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Flow-Induced Pressure Differentially Regulates Endothelin-1, Urotensin II, Adrenomedullin, and Relaxin in Pulmonary Vascular Endothelium

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We hypothesized that increased pulmonary vascular pressure—one of the characteristics of congestive heart failure—directly regulates pulmonary endothelial vasoconstrictors (endothelin-1, urotensin II) and vasodilators (adrenomedullin, relaxin). To this end, we subjected pulmonary artery endothelial cells in a novel flow-chamber model to different shear stresses (17, 29, and 46 dyn/cm^2) at low and elevated levels of downstream pressure (10 and 30 mm Hg). Application of elevated pressure over 16 h increased gene expression and peptide secretion of endothelin-1 at all shear levels, whereas secretion of adrenomedullin rose via decreased expression of its clearance receptor. In contrast, preprourotensin II mRNA and urotensin II peptide decreased in response to elevated pressure, and relaxin remained unaffected. This is the first study to identify pressure as key regulator of mediator synthesis by pulmonary vascular endothelium. Pressure-induced mediator regulation may represent an early event in the development of secondary pulmonary hypertension.

Key Words: **congestive heart failure; pulmonary hypertension; pulmonary endothelial cells; pressure; shear stress; endothelin-1; urotensin II; adrenomedullin; relaxin.**

Congestive heart failure (CHF) is characterized by complex neurohumoral activation associated with the upregulation of vasoconstricting and salt-retaining mediators (catecholamines, angiotensin II, endothelin-1, and vasopressin) and the compensatory rise of vasodilating and natriuretic hormones (atrial and brain natriuretic peptides as well as adrenomedullin [AM]) (1). Endothelin-1 (ET-1) represents the most potent vasoconstrictor identified until now in CHF (2, 3). We have recently demonstrated (4) that in patients suffering from severe CHF, pulmonary circulation is the major site of net release of ET-1 and, therefore, a contributor to circulating ETs, and that hemodynamic improvement in these patients restores pulmonary balance of ET-1 within hours.

In the present study, we investigated the hypothesis that increased pulmonary vascular pressure—one of the characteristics of severe (decompensated) CHF—directly regulates pulmonary endothelial gene expression of ET-1 and AM. We furthermore compared the response of these well-known mediators of CHF with that of urotensin II (U-II) and relaxin (RLX). U-II is a recently identified vasoactive peptide, which—depending on the anatomical site—may exert both constrictor and dilator effects (5, 6). Its vasoconstrictor potency is about one order of magnitude greater than that of ET-1 (5). The importance of U-II is currently unknown, but the peptide represents a novel candidate mediator for a number of pathophysiological states in view of its vasoactive properties and its abundance in cardiovascular tissues (7). RLX, on the other hand, represents a vasodilating, diuretic, and antifibrotic peptide that originates from the insulin superfamily (8), and that has recently been identified as novel compensatory mediator in human CHF (9).

MATERIALS AND METHODS

Flow chamber. In numerous laminar-flow chambers described in the literature, perfusion pressure and shear stress cannot be adjusted independently owing to the fixed geometry of the devices (10, 11). To work around this difficulty, we developed a chamber model according to Frangos *et al.* (10), but equipped with adjustable height. This rectangular laminar-flow chamber (6.0 cm length, 4.5 cm width) consists of a machine-milled polycarbonate plate, rectangular silastic gaskets of different heights (0.015, 0.025, and 0.040 cm), and the

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TABLE 1

Experimental Protocol—Adjustment of Flow-Related Parameters

Height: h (cm)	Flow: Q (mL/min)	Shear stress: τ (dyn/cm^2)	Pressure drop: Δp (mm Hg)	Reynolds number Re	Entry length: l (cm)
0.015	17.0	*17*	10.3	6	0.004
0.025	79.0	*29*	10.3	29	0.029
0.040	323.0	*46*	10.3	117	0.181

Note. Viscosity was $\eta = 1.02$ mPa s $= 0.0102$ dyn s/cm^2.

glass slide with the attached endothelial cell monolayer. These parts were held together by a vacuum maintained at the periphery of the slide.

The flow chamber was perfused with non-pulsatile flow delivered by a precision roller pump (PD-5201, Heidolph) with attached air reservoir that almost completely blunted pressure oscillation (amplitude <0.2 mm Hg for all conditions described in Table 1). We used a circulating volume of 300 mL cell culture medium permanently gassed with 95% air and 5% carbon dioxide. Shear stress τ (in dyn/cm^2) was adjusted according to the following formulas for a Newtonian fluid (10):

$$\tau = 6\ \eta\ Q/B\ h^2$$

$$\Delta p = 12\ \eta\ L\ Q/B\ h^3$$

(η, viscosity in dyn s/cm^2; Q, laminar flow in cm^3/s; B, chamber width in cm; h, chamber height in cm; Δp, pressure gradient across the chamber in dyn/cm^2; L, chamber length in cm). The flow-induced pressure gradient across the chamber was measured as the difference between inlet and outlet pressure and kept constant for varying values of τ by adopting the following procedure (see Table 1): To achieve a change from τ_1 to τ_2, we adjusted the chamber height h by τ_2/τ_1 and the flow Q by $(\tau_2/\tau_1)^3$ which yields a constant Δp and a linear rise of τ with h.

The Reynolds number of the flow through the chamber is given by:

$$\mathrm{Re} = Q\ \rho/\eta\ B = \tau(h^2\ \rho/6\ \eta^2)(\rho, \text{ density}).$$

The entry length l for plane Poiseuille flow can be estimated as (12):

$$l = 0.04\ h\ \mathrm{Re}.$$

For our maximum Reynolds number of 117, the entry length is approximately 0.18 cm (see Table 1).

Cell culture. Bovine pulmonary artery endothelial cells (PAEC, No. CCL-209, American Type Culture Collection), passage 6, were grown in Minimum Essential Medium (GIBCO) supplemented with 1.5 g/L sodium bicarbonate, 0.11 g/L sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5% FCS in a humidified 5% CO_2 atmosphere. Cells were used at complete confluence for the flow chamber experiments and at subconfluence for other experiments.

Experimental protocol. Each perfusion experiment lasted 16 h. We conducted experiments with three different shear stresses: 46 and 29 dyn/cm^2, both values covering the range reported for mean shear stresses in large arteries *in vivo* (13, 14), and 17 dyn/cm^2. In addition, each shear stress was applied in combination with either normal (10 mm Hg) or elevated downstream pressure (30 mm Hg) adjusted at the chamber outlet. The combination of low shear stress and high downstream pressure served to mimic the *in vivo* situation of decompensated severe CHF with consequent pulmonary congestion, i.e., a decrease in shear stress owing to compromised cardiac output and an increase in pulmonary wedge pressure (4). All experiments were performed in triplicate.

Analysis of gene expression. For total RNA isolation according to the method described by Chomczynski and Sacchi (15), cells were treated with 1.7 mL TRIZOL Reagent (Life Technologies) at 30°C for 5 min. Extraction of RNA was performed by addition of 0.35 mL chloroform, incubation at 30°C for 2 min, and subsequent centrifugation (10,000 rpm, 15 min, 4°C). RNA in the aqueous phase was precipitated by addition of 0.8 mL isopropyl alcohol. After centrifugation (10,000 rpm, 30 min, 4°C) and washing with 1.7 mL 70% ethanol, the pellet obtained was dissolved in diethylpyrocarbonate-treated water.

Total RNA (2 μg) was reverse-transcribed using avian myeloblastosis virus reverse transcriptase and dT_{15} primers according to the manufacturer's instructions (First Strand cDNA Synthesis Kit, Boehringer Mannheim). PCR amplification of single-stranded cDNA was then performed utilizing the following specific primer pairs:

- prepro-ET-1 (upstream: 5′ CTC CAG AGC ACG TTG TTC CGT ATG 3′; downstream: 5′ ACC AGG CAG GAT GCA TTT GCC TC 3′) (Maxim Biotech),
- endothelin-converting enzyme-1 (ECE-1) (upstream: 5′ CTA CAT GAT CTG GAA CCT GGT ACG G 3′; downstream: 5′ GTT CCC ATC CTT GTC GTA CTC TCG 3′) (Maxim Biotech),
- ECE-2 (up: 5′ CAA TTA TCT GAT CTG GAA CCT GGT ACA G 3′; down: 5′ GAT TCC CTT CCT TGT CAT ACT CGC 3′) (Maxim Biotech),
- prepro-U-II (up: 5′ AAC CCA AGA GGA AAT TTG AGA AAG TT 3′; down: 5′ CCA GGT AAC AAT GAA CAG GGT GTA G 3′) (TIB MOLBIOL), prepro-AM (up: 5′ GTG GAA TAA GTG GGC TCT GAG TCG T 3′; down: 5′ CCT GGA AGT TGT TCA TGC TCT GG 3′) (TIB MOLBIOL),
- calcitonin receptor-like receptor (CRLR, coding for the two functional AM receptors [16]) (up: 5′ GGA TGG CTC TGC TGG AAC GAT G 3′; down: 5′ GCA GTG ATG CAA TAG ACA ATC CGT 3′) (TIB MOLBIOL),
- prepro-RLX (5′ primer: 5′ TCT GTT TAC TAC TGA ACC AAT TT 3′; 3′ primer: 5′ AAC AAA TTC TGA CAT CAT ATT TAT G 3′) (TIB MOLBIOL),
- and glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) (up: 5′ TGA AGG TCG GAG TCA ACG GAT TTG GT 3′; down: 5′ CAT GTG GGC CAT GAG GTC CAC CAC 3′) (Clontech).

Southern blot hybridization was conducted for quantitation of the amplified sequences. To this end, PCR products were separated on 2% agarose gels, blotted onto nylon membranes (Hybond N, Amersham) and hybridized (Easy Hyb, Boehringer Mannheim) using radioactively labeled probes specific for prepro-ET-1, ECE-1, ECE-2, prepro-AM, CRLR, and GAPDH (250 bases each) (Maxim Biotech), and labeled oligos specific for prepro-U-II and prepro-RLX (TIB MOLBIOL). Finally, autoradiography was performed, and autoradiographs were quantified by use of the ImageMaster 1D Prime software (Pharmacia Biotech). All data were normalized to GAPDH mRNA expression.

Measurement of peptides in supernatants. We employed commercially available kits for ET-1 (ELISA; detection limit, 0.25 pg/mL), U-II (EIA; detection limit, 3 pg/mL) (both from Immundiagnostik),

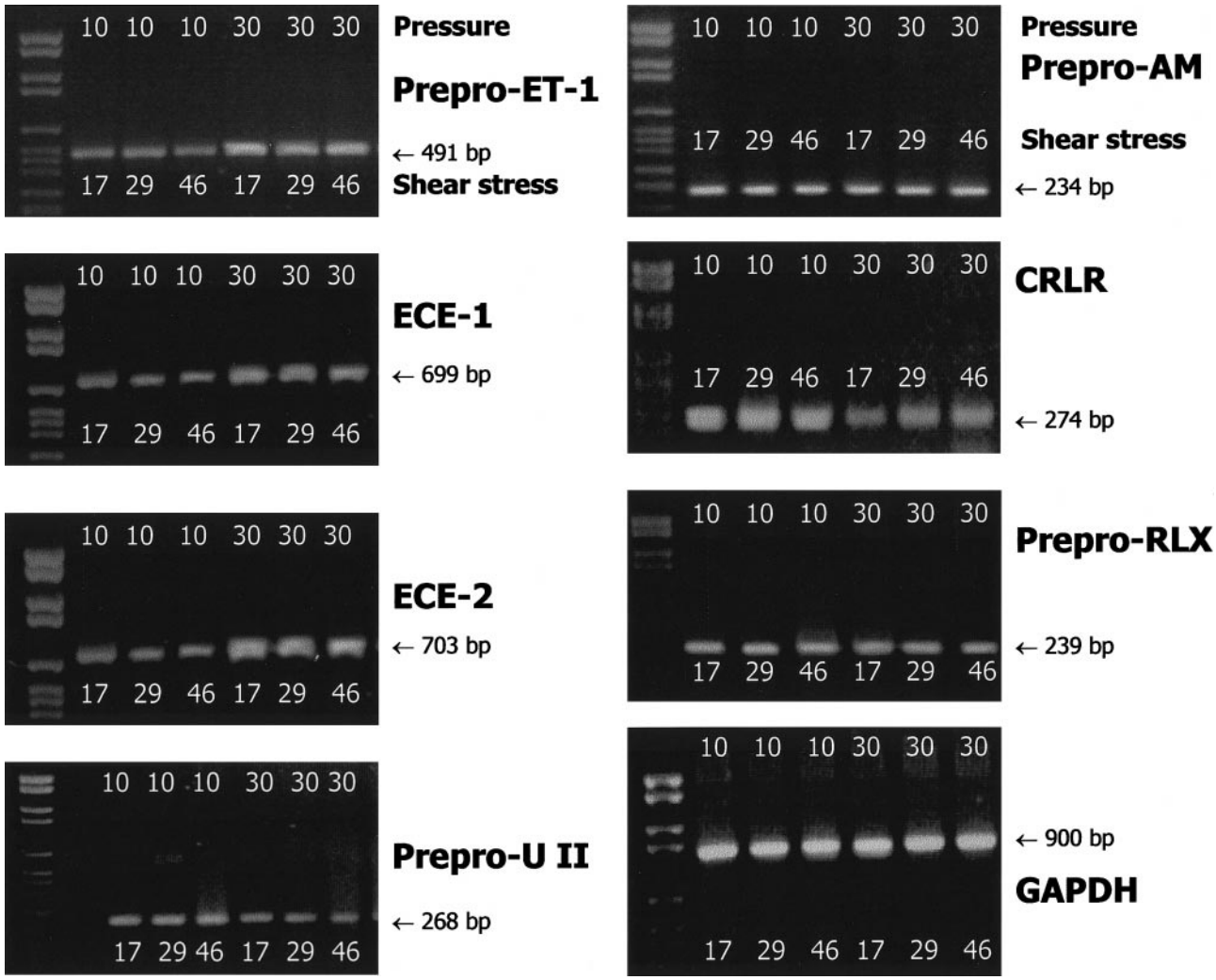

FIG. 1. RT-PCR analysis of the ET-1, U-II, AM, and RLX systems as well as GAPDH gene expression for three different shear stresses (17, 29, and 46 dyn/cm^2) under conditions of normal (10 mm Hg) and elevated downstream pressure (30 mm Hg). Experiments lasted 16 h and were performed in triplicate. The expected size of the amplification products is given for each mRNA determined. Quantitation of the amplified sequences by means of Southern blot hybridization is summarized in Tables 2 and 3.

AM (RIA; detection limit, 3 pg/mL) (Phoenix), and RLX (ELISA; detection limit, 0.40 pg/mL) (Immundiagnostik).

Statistics. Data are presented as mean ± SEM unless otherwise indicated. An error probability of $P < 0.05$ was regarded as significant.

Values were compared using a nonparametric two-way analysis of variance on ranks. After global testing, a multiple-comparison procedure with Bonferroni–Holm adjustment of P was carried out (17).

RESULTS

Gene Expression

Figure 1 shows representative examples of RT-PCR analyses of the ET-1, U-II, AM, and RLX systems, as well as the corresponding GAPDH gene expression. Results of Southern blot quantitation of the amplified sequences are summarized in Tables 2 and 3.

We found that application of elevated pressure significantly increased prepro-ET-1 mRNA (to 166–181% of the respective values for normal pressure), ECE-1 mRNA (to 203–230%), and ECE-2 mRNA (to 287–305%). Prepro-U-II mRNA significantly declined under conditions of elevated pressure, to 49–55% of the low-pressure values. Prepro-AM gene expression did not change, but CRLR mRNA significantly decreased in response to elevated pressure, to 50–63% of the values obtained under normal pressure. Prepro-RLX mRNA remained unaffected.

Secretion of Peptides

Figure 2 shows the secretion by PAEC of ET-1, U-II, AM, and RLX for the different shear stresses under

TABLE 2

Gene Expression of Endothelin-1, Endothelin-Converting Enzymes, and Urotensin II

Shear stress (dyn/cm^2)	Downstream pressure (mm Hg)	Prepro-ET-1 mRNA (%)	ECE-1 mRNA (%)	ECE-2 mRNA (%)	Prepro-U-II mRNA (%)
17	10	107 ± 17	115 ± 20	123 ± 23	94 ± 13
29	*10*	*100 ± 15*	*100 ± 12*	*100 ± 17*	*100 ± 14*
46	10	112 ± 13	93 ± 18	90 ± 14	119 ± 15
17	30	170 ± 21*	230 ± 29*	305 ± 34*	55 ± 10*
29	30	181 ± 23*	215 ± 25*	295 ± 26*	49 ± 12*
46	30	166 ± 16*	203 ± 16*	287 ± 30*	50 ± 15*

Note. Data (normalized to GAPDH expression) are expressed as percentage of the values determined at 29 dyn/cm^2 and 10 mm Hg. *, $P < 0.05$ vs 10 mm Hg.

conditions of low and high downstream pressure. Regarding ET-1, the concentrations yielded in culture supernatants over 16 h ranged between 5.8 ± 0.6 and 6.5 ± 0.8 pg/mL under conditions of low downstream pressure (10 mm Hg). Application of high downstream pressure significantly increased this secretion, to 217, 168, and 148% at 17, 29, and 46 dyn/cm^2, respectively. At a given level of pressure, we did not find a significant dependence on shear, although there was a tendency to higher secretion with decreasing shear stress at a downstream pressure of 30 mm Hg.

In contrast to ET-1, secretion of U-II significantly decreased under elevated compared with low pressure, to 55, 59, and 66% at 17, 29, and 46 dyn/cm^2, respectively. As with ET-1, we observed no significant shear dependence at a given pressure level.

Under conditions of low pressure, supernatant levels of AM amounted to 3.4 ± 0.5, 3.2 ± 0.6, and 2.6 ± 0.3 pg/mL at 17, 29, and 46 dyn/cm^2, respectively. Elevation of downstream pressure resulted in a significant rise of AM concentrations, to 250, 203, and 223% at the different shear levels. RLX levels—measured in the range between 6.9 ± 0.4 and 9.5 ± 0.8 pg/mL—were found to be independent of shear stress and pressure.

TABLE 3

Gene Expression of Adrenomedullin, the CRLR, and Relaxin

Shear stress (dyn/cm^2)	Downstream pressure (mm Hg)	Prepro-AM mRNA (%)	CRLR mRNA (%)	RLX mRNA (%)
17	10	97 ± 12	105 ± 20	120 ± 13
29	*10*	*100 ± 12*	*100 ± 10*	*100 ± 11*
46	10	102 ± 13	98 ± 16	96 ± 14
17	30	89 ± 14	50 ± 9*	102 ± 10
29	30	109 ± 10	61 ± 10*	110 ± 12
46	30	113 ± 13	63 ± 8*	99 ± 16

Note. Data (normalized to GAPDH expression) are expressed as percentage of the values determined at 29 dyn/cm^2 and 10 mm Hg. *, $P < 0.05$ vs. 10 mm Hg.

Role of the AM Receptor

With regard to the increased AM secretion in response to elevated pressure, we found an unchanged prepro-AM gene expression in combination with decreased expression of the CRLR (Fig. 1). Two functional AM receptors are expressed from the CRLR

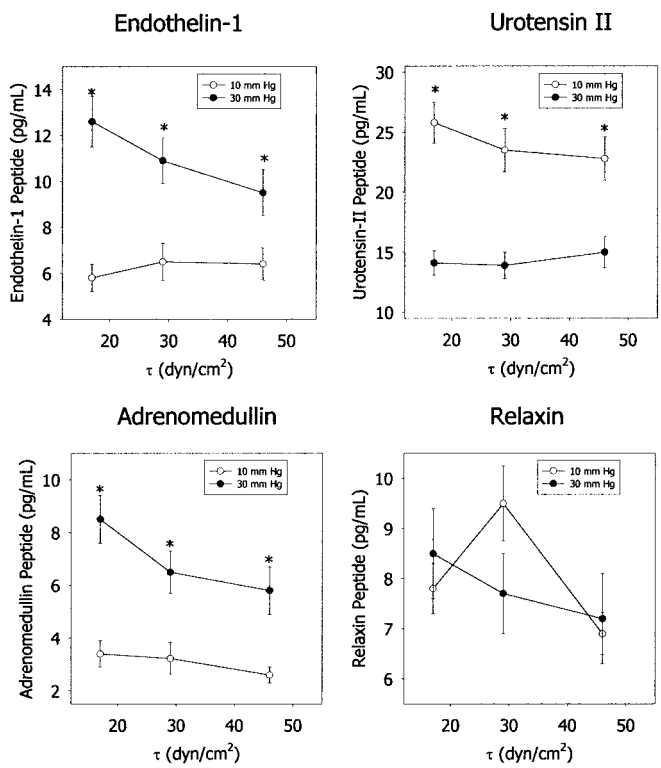

FIG. 2. Secretion by PAEC of ET-1, U-II, AM, and RLX for three different shear stresses (17, 29, and 46 dyn/cm^2) under conditions of normal (10 mm Hg) and elevated downstream pressure (30 mm Hg). Experiments lasted 16 h and were performed in triplicate. Peptides were measured in supernatants using ELISAs for ET-1 (detection limit 0.25 pg/mL) and RLX (detection limit 0.40 pg/mL), an EIA for U-II (detection limit 3 pg/mL), and a RIA for AM (detection limit 3 pg/mL). Data are means ± SEM. *, $P < 0.05$, 30 mm Hg vs 10 mm Hg.

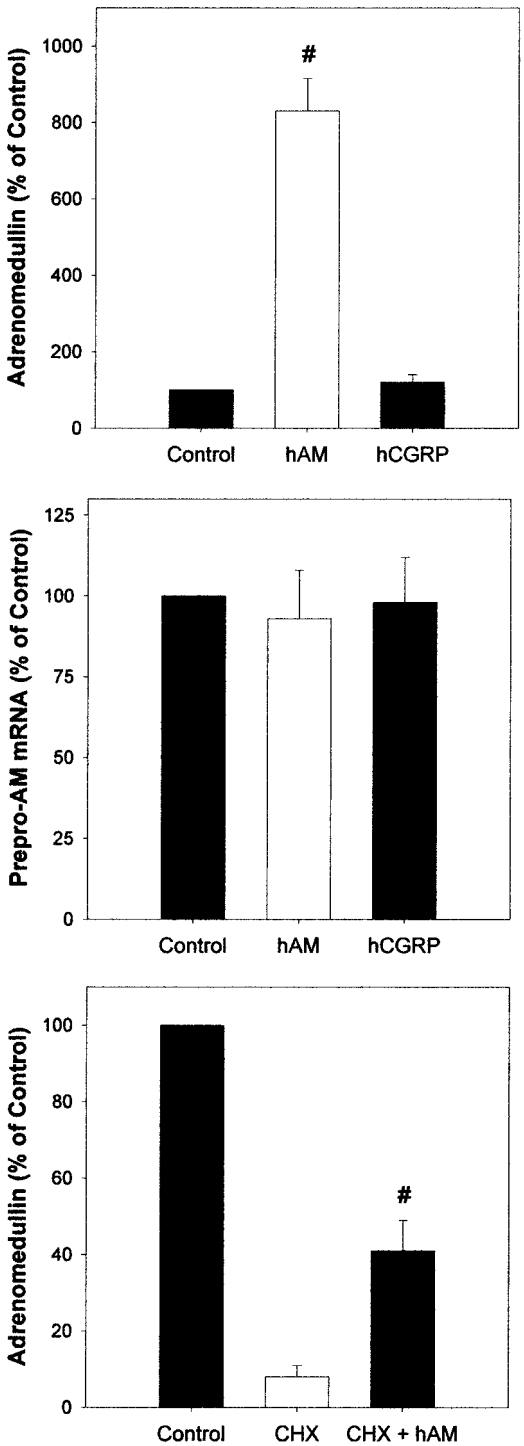

FIG. 3. The functional AM receptor is a clearance receptor in pulmonary vascular endothelium. Top: AM levels in PAEC supernatant after incubation over 4 h with the selective AM receptor antagonist hAM(22-52) (120 nmol/L) or with hCGRP(8–37) (1.2 μmol/L), a selective CGRP1R antagonist (n = 4 for each group). #, $P < 0.05$ vs control. Middle: Corresponding levels of GAPDH-normalized prepro-AM mRNA determined after 3 h. Bottom: Pretreatment of PAEC with the protein synthesis inhibitor cycloheximide (CHX) (0.01 mg/mL). Control indicates 6 h without treatment; CHX indicates no treatment over 2 h, then CHX for 4 h; CHX + hAM indicates no treatment over 2 h, then CHX for 4 h with hAM(22–52) administered 30 min after application of CHX for the rest of the experiment. #, $P < 0.05$ vs CHX.

gene: In the presence of the receptor activity-modifying protein-1 (RAMP1), the CRLR functions as a calcitonin gene-related peptide type-1 receptor (CGRP1R); in co-expression with RAMP2 or RAMP3, CRLR becomes a functional AM receptor (16). Our findings suggested a clearance function of one or both of these receptors.

To test for this hypothesis, we incubated PAEC over 4 h with the selective AM receptor antagonist hAM(22–52) (120 nmol/L) or with hCGRP(8–37) (1.2 μmol/L), a selective CGRP1R antagonist (16) (n = 4 for each group). Figure 3 shows that blockade of the functional AM receptor led to a marked rise of AM peptide concentrations in supernatant, to 830% of control values, although GAPDH-normalized prepro-AM mRNA determined after 3 h remained unchanged. Moreover, the effect of hAM (22–52) was still present after pretreatment of the cells with the protein synthesis inhibitor cycloheximide (CHX) (0.01 mg/mL), which excluded translational mechanisms to evoke this AM peptide elevation. In contrast to hAM (22–52), application of the CGRP1R antagonist had no effect. These results demonstrated that in the PAEC, the functional AM receptor acts as a clearance receptor.

DISCUSSION

The present study demonstrates that in pulmonary vascular endothelium, a differential regulation takes place of the two most potent vasoconstrictors currently known, ET-1 and U-II, in response to the elevation of flow-related pressure: The ET-1 system exhibited uniform upregulation both at the transcriptional (prepro-ET-1, ECE-1, and ECE-2) and at the peptide levels, whereas U-II was found to be downregulated at mRNA and peptide levels.

The implication of ET-1 in the development of pulmonary hypertension secondary to CHF is commonly accepted: ET-1 plasma values correlate closely with the extent of pulmonary hypertension (18), and increased pulmonary ET-1 gene expression has been demonstrated in experimental CHF (19). In addition, ET-1 antagonists have been shown to cause a pronounced decrease in pulmonary pressures and vascular resistance in animal models of CHF (20) and in CHF patients (21). In the light of these findings, pressure-induced ET-1 upregulation in pulmonary vascular endothelium initiated by left heart decompensation (i.e., pulmonary congestion) may constitute one of the early events leading to chronic hypertension with increased vascular resistance in the pulmonary vascular bed.

Owing to its vasoactivity, U-II represents a candidate regulator of vascular tone in physiological and

pathophysiological states (5, 7), but the real importance of this recently identified mediator is still unknown. In our experimental setup, U-II shows a pressure response completely distinct from that of ET-1, i.e., pressure elevation causes its pulmonary endothelial downregulation. If U-II behaved as a vasoconstrictor in pulmonary circulation, this finding would argue against an involvement of the peptide in the pathophysiology of secondary pulmonary hypertension. If U-II, on the other hand, functioned as pulmonary vasodilator as reported by Stirrat *et al.* (6) our results would suggest a pathophysiological contribution of the U-II system. Further studies have to be performed to clarify this item conclusively.

The vasodilatory, antifibrotic and natriuretic peptide AM is upregulated in plasma as well as in myocardium in human and experimental CHF, which renders it a potent counterregulator in CHF (22, 23). Here, we identified a potential mechanism of pressure-induced stimulation of pulmonary vascular AM: The downregulation of its clearance receptor leads to an increase in net synthesis of AM despite the fact that AM gene expression itself remains unchanged. Based on these findings, we may hypothesize a significant contribution of the pulmonary circulation to circulating AM in CHF.

In contrast to AM, RLX—recently described as counterregulatory mediator in CHF (9)—showed neither shear stress nor pressure-dependent regulation in our experimental setting. From these results, pulmonary endothelium does not appear to represent a major source of increased RLX in CHF.

Until now, two studies have investigated the effect of pressure on mediator synthesis in non-pulmonary endothelial cells. First, Hishikawa *et al.* reported that pure pressure up to 160 mm Hg without flow increases ET-1 release from umbilical vein endothelial cells and that this effect is mediated by phospholipase C and protein kinase C pathways (24). Second, Harrison *et al.* found no effect of flow-induced pressure (80–160 mm Hg) on ET-1 and ECE-1 in E. A. hy 926 cells (25). In our study, we attempted to mimic the *in vivo* situation in pulmonary circulation as closely as possible, which included the selection of pulmonary cells as well as the application of flow-related pressures and shear stresses that reflect *in vivo* data (4, 13, 14). This fact may account for the different observations.

The question arises as to which pathways are involved in this mechanotransduction in pulmonary vascular endothelium. Except for the study by Hishikawa *et al.*, most investigators have focused on *shear*-transducing pathways and compared in their studies shear-free conditions with different levels of shear. From these experiments, tyrosine kinase phosphorylation, alteration of the intracellular calcium activity, cytoskeletal changes, and activation of mechanosensitive ion channels have to be considered candidate pathways (26). It remains to be examined whether pressure-related mechanotransduction shares distinct pathways with the signal transduction involved in the shear-dependent regulation of cellular processes.

In conclusion, we have shown that pathophysiologically relevant values of flow-induced pressure represent a shear-independent regulator of ET-1, U-II, and AM in pulmonary vascular endothelium. This pressure-induced mechanotransduction may be involved in the development of secondary pulmonary hypertension.

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