993), is a vasodilatory peptide with antimitogenic and natriuretic properties. It consists of 52 amino acids in humans, and it is classified into the calcitonin gene-related peptide (CGRP) family (Brain and Grant, 2004). From a functional point of view, three different receptors, all expressed from the calcitonin receptor-like receptor (CL) gene and chaperoned to the cell surface by different receptor activity-modifying proteins (RAMPs), mediate the majority of AM actions (McLatchie et al., 1998; Brain and Grant, 2004; Kuwasako et al., 2004): RAMP1 enables CL to form the CGRP(8-37)-sensitive CGRP type-1 receptor (CGRP1 receptor), which binds both α-CGRP, and, with somewhat weaker affinity, AM itself. RAMP2 enables CL to act as AM(22-52)-sensitive AM1 receptor; and in the presence of RAMP3, the CL becomes an AM2 receptor sensitive to both CGRP(8-37) and AM(22-52).

The important role of AM as pulmonary vasodilator is now widely accepted (Brain and Grant, 2004). AM compensates

This work was supported by a university grant (to T.D.).
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.106.114298.

ABBREVIATIONS: AM, adrenomedullin; CGRP, calcitonin gene-related peptide; CL, calcitonin receptor-like receptor; RAMP, receptor activity-modifying proteins; CGRP1 receptor, calcitonin gene-related peptide type-1 receptor; ET-1, endothelin-1; IRL, isolated rat lung(s); RPAEC, rat pulmonary artery endothelial cell(s); h, human,; r, rat; H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; L-NOARG, N^G-nitro-L-arginine; KT-5720, (8*R**,9*S**,11*S**)-(−)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-triazadi benzo[*a*,*g*]-cycloocta[*c*,*d*,*e*]triden-1-one; DMSO, dimethyl sulfoxide; RIA, radioimmunoassay; ECE, endothelin-converting enzyme; E-64, *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide; siRNA, small interference RNA; KT-5823, (8*R**,9*S**,11*S**)-(−)-9-hydroxy-8-methyl-2,3,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*-triazadibenzo[*a*,*g*]cycloocta[*c*,*d*,*c*]triden-1-one; A-127722, (2*R**,3*R**,4*S**)-1-(*N*,*N*-dibutylacetamido)-4-(1,3-benzodioxol-5-yl)-2-(4-methoxyphenyl)pyrrolidine-3-carboxylic acid; A192621, (±)-*trans*, *trans*-2-(4-*n*-propoxyphenyl)-4-(1,3-benzodioxol-5-yl)-1-[(2,6-dienthylphenyl) aminocarbonylmethyl]pyrrolidine-3-carboxylic acid.

for pulmonary vasoconstriction in different states of disease, e.g., in human congestive heart failure (Jougasaki et al., 1995), and it acts as a player in sepsis and sepsis-related acute respiratory distress syndrome (Nishio et al., 1997; Stangl et al., 2001). In rats with hypoxia-induced pulmonary hypertension, AM mRNA and protein were found to be upregulated in right heart and lung (Nakanishi et al., 2004). Recently, it was reported that inhaled AM improved hemodynamics and gas exchange in a piglet model of pulmonary hypertension (Kandler et al., 2003). Moreover, inhaled AM also showed beneficial effects on pulmonary hemodynamics and exercise capacity in patients suffering from idiopathic pulmonary arterial hypertension (Nagaya et al., 2004). The above-mentioned study in piglets (Kandler et al., 2003) demonstrated a marked reduction of pulmonary gene expression of the potent vasoconstrictor endothelin-1 (ET-1) after AM inhalation. Because ET-1 is clearly implicated in the pathogenesis of pulmonary hypertension (Giaid et al., 1993; Keith, 2000), this finding may pose one essential explanation for the described AM effect.

In this study, we used isolated rat lungs (IRL) and rat pulmonary artery endothelial cells (RPAEC) to investigate the regulation by AM of pulmonary ET-1 under baseline and hypoxic conditions. We also focused on the question which of the established AM signaling pathways—cAMP or nitric oxide/cGMP signaling (Brain and Grant, 2004)—was involved in regulating ET-1 under different conditions.

Materials and Methods

Isolated Rat Lungs. The study conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication 85-23, revised 1996) and was approved by the Local Ethical and Animal Care Committee. Male Wistar rats, weighing 300 to 400 g, were used. After intraperitoneal anesthesia (thiopental sodium, 40–80 mg/kg body weight), lungs were excised, suspended, and perfused as described previously (Dschietzig et al., 2000). Within 20 min, pulmonary flow was gradually increased to 14 ml/min. Thereafter, the lungs were perfused in nonrecirculatory mode and controlled for constant pulmonary artery pressure and constant weight over 45 min. At last, lungs were perfused in recirculatory mode with 80 ml of buffer containing 1 g% endotoxin-free bovine serum albumin.

Cells. Primary RPAEC were obtained as described previously (Kelly et al., 1998) and grown in minimum essential medium (Invitrogen, Karlsruhe, Germany) supplemented with 1.5 g/l sodium bicarbonate, 0.11 g/l sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% fetal calf serum in a humidified 5% CO_2 atmosphere. Cells at passages 6 to 8 were used shortly before confluence.

Protocol. Experiments performed in recirculatory perfusion lasted 6 h in IRL and 6 or 12 h in RPAEC and were performed under normoxic or normobaric hypoxic (10% oxygen) conditions as indicated. We used the following drugs (all from Sigma-Aldrich, Munich, Germany) that, for IRL, were always given at the beginning of recirculatory perfusion: AM1 and AM2 receptor antagonist hAM(22-52) (500 nM; K_i/IC$_{50}$ in nontransfected cells 3–8 nM) (Kuwasako et al., 2004), CGRP1 receptor and AM2 receptor antagonist hCGRP(8-37) (500 nM; K_i/IC$_{50}$ in nontransfected cells 2–10 nM) (Kuwasako et al., 2004), protein kinase A inhibitors H89 (5 μM; K_i = 50 nM) (Chijiwa et al., 1990) and KT-5720 (2.5 μM; K_i = 50 nM) (Kase et al., 1987), nitric-oxide synthase inhibitor N^G-nitro-L-arginine (L-NOARG) (100 μM; IC$_{50}$ = 1.4 μM) (Pfeiffer et al., 1996), and the protein kinase G inhibitor KT-5823 (1 μM; K_i = 12 nM) (Kase et al., 1987). In general, the concentrations of inhibitors and antagonists were chosen to exceed the aforementioned K_i or IC$_{50}$ values by the factor of 50 to 100. Some experiments were performed in the presence of exogenous hAM (Sigma-Aldrich).

Drugs and Solvents. Drugs were stored as stock solutions as follows: hAM, 2 mM in deionized water; hAM(22-52) and hCGRP(8-37), 5 mM in deionized water; H89, 5 mM in DMSO; KT-5720, 2.5 mM in DMSO; L-NOARG, 100 mM in DMSO; and KT-5823, 1 mM in DMSO. Before experiments, all drugs were diluted freshly in perfusion buffer at the final concentrations mentioned above. The solvent content of the perfusate amounted to the following values: hAM, <0.0001% deionized water; hAM(22-52) and hCGRP(8-37), 0.01% deionized water; and H89, KT-5720, L-NOARG, and KT-5823, 0.1% DMSO.

Measurement of ET-1, AM, and CGRP. The concentration of rat ET-1 in IRL perfusate and RPAEC-conditioned media was determined by RIA using a commercial kit (Peninsula Laboratories, Belmont, CA) as described previously (Brunner, 1995). The detection limit was ~0.15 pg/tube; the cross-reactivity of other endothelin isomers and big ET-1 in this assay was less than 5 and 37%, respectively, according to the supplier. To detect AM, we used a commercially available radioimmunoassay kit for rat AM (detection limit, 3 pg/ml or 0.5 pM) (Phoenix Pharmaceuticals, Belmont, CA), which showed no relevant cross-reactivity to rat proadrenomedullin N-terminal 20 peptide, human amylin, and rat endothelin-1 (<0.1%). We measured rat CGRP with a sandwich enzyme-linked immunosorbent assay kit (Cayman Chemical, Ann Arbor, MI) that had a detection limit of 1 pg/ml and displayed no relevant cross-reactivity to amylin, calcitonin, and adrenomedullin (<0.1%).

mRNA Analysis. To determine mRNA expression of preproET-1 in RPAEC, total RNA was extracted and processed using reverse transcription-polymerase chain reaction and subsequent Southern blotting of the polymerase chain reaction fragments as described previously (Dschietzig et al., 2001). Autoradiographs were quantified using ImageMaster 1D Prime software (Pharmacia Biotech, Munich, Germany). All data were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA expression.

Western Blotting. RAMP expression was determined semiquantitatively by Western blotting as described previously (Dschietzig et al., 2001) using rabbit monoclonal antibodies against rat RAMP1–3 (Acris Antibodies, Herford, Germany). For normalization of RAMP expression, we used a monoclonal mouse anti-actin antibody (Acris Antibodies).

Determination of Endothelin Type-B Receptor and AM Receptor Sites. Cell membranes from RPAEC were prepared and ^{125}I-rET-1 (2000 Ci/mmol; GE Healthcare (Little Chalfont, Buckinghamshire, UK) saturation binding (0.1 pM–1 nM) was performed following established methods (Elshourbagy et al., 1993). Nonspecific binding determined by coincubation with 100 nM unlabeled ET-1 was less than 15% of total binding at all concentrations used. The RPAEC used in this study contained no ET$_A$ receptors as evident from competition binding experiments (^{125}I-ET-1 concentration, 150 pM) in which the ET$_B$-selective antagonist A-192621 potently displaced radiolabel ($-\log[\text{IC}_{50}] = 9.3 \pm 0.04$; $n = 4$), whereas the ET$_A$-selective antagonist A-127722 was ineffective up to 10 μM (both endothelin antagonists from Abbott Laboratories, Abbott Park, IL).

For determination of AM receptor binding sites, we used ^{125}I-rAM(1-52) (2000 Ci/mmol; GE Healthcare) according to established methods (Chakravarty et al., 2000). Nonspecific binding, determined by coincubation with 100 nM unlabeled AM, was less than 10% of total binding. The number of AM receptor sites was detected by blocking CGRP1 receptors with 10 nM unlabeled β-CGRP (Sigma-Aldrich), because this peptide does not bind AM receptors (Kuwasako et al., 2004).

Measurement of Endothelin-Converting Enzyme Activity. Phosphoramidon-sensitive endothelin-converting enzyme (ECE) activity in RPAEC was determined on the basis of previous reports (Takaoka et al., 1991; Ehrenreich et al., 1999), with major modifications. Cultured cells were lysed and centrifuged at 1000*g* for 10 min.

The supernatants were then further centrifuged at 45,000*g* for 30 min. The resulting pellet was resuspended in 2 ml of ice-cold buffer A and sonicated for 15 s. The suspension was frozen at −70°C for subsequent measurement of ECE activity. Suspensions obtained from cells were diluted to 0.8 mg of protein per milliliter using 50 mM phosphate buffer, pH 6.5, containing 0.1% (w/v) bovine serum albumin and 0.1% (w/v) Triton X-100 (buffer B). Approximately 25 μg of protein was preincubated in buffer B containing inhibitor cocktail (0.1 mM phenylmethylsulfonyl fluoride, 0.02 mM E-64, and 1 mM *N*-ethyl-maleimide) and 0.01 mM thiorphan (to inhibit ET-1 degradation) in absence or presence of 0.1 mM phosphoramidon (all concentrations are final) in a total volume of 0.09 ml for 30 min at 37°C before addition of 0.03 ml of big ET-1 (final concentration, 100 nM) to start the assay. After 2 h at 37°C, 10 mM ice-cold EDTA (final concentration) was added to stop ECE activity. The mixture was left to stand for 15 min at 4°C, diluted 19-fold with RIA buffer, pH 7.4, containing 100 mM Na-phosphate, 50 mM NaCl, 0.1% Triton X-100, 0.1% bovine serum albumin, and 0.01% NaN_3. The RIA assay contained 0.1 ml of the diluted ECE assay mixture, 0.1 ml of ^{125}I-ET-1, and 0.025 ml of anti-ET-1 antibody (total RIA assay volume, 0.225 ml). The anti-rat ET-1 antibody (Ehrenreich et al., 1999) used for measuring ECE was highly specific for ET-1 without cross-reactivity with big ET-1. The IC_{50} was 35 to 50 pg/assay tube (antibody dilution, 1:10,000). Specific ECE activity was defined as phosphoramidon-inhibitable activity (~90% of total activity) and was calculated as ng of ET-1 formed per milligram of protein per hour. Each measurement was performed in triplicate (total and phosphoramidon-inhibitable activity).

Small Interference RNA Experiments. To silence RAMP2 expression in RPAEC, we used three different rat RAMP2 siRNAs (siRNA1, number 199536; siRNA2, number 199535; and siRNA3, number 199534) and a Silencer negative control siRNA (Ambion, Austin, TX). Cells were transfected using the siPORT lipid transfection reagent (Silencer siRNA transfection kit; Ambion) according to the manufacturer's instructions. Eighty nanomoles of siRNA was diluted in 1.5 μl of transfection reagent, and cells were incubated with transfection mixture for 8 h. Cells were used for determination of RAMP2 expression or subsequent experiments 48 h after transfection.

Statistics. Data are presented as mean ± S.E.M. Different groups (of IRL) were compared using the Kruskal-Wallis analysis of variance on ranks. Differences between groups of RPAEC (over time or after different transfections) were analyzed with a nonparametric multiple analysis of variance for repeated measures. After global testing, post hoc tests were carried out using the sequentially rejective test procedure according to Bonferroni-Holm (Holm, 1979). An error probability of $P < 0.05$ was regarded as significant.

Results

Regulation of Pulmonary ET-1: Effect of Different AM Concentrations. The corresponding ET-1 data are depicted in Figs. 1 (IRL) and 2 (RPAEC). The investigation of ET-1 gene expression, ECE activity, and ET_B sites in RPAEC is shown in Tables 1 to 3. Effects of the different inhibitors alone and of the solvent DMSO are summarized in Table 5. IRL hemodynamics is shown in Table 6.

Control IRL released 0.36 ± 0.02 pg/ml ET-1 over 6 h into the perfusate; control RPAEC produced medium concentrations of 136 ± 15 pg/ml over 6 h and 240 ± 21 pg/ml over 12 h. In the presence of hAM(22-52), ET-1 release changed neither in IRL nor in RPAEC. In contrast, hCGRP(8-37) significantly decreased ET-1 levels to 61% in IRL and to 68 and 64% after 6 and 12 h, respectively, in RPAEC. None of these interventions produced a significant alteration of mean pulmonary

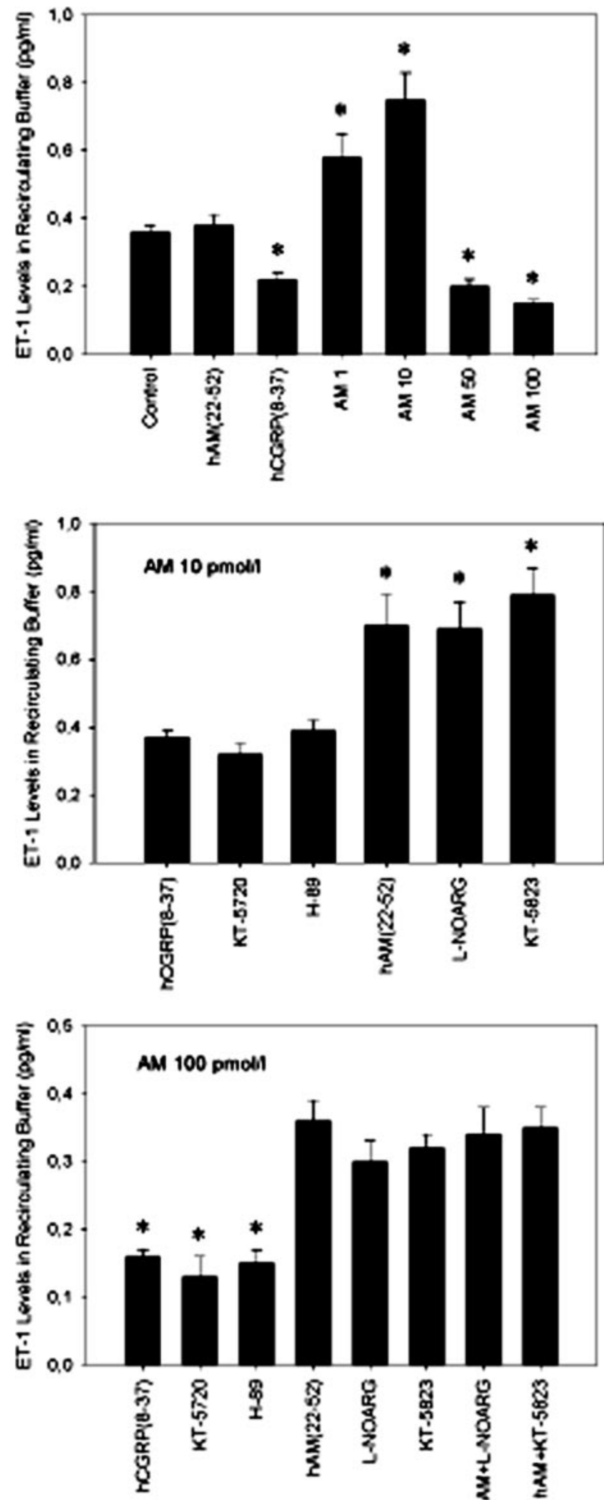

Fig. 1. Release of ET-1 into recirculating buffer of isolated, blood-free perfused rat lungs over 6 h. ET-1, in picograms per milliliter, was measured using a radioimmunoassay (detection limit, 0.15 pg/tube). IRL were perfused with buffer (top), in the presence of 10 pM exogenous AM (middle) or in the presence of 100 pM AM (top), with the following pharmacological interventions: the AM1 and AM2 receptor antagonist hAM(22-52) (500 nM), the CGRP1 receptor and AM2 receptor antagonist hCGRP(8-37) (500 nM), the protein kinase A inhibitors H89 (5 μM) and KT-5720 (2.5 μM), the nitric-oxide synthase inhibitor L-NOARG (100 μM), and the protein kinase G inhibitor KT-5823 (1 μM). The effects of H89, KT-5720, L-NOARG, and KT-5823 alone and of the solvent DMSO (0.1% in perfusate) are summarized in Table 5. Number of experiments is $n = 6$ in each group. *, $P < 0.05$ versus control.

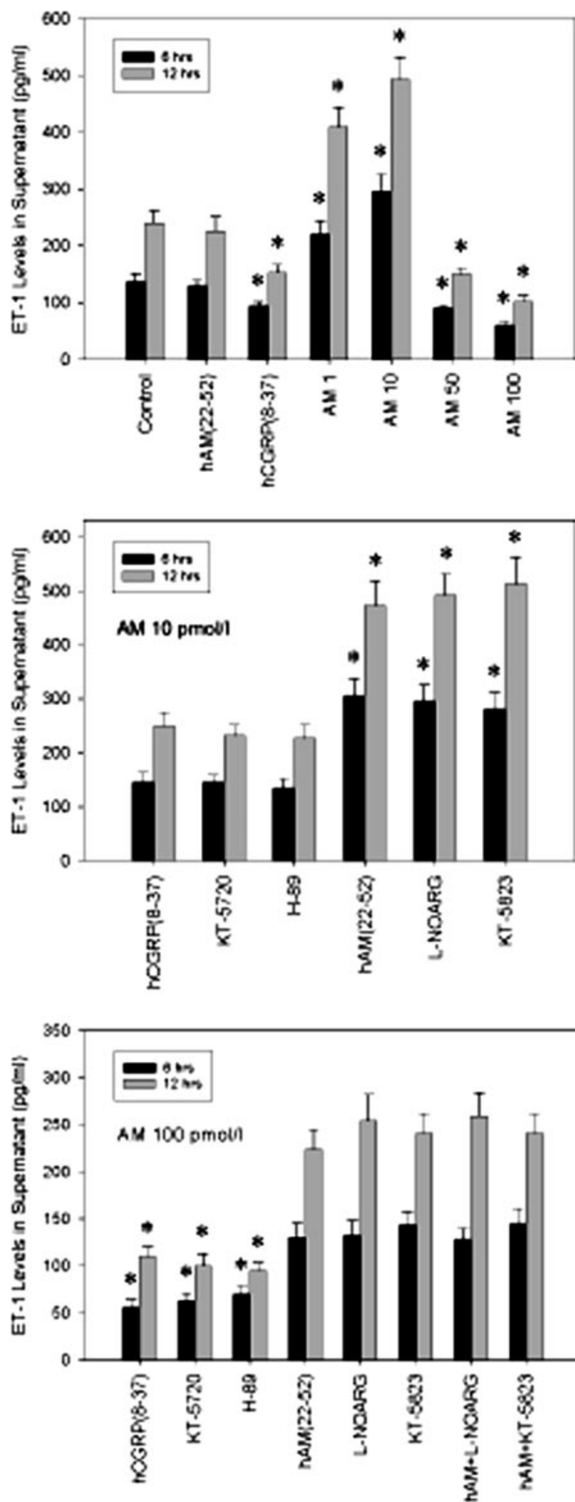

Fig. 2. Six- and 12-h release of ET-1 into supernatant by RPAEC. ET-1, in picograms per milliliter, was measured using a radioimmunoassay (detection limit, 0.15 pg/tube). RPAEC were cultured with medium (top), in the presence of 10 pM exogenous AM (middle), or in the presence of 100 pM AM (bottom), with the following pharmacological interventions: the AM1 and AM2 receptor antagonist hAM(22-52) (500 nM), the CGRP1 receptor and AM2 receptor antagonist hCGRP(8-37) (500 nM), the protein kinase A inhibitors H89 (5 μM) and KT-5720 (2.5 μM), the nitric-oxide synthase inhibitor L-NOARG (100 μM), and the protein kinase G inhibitor KT-5823 (1 μM). The effects of H89, KT-5720, L-NOARG, and KT-5823 alone and of the solvent DMSO (0.1% in perfusate) are summarized in Table 5. Number of experiments is $n = 6$ in each group. *, $P < 0.05$ versus control.

arterial pressure of IRL, which amounted to 12.4 ± 1.0 mm Hg in controls.

Exogenous AM showed a clearly biphasic effect on ET-1 levels. At concentrations of 1 and 10 pM, AM resulted in increased ET-1 production (AM 1 pM/AM 10 pM: IRL, 161%/208% baseline ET-1; RPAEC 6 h, 162%/218%; and RPAEC 12 h, 170%/205%). At higher AM levels, ET-1 release was inhibited (AM 50 pM/AM 100 pM: IRL, 55%/42% baseline ET-1; RPAEC 6 h, 66%/44%; and RPAEC 12 h, 62%/42%). In IRL, higher levels of exogenous AM evoked a significant drop in mean pulmonary arterial pressure to 9.2 ± 1.2 mm Hg at 50 pM and to 7.6 ± 1.0 mm Hg at 100 pM.

Subsequently, we sought to elucidate the effects of exogenously administered AM. The ET-1-increasing action of low AM (10 pM) was blocked by hCGRP(8-37) as well as by the protein kinase A inhibitors KT-5720 and H89, both in IRL and in RPAEC; hAM(22-52), L-NOARG, and KT-5823 had no effect. Mean pulmonary arterial pressure in IRL was altered by none of these drugs compared with baseline.

In contrast to the experiments with low exogenous AM, hCGRP(8-37) and the protein kinase A inhibitors failed to affect the ET-1-decreasing effect of higher AM concentrations (100 pM) in both settings, with this effect being susceptible to hAM(22-52), the NO synthase inhibitor L-NOARG, the protein kinase G inhibitor KT-5823, or combinations thereof. In IRL, hAM(22-52), L-NOARG, KT-5823, and combinations thereof also elevated mean pulmonary arterial pressure to baseline levels, i.e., these interventions abolished the pulmonary vasodilation induced by 100 pM AM. To further elucidate the effects observed in IRL and RPAEC, we also investigated the processes that may govern ET-1 release, i.e., gene expression, activity of the endothelin-converting enzyme, and the density of the endothelial clearance receptor ET_B.

Consistent with its decreasing effect on ET-1 release (see above), hCGRP(8-37) decreased ET-1 gene expression and inhibited ECE activity (Table 1), whereas hAM(22-52) showed no effect over 6 or 12 h. The data furthermore closely reflected the biphasic action of exogenous AM: low levels (1 and 10 pM) up-regulated ET-1 gene expression and promoted ECE activity. Higher AM concentrations (50 and 100 pM) had some opposite effects, namely, down-regulation of gene expression and additional increase in the number of ET_B sites without affecting their affinity, both leading to the observed fall of ET-1 production in the above-mentioned experiments. In the presence of low AM (10 pM), hCGRP(8-37) and the protein kinase A inhibitors KT-5720 and H89 resulted in complete return of ET-1 gene expression and ECE activity to untreated control levels (Table 2). hAM(22-52), L-NOARG, or KT-5823 had no effect. In contrast, hAM(22-52), the NO synthase inhibitor L-NOARG, the protein kinase G inhibitor KT-5823, or combinations thereof clearly abolished the changes related to high AM (100 pM) (Table 3), i.e., these interventions prevented both down-regulation of ET-1 gene expression and up-regulation of ET_B sites.

Regulation of Pulmonary ET-1: Oxygen-Dependent Effects. The release of ET-1 in IRL and RPAEC is depicted in Fig. 3. The investigation of ET-1 gene expression, ECE activity, and ET_B sites in RPAEC is shown in Table 4. IRL hemodynamics is shown in Table 6. Mean pulmonary arterial pressure of IRL, which amounted to 12.4 ± 1.0 mm Hg in controls, rose to 20.4 ± 1.8 mm Hg at the end of the 6-h perfusion period under hypoxia.

TABLE 1

Preproendothelin-1 gene expression, ECE activity, and ET_B sites in RPAEC cultured under control conditions or in the presence of hAM(22-52), hCGRP(8-37), and different concentrations of exogenous AM

For each group, $n = 6$.

h	Control	AM(22-52)	CGRP(8-37)	AM1	AM10	AM50	AM100
			Preproendothelin-1 mRNA (% control)				
6	100 ± 7	92 ± 9	68 ± 8*	155 ± 13*	209 ± 26*	71 ± 10*	51 ± 8*
12	100 ± 10	98 ± 11	63 ± 10*	201 ± 19*	291 ± 19*	65 ± 7*	41 ± 6*
			ECE activity (ng ET-1/mg protein/h)				
6	183 ± 17	192 ± 19	106 ± 12*	271 ± 35*	269 ± 29*	173 ± 17	195 ± 25
12	166 ± 10	178 ± 11	93 ± 10*	300 ± 28*	310 ± 40*	186 ± 20	159 ± 21
			Maximal no. of ET_B sites (fmol/mg protein)				
6	173 ± 17	162 ± 19	160 ± 12	173 ± 17	186 ± 16	321 ± 30*	350 ± 35*
12	169 ± 12	158 ± 15	163 ± 10	169 ± 12	170 ± 16	333 ± 36*	375 ± 37*

* $P < 0.05$ vs. control.

TABLE 2

Preproendothelin-1 gene expression, ECE activity, and ET_B sites in RPAEC cultured under control conditions or in the presence of 10 pM exogenous AM given in combination with antagonists and inhibitors

For each group, $n = 6$.

h	CGRP(8-37)	H89	KT-5720	hAM(22-52)	L-NOARG	KT-5823
			Preproendothelin-1 mRNA (% control)			
6	109 ± 16	101 ± 10	89 ± 16	192 ± 22*	212 ± 20*	229 ± 36*
12	91 ± 9	99 ± 9	111 ± 9	299 ± 19*	280 ± 29*	275 ± 27*
			ECE activity (ng ET-1/mg protein/h)			
6	180 ± 17	193 ± 17	196 ± 20	289 ± 29*	249 ± 29*	261 ± 28*
12	160 ± 17	144 ± 16	166 ± 18	319 ± 40*	300 ± 40*	290 ± 30*
			Maximal no. of ET_B sites (fmol/mg protein)			
6	178 ± 16	175 ± 17	180 ± 16	188 ± 16	173 ± 18	182 ± 18
12	175 ± 16	161 ± 12	179 ± 16	199 ± 26	156 ± 15	170 ± 19

* $P < 0.05$ vs. untreated control (no AM, no drugs; see Table 1).

TABLE 3

Preproendothelin-1 gene expression, ECE activity, and ET_B sites in RPAEC cultured under control conditions or in the presence of 100 pmol/l exogenous AM given in combination with antagonists and inhibitors

For each group, $n = 6$.

h	CGRP(8-37)	H89	KT-5720	AM(22-52)	L-NOARG	KT-5823	L-NOARG + AM(22-52)	KT-5823 + AM(22-52)
				Preproendothelin-1 mRNA (% control)				
6	50 ± 5*	41 ± 8*	56 ± 8*	90 ± 13	91 ± 10	100 ± 10	95 ± 10	89 ± 10
12	40 ± 8*	46 ± 6*	51 ± 6*	109 ± 10	110 ± 14	93 ± 13	93 ± 11	109 ± 13
				ECE activity (ng ET-1/mg protein/h)				
6	183 ± 17	185 ± 20	173 ± 19	190 ± 25	183 ± 15	188 ± 25	177 ± 17	163 ± 19
12	166 ± 20	179 ± 23	182 ± 22	151 ± 28	186 ± 20	166 ± 21	180 ± 20	172 ± 22
				Maximal no. of ET_B sites (fmol/mg protein)				
6	350 ± 35*	350 ± 35*	350 ± 35*	170 ± 14	179 ± 17	163 ± 18	180 ± 19	159 ± 15
12	375 ± 37*	375 ± 37*	375 ± 37*	179 ± 16	189 ± 20	160 ± 15	179 ± 18	169 ± 16

* $P < 0.05$ vs. untreated control (no AM, no drugs; see Table 1).

For endogenous AM, normobaric hypoxia induced a significant increase in IRL release, from 6.6 ± 1.6 pM in controls to 17.5 ± 2.5 pM, and in RPAEC production, from control values of 39 ± 3.3 and 63 ± 6.7 pM to 81 ± 6.8 and 148 ± 15 pM over 6 and 12 h, respectively. CGRP levels in recirculating buffer of IRL rose from 1.7 ± 0.09 pg/ml under normoxia to 4.6 ± 0.41 pg/ml under hypoxia. In contrast, we detected relevant amounts of CGRP neither in supernatant nor in lysate of RPAEC; even the approximately 100-fold enrichment of peptide over C2 columns yielded no detectable traces of CGRP.

Hypoxic IRL (Fig. 3) released 0.82 ± 0.06 pg/ml ET-1 over 6 h into the perfusate, which posed a significant elevation compared with control IRL (0.36 ± 0.02 pg/ml). Likewise, hypoxic RPAEC also produced significantly heightened medium concentrations: 256 ± 21 pg/ml (compared with 136 ± 15 pg/ml in controls) over 6 h and 510 ± 55 pg/ml (compared with 240 ± 21 pg/ml in controls) over 12 h. In both models, ET-1 levels were further elevated in the presence of hAM(22-52), the NO synthase inhibitor L-NOARG, the protein kinase G inhibitor KT-5823, or combinations thereof. This effect was accompanied by further significant increases in mean pulmonary arterial pressure in IRL: to 26.9 ± 2.0 mm Hg in the presence of hAM(22-52), 28.9 ± 3.4 mm Hg in the presence of L-NOARG, 26.2 ± 2.3 mm Hg in the presence of KT-5823, 29.6 ± 2.9 mm Hg in the presence of hAM(22-52) plus L-NOARG, and 27.9 ± 2.4 mm Hg in the presence of hAM(22-52) plus KT-5823. In contrast, hCGRP(8-37) and the protein

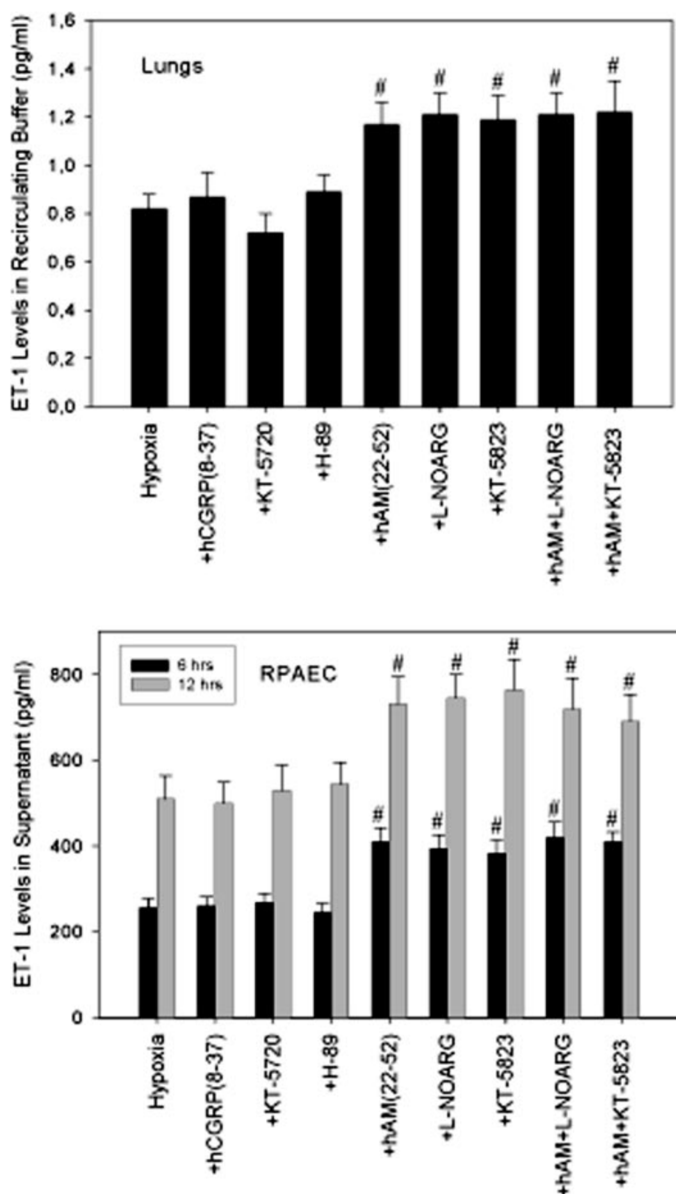

Fig. 3. ET-1 release under normobaric hypoxia (10% oxygen) into recirculating buffer of isolated, blood-free perfused rat lungs (top) over 6 h and into supernatant of RPAEC over 6 and 12 h (bottom). ET-1, in picograms per milliliter, was measured using a radioimmunoassay (detection limit, 0.15 pg/tube). Experiments were conducted using the following drugs: the CGRP1 receptor and AM2 receptor antagonist hCGRP(8-37) (500 nM), the protein kinase A inhibitors H89 (5 μM) and KT-5720 (2.5 μM), the AM1 and AM2 receptor antagonist hAM(22-52) (500 nM), the nitric-oxide synthase inhibitor L-NOARG (100 μM), the protein kinase G inhibitor KT-5823 (1 μM), and combinations thereof. Number of experiments is n = 6 in each group. #, $P < 0.05$ versus hypoxia without intervention.

kinase A inhibitors KT-5720 and H89 had no effect on ET-1 release or IRL hemodynamics under hypoxia.

Hypoxia significantly promoted ET-1 gene expression and the number of ET_B sites, but it did not change ECE activity compared with control conditions (Table 4). ET-1 mRNA levels and the number of ET_B sites were further elevated in the presence of hAM(22-52), the NO synthase inhibitor L-NOARG, the protein kinase G inhibitor KT-5823, or combinations thereof, whereas ECE activity remained unchanged. Corresponding to the findings concerning ET-1 release and pulmonary hemodynamics (see above), hCGRP(8-37) and the protein kinase A inhibitors KT-5720 and H89 showed no effects.

Influence of Different AM and Oxygen Levels on RAMP Expression and Number of AM Receptor Sites. To account for the obvious switch of AM signal transduction observed in the presence of high levels of exogenous AM and under hypoxia, we investigated potential changes of the number of AM receptor sites. Radioligand assays performed in the presence of 10 nM unlabeled β-CGRP demonstrated a remarkable increase in the number of AM receptor sites after 6 h of 100 nM AM, from 590 ± 53 in controls to 1025 ± 90 fmol/mg protein in the AM group, and after 6 h of hypoxia, to 1587 ± 130 fmol/mg protein (Fig. 4, top). Low exogenous AM (10 nM) did not affect AM receptor sites. We furthermore observed no change of K_D values among the different groups.

In Western blot analyses, we detected that both high levels of AM and hypoxia significantly altered the RAMP expression pattern of RPAEC within 6 h of exposure (Fig. 4, middle and bottom): 100 nM of AM down-regulated RAMP1 to 44 ± 12% of control levels and up-regulated RAMP2 to 210 ± 24%, while leaving RAMP3 unaffected. Hypoxia similarly lowered RAMP1, to 31 ± 10% of control levels, and it elevated RAMP2 to 321 ± 23%, without regulating RAMP3. Low levels of exogenous AM (10 nM) had no effect.

To confirm the causal relation between RAMP2 regulation and the switch of AM signaling, we also performed siRNA experiments to silence RAMP2 protein expression (Fig. 5). First, we found that RAMP2 siRNAs 1 and 3 completely suppressed RAMP2 expression, whereas the negative control siRNA had no influence. The RAMP2 siRNA2 was ineffective at the dosage used (Fig. 5A). Resorting to RAMP2 siRNA1 as a representative example for RAMP2 silencing, we then demonstrated that in RPAEC with silenced RAMP2, in comparison with cells transfected with negative control siRNA, the basal expression of AM binding sites was significantly lower and the increase in binding sites induced by 100 pM exogenous AM or exposure to hypoxia was abolished (Fig. 5B). Correspondingly, the cells with silenced RAMP2 did not show a decrease in ET-1 secretion in response to 100 pM AM but, instead, a moderate increase (Fig. 5C) that proved sensitive to hCGRP(8-37) and the protein kinase A inhibitors (Fig. 5D); in contrast, RPAEC with functioning RAMP2 expression (after transfection with negative control siRNA or RAMP2 siRNA2) reacted, like nontransfected cells, with an ET-1 decrease susceptible to hAM(22-52), NO inhibition, and protein kinase G inhibition. Eventually, RPAEC with silenced RAMP2, in comparison with cells with nonsilenced RAMP2, produced significantly higher ET-1 levels under hypoxia, which were insensitive to hAM(22-52), L-NOARG, and KT-5823 (Fig. 5E).

Time Course of Endogenous AM and ET-1 Production by IRL and RPAEC. It remained to be clarified whether the exogenous AM concentrations used in the aforementioned experiments corresponded with the endogenous AM levels present under normoxic or hypoxic conditions. Figure 6, top, shows the time course of endogenous AM under normoxia and hypoxia. In both models, there is a 2- to 3-h delay regarding the appearance of detectable amounts of AM. We thereafter observed a quasilinear AM increase in IRL over 6 h and a sigmoid-like AM increment in RPAEC over 12 h. In the presence of hAM(22-52), the rise of AM was significantly steeper, with the effect being clearly more pro-

TABLE 4
Preproendothelin-1 gene expression, ECE activity, and ET_B sites in RPAEC cultured under hypoxic conditions
For each group, $n = 6$.

h	Hypoxia	+ CGRP(8-37)	+ H89	+ KT-5720	+ AM(22-52)	+ L-NOARG	+ KT-5823	+ L-NOARG + AM(22-52)	+ KT-5823 + AM(22-52)
	Preproendothelin-1 mRNA (% control)								
6	185 ± 17*	195 ± 15*	188 ± 21*	174 ± 18*	270 ± 23†	255 ± 20†	281 ± 30†	280 ± 25†	281 ± 32†
12	230 ± 20*	210 ± 20*	213 ± 22*	209 ± 19*	310 ± 33†	315 ± 30†	324 ± 35†	319 ± 30†	321 ± 35†
	ECE activity (ng ET-1/mg protein/h)								
6	208 ± 19	218 ± 18	198 ± 19	185 ± 21	179 ± 29	200 ± 19	203 ± 21	213 ± 16	190 ± 19
12	154 ± 15	144 ± 18	140 ± 20	166 ± 19	160 ± 15	171 ± 24	140 ± 18	155 ± 17	152 ± 18
	Maximal no. of ET_B sites (fmol/mg protein)								
6	370 ± 37*	380 ± 35*	355 ± 37*	392 ± 40*	238 ± 19*†	240 ± 20*†	223 ± 19*†	229 ± 20*†	222 ± 19*†
12	355 ± 32*	385 ± 39*	348 ± 36*	390 ± 42*	244 ± 18*†	254 ± 25*†	256 ± 28*†	250 ± 25*†	266 ± 28*†

* $P < 0.05$ vs. untreated control (no AM, no drugs).
† $P < 0.05$ vs. hypoxia.

TABLE 5
Release of endothelin-1 in isolated lungs; release of endothelin-1, preproendothelin-1 gene expression, ECE activity, and ET_B sites in RPAEC under control conditions: effects of different pharmacological inhibitors and of solvent
For each group, $n = 5$.

h	Control	H89	KT-5720	L-NOARG	KT-5823	0.1% DMSO
	EC Isolated lungs: release of endothelin-1 into buffer (pg/ml)					
6	0.36 ± 0.02	0.34 ± 0.04	0.33 ± 0.04	0.39 ± 0.03	0.34 ± 0.02	0.35 ± 0.03
	EC RPAEC: release of endothelin-1 into supernatant (pg/ml)					
6	136 ± 15	146 ± 13	131 ± 12	148 ± 17	140 ± 10	148 ± 15
12	240 ± 21	220 ± 24	249 ± 19	254 ± 20	240 ± 14	230 ± 23
	EC Preproendothelin-1 mRNA (% control)					
6	100 ± 7	110 ± 10	90 ± 11	116 ± 13	91 ± 8	100 ± 9
12	100 ± 10	106 ± 8	111 ± 10	109 ± 10	94 ± 10	112 ± 14
	EC ECE activity (ng ET-1/mg protein/h)					
6	183 ± 17	203 ± 21	180 ± 15	188 ± 10	193 ± 14	185 ± 17
12	166 ± 10	160 ± 12	156 ± 19	172 ± 12	148 ± 18	151 ± 16
	EC Maximal no. of ET_B sites (fmol/mg protein)					
6	173 ± 17	150 ± 22	173 ± 17	199 ± 19	179 ± 21	173 ± 18
12	169 ± 12	159 ± 10	192 ± 18	169 ± 10	183 ± 13	149 ± 17

TABLE 6
Pulmonary arterial pressure (in mm Hg) in isolated rat lungs under normoxia and hypoxia
For each group, $n = 6$.

	Normoxia	Normoxia + 10 pM AM	Normoxia + 100 pM AM	Hypoxia
No intervention	12.4 ± 1.0	11.3 ± 1.3	7.6 ± 1.0*	20.4 ± 1.8*
hCGRP(8-37)	13.1 ± 0.8	11.9 ± 1.2	7.0 ± 1.2*	19.4 ± 1.9*
H89	12.1 ± 0.9	12.1 ± 0.9	7.9 ± 1.0*	21.4 ± 2.2*
KT-5720	12.7 ± 1.3	12.5 ± 1.0	8.2 ± 1.1*	20.9 ± 1.7*
hAM(22-52)	11.9 ± 1.1	12.3 ± 1.1	11.1 ± 1.5	26.9 ± 2.0*†
L-NOARG	13.0 ± 0.8	13.3 ± 1.5	11.0 ± 1.5	28.9 ± 3.4*†
KT-5823	11.8 ± 1.2	12.0 ± 1.3	11.4 ± 1.4	26.2 ± 2.3*†
hAM(22-52) + L-NOARG	12.8 ± 1.2	11.8 ± 1.2	11.8 ± 1.1	29.6 ± 2.9*†
hAM(22-52) + KT-5823	12.6 ± 1.0	11.6 ± 0.8	12.1 ± 1.2	27.9 ± 2.4*†
0.1% DMSO	12.0 ± 1.0	11.8 ± 1.0	7.4 ± 1.0*	20.0 ± 1.9*

* $P < 0.05$ vs. control (normoxia, no intervention).
† $P < 0.05$ vs. hypoxia.

nounced under hypoxic conditions. In IRL, the absolute AM levels ranged below 10 pM under normoxia but rose to almost 70 pM under hypoxia plus hAM(22-52). In the RPAEC model, AM concentrations, due to permanent secretion into supernatant, covered a remarkable range, from below 10 pM during the first 3 h under normoxia to values of >400 pM after 12 h of hypoxia plus hAM(22-52).

Concerning ET-1 production (Fig. 6, bottom), there was a 2- to 3-h delay to the appearance of detectable amounts of peptide in both models. Moreover, the ET-1-increasing effects of low exogenous AM (1 and 10 pM) became noticeable after 3 and 4 h in IRL and RPAEC, respectively; the ET-1-decreasing action of 50 and 100 pM AM was detectable after 4 h (IRL) and 5 to 6 h (RPAEC).

Discussion

This study represents the first detailed investigation into the regulation of ET-1 by AM in lungs and pulmonary vascular endothelium. The most relevant findings are as follows. 1) Under physiological, normoxic conditions, low levels of endogenous AM promote pulmonary ET-1 production by up-

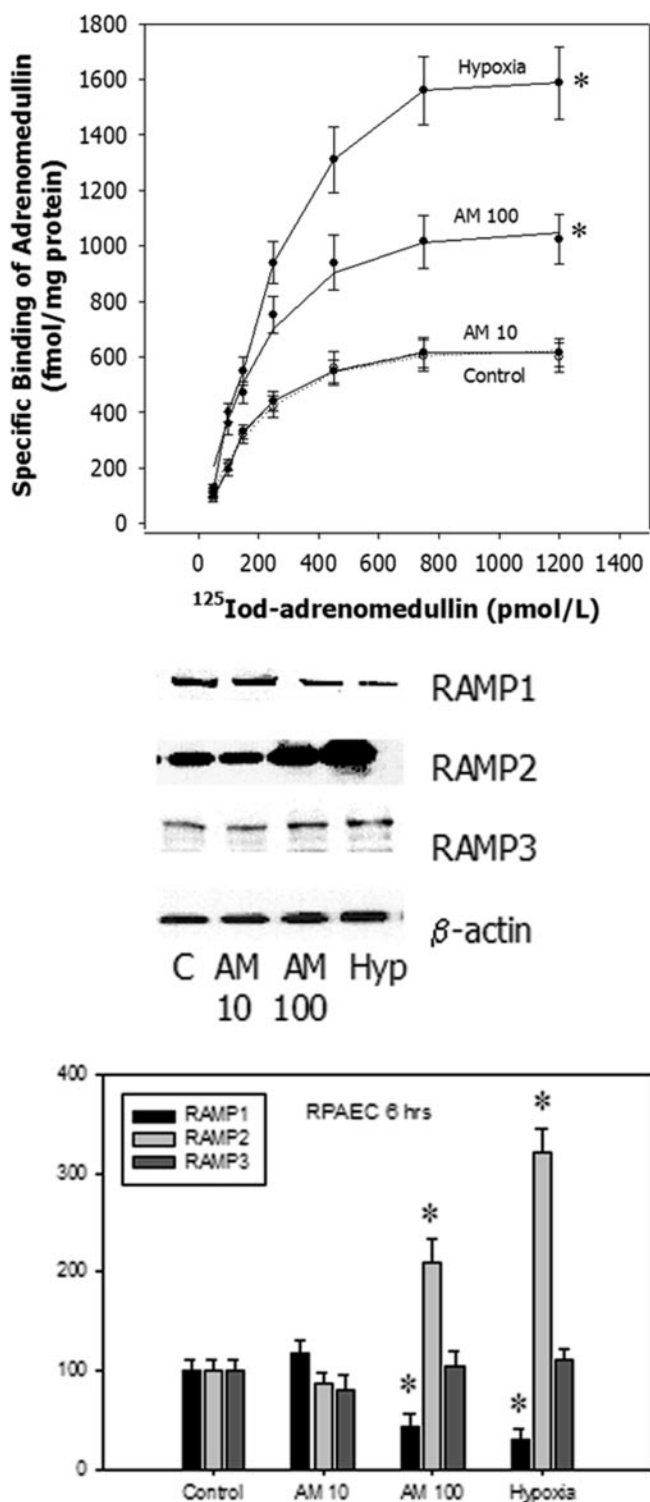

Fig. 4. Number of AM receptor sites (top) and expression of RAMP1–3 (middle and bottom) in RPAEC cultured over 6 h with medium (controls, C) in the presence of 10 and 100 pM exogenous AM (AM10 and AM100) and under hypoxic conditions (Hyp) (10% oxygen). For determination of AM receptor binding sites in membrane preparations of RPAEC, we used ^{125}I-rAM(1-52) (2000 Ci/mmol); nonspecific binding was determined by coincubation with 100 nM unlabeled AM and was less than 10% of total binding. The number of AM receptor sites was detected by blocking CGRP1 receptors with 10 nM unlabeled β-CGRP. Western blots were performed using rabbit monoclonal antibodies against rat RAMP1–3. For normalization of RAMP expression, we used a monoclonal mouse anti-actin antibody. Number of experiments was $n = 4$ for each group. *, $P < 0.05$ versus control.

regulating ET-1 gene expression as well as ECE activity via a pathway involving CGRP1 (or AM2) receptors and protein kinase A. 2) Under hypoxia, high levels of endogenous AM inhibit pulmonary ET-1 production by down-regulating its gene expression and increasing the number of endothelial ET_B (clearance) receptors, via AM1 receptors and nitric oxide/protein kinase G, and 3) this switch of signaling mode is attributable to changes of the RAMP1/RAMP2 expression ratio. Because hypoxia is an established model for pulmonary hypertension, these results may substantiate recent reports on the therapeutic value of inhaled AM in pulmonary hypertension (Kandler et al., 2003; Nagaya et al., 2004), and in particular, on the notion (Kandler et al., 2003) that AM-mediated inhibition of the potent pulmonary mitogen and vasoconstrictor ET-1 may greatly contribute to this action.

We cannot ultimately decide here whether the pathway promoting ET-1 production under normoxia involves CGRP1 or AM2 receptors. Both receptors are obviously present in the RPAEC used (Fig. 4). In rats and mice, in contrast to humans, pigs, and cows, the AM2 receptor displays a pharmacological profile very similar to the CGRP1 receptor, i.e., it is preferentially antagonized by hCGRP(8-37), and AM antagonists are significantly weaker at this receptor (Kuwasako et al., 2004).

Since the CGRP1 receptor binds both AM and α-CGRP and we found relevant concentrations of the latter in IRL, it is also possible that part of the ET-1-decreasing effect of hCGRP(8-37) in IRL under normoxia is attributable to inhibition of endogenous α-CGRP. This uncertainty, however, bears no impact on the findings obtained with exogenous AM and particularly under hypoxia, because the hypoxia-related effects were exclusively mediated by the AM1 receptor, which does not interact with α-CGRP (Kuwasako et al., 2004). In RPAEC, it seems very unlikely that CGRP contributed to any of the observed effects, because we did not find relevant CGRP levels. This coincides well with the general notion that CGRP is prevailingly released by sensory nerve fibers (Brain and Grant, 2004).

Next, the question arises whether the different dosages of exogenous AM (1, 10, 50, and 100 pM) used under normoxic conditions corresponded with the endogenous AM levels present under normoxia or hypoxia and therefore whether the results obtained with exogenous AM are representative for normoxic or hypoxic situations. To this end, we conducted detailed investigations into the time courses of endogenous ET-1 and AM production (Fig. 6), and we also included experiments with the AM antagonist hAM(22-52). We have previously shown that the AM receptor functions as clearance receptor in pulmonary endothelium (Dschietzig et al., 2002), the blockade of which may unmask the real extent of AM secretion. The data summarized in Fig. 6 demonstrate that there is 1) a delayed ET-1 response to exogenous AM (explained by the time required for peptide synthesis, and, in high AM, also by the time needed for synthesis of new AM1 receptors); and 2) a delayed rise of endogenous AM because of certain model characteristics (nonrecirculatory rinsing of IRL and medium change in RPAEC before experiments). So, low concentrations of exogenous AM (1 and 10 pM) were in the range of normoxic endogenous levels throughout the IRL experiments and during the first 3 h in RPAEC; but even in RPAEC experiments lasting 12 h, the threshold to ET-1-inhibiting levels (~35–40 pM in this model; data not shown)

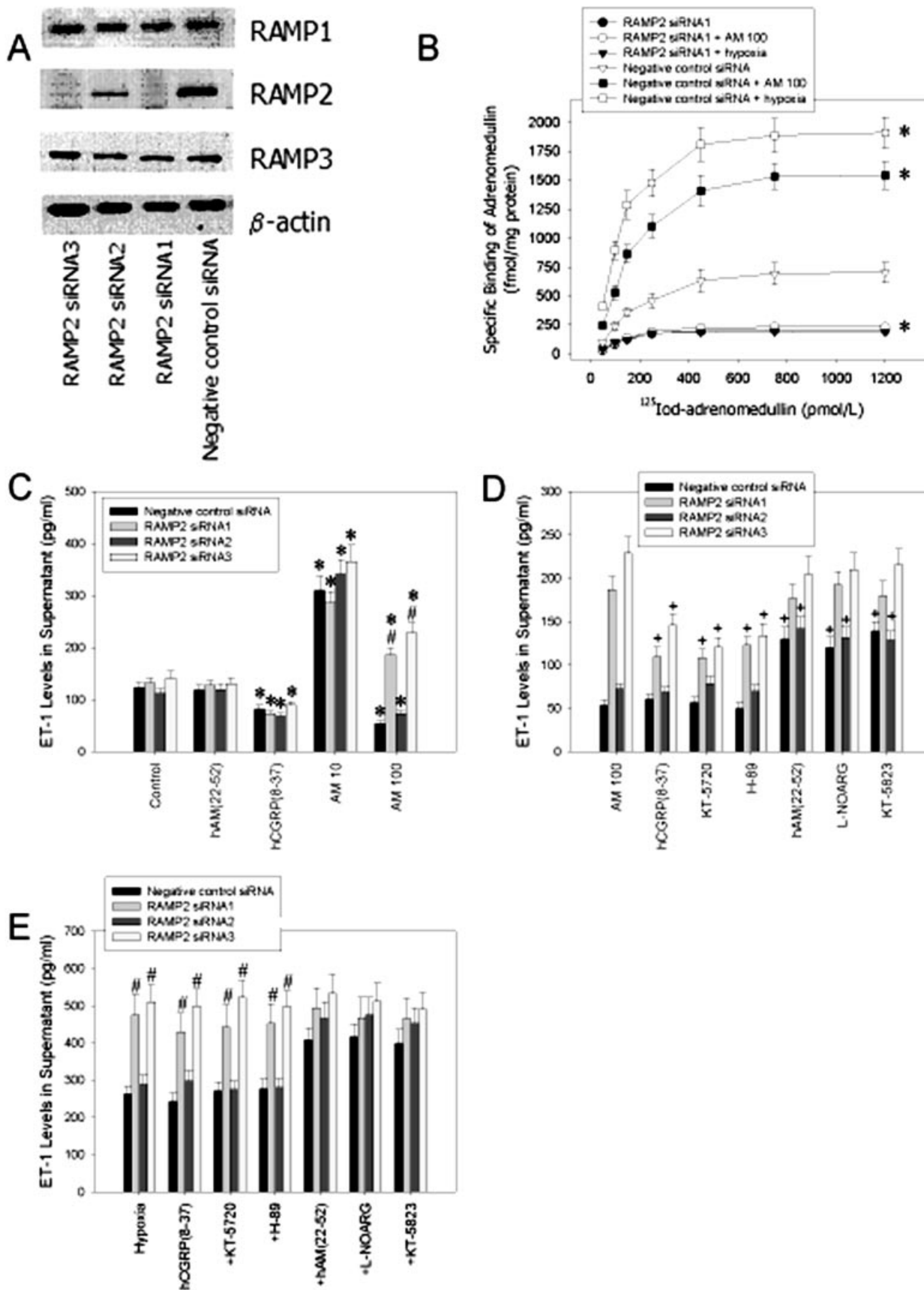

Fig. 5. Expression of RAMP1–3 in RPAEC transfected with different siRNAs. A, Western blots were performed using rabbit monoclonal antibodies against rat RAMP1–3. For normalization of RAMP expression, we used a monoclonal mouse anti-actin antibody. B, number of AM receptor sites in RPAEC transfected with RAMP2 siRNA1 and negative control siRNA, cultured over 6 h with medium alone, in the presence of 100 pM exogenous AM, or under hypoxic conditions (10% oxygen). For determination of AM receptor binding sites in membrane preparations of RPAEC, we used ^{125}I-rAM(1-52) (2000 Ci/mmol); nonspecific binding was determined by coincubation with 100 nM unlabeled AM and was less than 10% of total binding. The number of AM receptor sites was detected by blocking CGRP1 receptors with 10 nM unlabeled β-CGRP. *, $P < 0.05$ versus negative control siRNA. C to E, 6-h release of ET-1 by RPAEC transfected with different siRNAs (one negative control and three RAMP2 siRNAs). ET-1, in picograms per milliliter, was measured using a radioimmunoassay (detection limit, 0.15 pg/tube). C, experiments were performed with medium alone (control), in the presence of the AM1 and AM2 receptor antagonist hAM(22-52) (500 nM) or the CGRP1 receptor and AM2 receptor antagonist hCGRP(8-37) (500 nM), or in the presence of 10 or 100 pM exogenous AM. $n = 3$ in each group. *, $P < 0.05$ versus control; #, $P < 0.05$ versus negative control siRNA. D, experiments were performed in the presence of 100 pM AM, with the following pharmacological interventions: hCGRP(8-37) (500 nM), the protein kinase A inhibitors KT-5720 (2.5 μM) and H89 (5 μM), hAM(22-52) (500 nM), the nitric-oxide synthase inhibitor L-NOARG (100 μM), and the protein kinase G inhibitor KT-5823 (1 μM). $n = 3$ in each group. +, $P < 0.05$ versus AM100 without intervention. E, experiments were performed under hypoxia (oxygen 10%), with the same interventions as described in B. $n = 3$ in each group. #, $P < 0.05$ versus negative control siRNA.

was reached only after 6 h, which was too late to essentially affect the cumulative 12-h value. High dosages of exogenous AM (50 and 100 pM), in contrast, were in the range of endogenous hypoxia-induced AM production, but this was only evident, at least for the IRL and the 6-h RPAEC experiments, after inhibition, by hAM(22-52), of the increasing clearance function of AM receptors.

We are not acquainted with other reports dealing with AM-related ET-1 regulation in the lung and in pulmonary endothelium; the topic has scarcely been investigated until now. In 1995, experiments conducted in rat vascular smooth muscle cells indicated that AM inhibits not basal but thrombin- and PDGF-induced ET-1 production, probably through a cAMP-dependent process (Kohno et al., 1995). Furthermore, AM was demonstrated to down-regulate basal and stimulated ET-1 expression in bovine aortic endothelial cells by cAMP-independent mechanisms (Barker et al., 2002). Conversely, the potential role of the ET_B receptor for ET-1-mediated AM stimulation has been shown in canine aortic endothelial cells under control conditions (Jougasaki et al., 1998), and in rat cardiomyocytes, exogenous ET-1 elevated AM secretion (Mishima et al., 2001).

In our siRNA experiments, we established that the up-regulation of RAMP2 expression poses the basic mechanism ensuing the switch from CGRP1 (AM2) receptor to AM1 receptor signaling: RAMP2 silencing was followed by com-

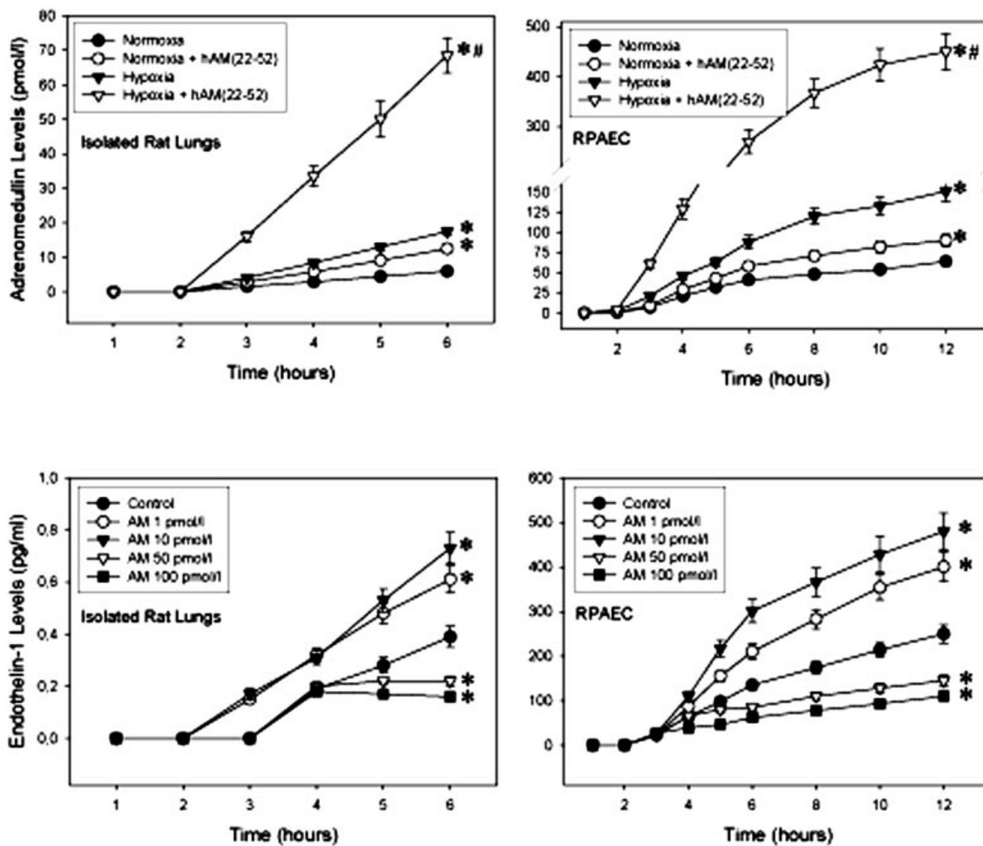

Fig. 6. Time course of endogenous AM levels (top) in IRL recirculate (6 h) and RPAEC supernatant (12 h) under normoxia and hypoxia, in presence or absence of the AM1 and AM2 receptor antagonist hAM(22-52) (500 nM). AM, in picomoles per liter, was measured using a radioimmunoassay (detection limit, 0.5 pM). Number of experiments was $n = 3$ for each group. *, $P < 0.05$ versus normoxia; #, $P < 0.05$ versus hypoxia. Bottom, time course of ET-1 release into IRL recirculate (6 h) and RPAEC supernatant (12 h) under normoxia, in presence or absence of 1, 10, 50, and 100 pM exogenous AM. ET-1, in picograms per milliliter, was measured using a radioimmunoassay (detection limit, 0.15 pg/tube). *, $P < 0.05$ versus control.

plete insensitivity to hAM(22-52), NO inhibition, and inhibition of protein kinase G; loss of the ET-1-decreasing effect of high exogenous AM (with the remaining CGRP1/AM2 receptor population causing moderate ET-1 up-regulation); and significantly elevated ET-1 secretion under hypoxia.

There is no other report on investigations of RAMP expression in pulmonary cells until now. In human coronary artery smooth muscle cells, short-term hypoxia was demonstrated to heighten RAMP2 and AM mRNA levels, suggesting increased AM-AM1 receptor coupling (Cueille et al., 2005). Others observed elevated RAMP2 and RAMP3 mRNA levels in cardiomyocytes obtained from rats with heart failure (Oie et al., 2005). The opposite finding—hypoxia-related down-regulation of RAMP2 despite AM up-regulation—was reported from experiments using human neuroblastoma cells (Kitamuro et al., 2001), indicating pronounced cell specificity of RAMP regulation during hypoxia.

Other findings presented here are in good accordance with previous reports. First, it is generally accepted that both CGRP1 and AM receptors may act via cAMP/protein kinase A or nitric oxide/protein kinase G pathways (Brain and Grant, 2004). We were also able to confirm the up-regulation of ET-1 and AM during hypoxia: a Chinese group found an increment in AM expression in the lungs of chronic hypoxic rats (Cheng et al., 2002), and Japanese authors described AM induction during 6- and 12-h hypoxia in human coronary artery endothelial cells (Nakayama et al., 1999). With ET-1, it has been shown in various models, including endothelial cells, that hypoxia, via hypoxia-inducible factor-1, up-regulates the peptide (Maemura et al., 1992). Moreover, our finding that hypoxia leads to increased ET_B receptor density in RPAEC (Table 4) corresponds well with previous reports (Muramatsu et al., 1999; Sato et al., 1999).

Brunner and coworkers demonstrated previously that ET-1 gene expression and secretion generally depend of intracellular calcium levels and that there is no linear but instead a bell-shaped relationship between intracellular calcium and ET-1 (Brunner, 1995), implying the existence of a cell-specific optimal intracellular calcium level for ET-1 production. In our models, the AM-protein kinase A pathway effective under normoxia obviously changes calcium levels toward this optimum, presumably through protein kinase A-mediated phosphorylation of inositol-1,4,5-trisphosphate receptors, which pose the key effectors of calcium tuning in electrically nonexcitable cells (Straub et al., 2004). It is, in contrast, long known that nitric oxide-protein kinase G-coupled events also interfere with calcium handling via inositol-1,4,5-trisphosphate receptors (Neylon et al., 1990) and that this pathway usually diminishes ET-1 production (Brunner et al., 2006). This, in turn, may reflect the situation in our hypoxia experiments.

We doubtlessly have to consider some limitations of the approach used here. First, we investigated AM-ET-1 interactions in the acute setting, up to 12 h, which may not accurately reflect the situation encountered in the long-term course of pulmonary disease. Our models, in contrast, exhibit key characteristics of pulmonary hypertension, e.g., AM, ET-1, and ET_B receptor up-regulation, which substantiates these results. Second, we used blood-free perfusion for IRL, which excludes many cell-mediated events that affect pulmonary perfusion pressure and permeability. Thus, our findings have to be confirmed in chronic in vivo models.

We conclude that we have found a hypoxia-related switch of pulmonary AM signaling, attributable to up-regulation of the RAMP2/AM1 receptor system, which turns normoxic promotion of ET-1 levels into down-regulation of ET-1 gene expression and enhanced ET-1 clearance and may thereby contribute to beneficial effects of AM observed in the clinical setting.

References

Barker S, Lees DM, Wood EG, and Corder R (2002) Inhibitory effect of adrenomedullin on basal and tumour necrosis factor alpha-stimulated endothelin-1 synthesis in bovine aortic endothelial cells is independent of cyclic AMP. *Biochem Pharmacol* **63:**149–156.

Brain SD and Grant AD (2004) Vascular actions of calcitonin gene-related peptide and adrenomedullin. *Physiol Rev* **84:**903–934.

Brunner F (1995) Dependence of endothelin-1 secretion on Ca^{2+}. *Biochem Pharmacol* **49:**1785–1791.

Brunner F, Bras-Silva C, Cerdeira AS, and Leite-Moreira AF (2006) Cardiovascular endothelins: essential regulators of cardiovascular homeostasis. *Pharmacol Ther* **111:**508–531.

Chakravarty P, Suthar TP, Coppock HA, Nicholl CG, Bloom SR, Legon S, and Smith DM (2000) CGRP and adrenomedullin binding correlates with transcript levels for calcitonin receptor-like receptor (CRLR) and receptor activity modifying proteins (RAMPs) in rat tissues. *Br J Pharmacol* **130:**189–195.

Cheng D, Tian W, Chen W, and Xiao X (2002) Expression of adrenomedullin and its receptor in lungs of rats with hypoxic pulmonary hypertension. *Chin Med J (Engl)* **115:**1806–1808.

Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, and Hidaka H (1990) Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J Biol Chem* **265:**5267–5272.

Cueille C, Birot O, Bigard X, Hagner S, and Garel JM (2005) Post-transcriptional regulation of CRLR expression during hypoxia. *Biochem Biophys Res Commun* **326:**23–29.

Dschietzig T, Alexiou K, Laule M, Becker R, Schror K, Baumann G, Brunner F, and Stangl K (2000) Stimulation of pulmonary big endothelin-1 and endothelin-1 by antithrombin III: a rationale for combined application of antithrombin III and endothelin antagonists in sepsis-related acute respiratory distress syndrome? *Crit Care Med* **28:**2445–2449.

Dschietzig T, Azad HA, Asswad L, Bohme C, Bartsch C, Baumann G, and Stangl K (2002) The adrenomedullin receptor acts as clearance receptor in pulmonary circulation. *Biochem Biophys Res Commun* **294:**315–318.

Dschietzig T, Richter C, Bartsch C, Laule M, Armbruster FP, Baumann G, and Stangl K (2001) The pregnancy hormone relaxin is a player in human heart failure. *FASEB J* **15:**2187–2195.

Ehrenreich H, Loffler BM, Hasselblatt M, Langen H, Oldenburg J, Subkowski T, Schilling L, and Siren AL (1999) Endothelin converting enzyme activity in primary rat astrocytes is modulated by endothelin B receptors. *Biochem Biophys Res Commun* **261:**149–155.

Elshourbagy NA, Korman DR, Wu HL, Sylvester DR, Lee JA, Nuthalaganti P, Bergsma DJ, Kumar CS, and Nambi P (1993) Molecular characterization and regulation of the human endothelin receptors. *J Biol Chem* **268:**3873–3879.

Giaid A, Yanagisawa M, Langleben D, Michel RP, Levy R, Shennib H, Kimura S, Masaki T, Duguid WP, and Stewart DJ (1993) Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N Engl J Med* **328:**1732–1739.

Holm S (1979) A simple sequentially rejective multiple test procedure. *Scand J Stat* **6:**65–70.

Jougasaki M, Schirger JA, Simari RD, and Burnett JC Jr (1998) Autocrine role for the endothelin-B receptor in the secretion of adrenomedullin. *Hypertension* **32:** 917–922.

Jougasaki M, Wei CM, McKinley LJ, and Burnett JC Jr (1995) Elevation of circulating and ventricular adrenomedullin in human congestive heart failure. *Circulation* **92:**286–289.

Kandler MA, von der HK, Mahfoud S, Chada M, Schoof E, Papadopoulos T, Rascher W, and Dotsch J (2003) Pilot intervention: aerosolized adrenomedullin reduces pulmonary hypertension. *J Pharmacol Exp Ther* **306:**1021–1026.

Kase H, Iwahashi K, Nakanishi S, Matsuda Y, Yamada K, Takahashi M, Murakata C, Sato A, and Kaneko M (1987) K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem Biophys Res Commun* **142:**436–440.

Keith IM (2000) The role of endogenous lung neuropeptides in regulation of the pulmonary circulation. *Physiol Res* **49:**519–537.

Kelly JJ, Moore TM, Babal P, Diwan AH, Stevens T, and Thompson WJ (1998) Pulmonary microvascular and macrovascular endothelial cells: differential regulation of Ca^{2+} and permeability. *Am J Physiol* **274:**L810–L819.

Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, and Eto T (1993) Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun* **192:**553–560.

Kitamuro T, Takahashi K, Totsune K, Nakayama M, Murakami O, Hida W, Shirato K, and Shibahara S (2001) Differential expression of adrenomedullin and its receptor component, receptor activity modifying protein (RAMP) 2 during hypoxia in cultured human neuroblastoma cells. *Peptides* **22:**1795–1801.

Kohno M, Kano H, Horio T, Yokokawa K, Yasunari K, and Takeda T (1995) Inhibition of endothelin production by adrenomedullin in vascular smooth muscle cells. *Hypertension* **25:**1185–1190.

Kuwasako K, Cao YN, Nagoshi Y, Kitamura K, and Eto T (2004) Adrenomedullin receptors: pharmacological features and possible pathophysiological roles. *Peptides* **25:**2003–2012.

Maemura K, Kurihara H, Morita T, Oh-hashi Y, and Yazaki Y (1992) Production of endothelin-1 in vascular endothelial cells is regulated by factors associated with vascular injury. *Gerontology* **38 (Suppl 1):**29–35.

McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, and Foord SM (1998) RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature (Lond)* **393:**333–339.

Mishima K, Kato J, Kuwasako K, Ito K, Imamura T, Kitamura K, and Eto T (2001) Effects of endothelin on adrenomedullin secretion and expression of adrenomedullin receptors in rat cardiomyocytes. *Biochem Biophys Res Commun* **287:**264–269.

Muramatsu M, Oka M, Morio Y, Soma S, Takahashi H, and Fukuchi Y (1999) Chronic hypoxia augments endothelin-B receptor-mediated vasodilation in isolated perfused rat lungs. *Am J Physiol* **276:**L358–L364.

Nagaya N, Kyotani S, Uematsu M, Ueno K, Oya H, Nakanishi N, Shirai M, Mori H, Miyatake K, and Kangawa K (2004) Effects of adrenomedullin inhalation on hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension. *Circulation* **109:**351–356.

Nakanishi K, Osada H, Uenoyama M, Kanazawa F, Ohrui N, Masaki Y, Hayashi T, Kanatani Y, Ikeda T, and Kawai T (2004) Expressions of adrenomedullin mRNA and protein in rats with hypobaric hypoxia-induced pulmonary hypertension. *Am J Physiol* **286:**H2159–H2168.

Nakayama M, Takahashi K, Murakami O, Shirato K, and Shibahara S (1999) Induction of adrenomedullin by hypoxia in cultured human coronary artery endothelial cells. *Peptides* **20:**769–772.

Neylon CB, Hoyland J, Mason WT, and Irvine RF (1990) Spatial dynamics of intracellular calcium in agonist-stimulated vascular smooth muscle cells. *Am J Physiol* **259:**C675–C686.

Nishio K, Akai Y, Murao Y, Doi N, Ueda S, Tabuse H, Miyamoto S, Dohi K, Minamino N, Shoji H, et al. (1997) Increased plasma concentrations of adrenomedullin correlate with relaxation of vascular tone in patients with septic shock. *Crit Care Med* **25:**953–957.

Oie E, Vinge LE, Andersen GO, Yndestad A, Krobert KA, Sandberg C, Ahmed MS, Haug T, Levy FO, Skomedal T, et al. (2005) RAMP2 and RAMP3 mRNA levels are increased in failing rat cardiomyocytes and associated with increased responsiveness to adrenomedullin. *J Mol Cell Cardiol* **38:**145–151.

Pfeiffer S, Leopold E, Schmidt K, Brunner F, and Mayer B (1996) Inhibition of nitric oxide synthesis by NG-nitro-L-arginine methyl ester (L-NAME): requirement for bioactivation to the free acid, NG-nitro-L-arginine. *Br J Pharmacol* **118:**1433–1440.

Sato K, Rodman DM, and McMurtry IF (1999) Hypoxia inhibits increased ETB receptor-mediated NO synthesis in hypertensive rat lungs. *Am J Physiol* **276:** L571–L581.

Stangl K, Laule M, Richter C, Stangl V, Koch J, Goktas O, Baumann G, and Dschietzig T (2001) Pulmonary adrenomedullin counteracts deterioration of coronary flow and myocardial performance evoked by pulmonary endothelins in experimental acute respiratory distress syndrome. *Crit Care Med* **29:**1027–1032.

Straub SV, Wagner LE, Bruce JI, and Yule DI (2004) Modulation of cytosolic calcium signaling by protein kinase A-mediated phosphorylation of inositol 1,4,5-trisphosphate receptors. *Biol Res* **37:**593–602.

Takaoka M, Shiragami K, Fujino K, Miki K, Miyake Y, Yasuda M, Wang GF, Hisaki K, Matsumura Y, and Morimoto S (1991) Phosphoramidon-sensitive endothelin converting enzyme in rat lung. *Biochem Int* **25:**697–704.

Address correspondence to: Dr. Thomas Dschietzig, Department of Cardiology and Angiology, University Medical Centre Charité, Campus Mitte, Schumannstr. 20/21, 10117 Berlin, Germany. E-mail: thomas.dschietzig@charite.de