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Parathyroid Hormone-Related Peptide Is Induced by Stimulation of α_{1A}-Adrenoceptors and Improves Resistance against Apoptosis in Coronary Endothelial Cells

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ABSTRACT

Parathyroid hormone-related peptide (PTHrP) is expressed throughout the vascular system, including coronary endothelial cells. The regulation of endothelial PTHrP expression and the role of PTHrP expression in endothelial cells is not clear. This study investigates the question of whether the stimulation of α-adrenergic or angiotensin II receptors increases endothelial expression of PTHrP and whether endogenously expressed PTHrP exerts intracrine effects in coronary endothelial cells. We found that the stimulation of α_{1A}-adrenoceptors, but not that of angiotensin II, increases cellular expression of PTHrP in growing, but not in growth-arrested, coronary endothelial cells. Angiotensin II increases the expression of PTHrP in smooth muscle cells but not in endothelial cells. PTHrP enters the nucleus of endothelial cells at the stadium of confluence, which suggests an intracrine effect of PTHrP. It was further investigated whether the down-regulation of endogenous PTHrP expression by transfection with antisense oligonucleotides alters cell proliferation or apoptosis resistance in growing or nongrowing endothelial cells. Down-regulation of PTHrP did not modify cell proliferation, but it increased the amount of UV-induced apoptosis. An increased expression of PTHrP in cells pretreated with an α-adrenoceptor agonist reduced the basal rate of apoptosis and improved resistance against UV-induced apoptosis. These results indicate a novel intracrine effect of PTHrP in coronary endothelial cells that improves cell survival. In endothelial cells, its expression is regulated by α-adrenoceptor stimulation in a cell-cycle–dependent and cell-type–specific manner.

Parathyroid hormone-related peptide (PTHrP) has structural similarities to parathyroid hormone (PTH). Unlike PTH, PTHrP is widely expressed throughout the body, including the vascular system. Within the vascular system, PTHrP is expressed in smooth muscle and endothelial cells (Thiede et al., 1990; Hongo et al., 1991; Ishikawa et al., 1994; Rian et al., 1994; Schluter et al., 2000). Smooth muscle cells, but not endothelial cells, also express a corresponding PTHrP receptor (Mok et al., 1989). PTHrP induces the relaxation of smooth muscle cells and lowers the blood pressure (Nickols et al., 1989; DiPette et al., 1992; Maeda et al., 1999). In addition, PTHrP either can inhibit smooth muscle cell proliferation by binding to PTHrP receptors or increase proliferation via a novel intracrine effect, which depends on nuclear shuttle of PTHrP (Massfelder et al., 1997). The regulation of PTHrP expression has been evaluated in smooth muscle cells. It was shown that factors leading to vasoconstriction, such as angiotensin II or norepinephrine, increase PTHrP expression in smooth muscle cells (Pirola et al., 1993; Massfelder et al., 1996). A subsequent increased release of PTHrP may be considered as a compensatory mechanism, although direct evidence for such compensation is lacking.

The role of PTHrP expression in endothelial cells is less clear. These cells do not express a corresponding receptor (Rian et al., 1994). Therefore, they might been considered simply as an additional source for PTHrP in the vascular bed. Mechanisms that characterize the regulation of cellular PTHrP expression in endothelial cells have not been evaluated in great detail. Nevertheless, initial studies suggested that PTHrP expression in smooth muscle and endothelial cells might be regulated in a different way (Ishikawa et al., 1994). The regulation of PTHrP expression in nonmalignant cells is often cell cycle-dependent, and the underlying controlling mechanisms seem to be disrupted in carcinoma cell lines displaying pathological overexpression of PTHrP (Okano et al., 1995). Whether the regulation of PTHrP expression in coronary endothelial cells is cell cycle-dependent is not known. Our study focuses on coronary endothelial cells isolated from ventricles of rat hearts, because in the ventri-

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ABBREVIATIONS: PTHrP, parathyroid hormone-related peptide; PTH, parathyroid hormone; TGF, transforming growth factor; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine B isothiocyanate; HOE33258, Hoechst 33258; CEC, chloroethylclonidine; 5-MU, 5-methyl-urapidil; BMY7378, 8-[2-]4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione.

cle, coronary endothelial cells represent the main source for the release of PTHrP. There are several lines of evidence that PTHrP, locally produced by coronary endothelial cells, is part of a specific regulatory mechanism in the heart. First, cardiac effects of PTHrP differ from systemic effects of PTHrP in circulation, because its effect on coronary resistance and cardiac function exceeds that of PTH in the rat heart (Nickols et al., 1989). Second, TGF-β_1, a factor that plays an important role in the transition from hypertrophy to heart failure, down-regulates ventricular expression of PTHrP and causes a loss of ventricular PTHrP expression in spontaneously hypertensive rats that are stroke prone, which develop heart failure (Wenzel et al., 2001).

Irrespective of these questions, it is also unknown whether PTHrP exerts an intracrine effect in coronary endothelial cells, such as smooth muscle cells. Intracrine effects of PTHrP depend on nuclear shuttle of PTHrP (Lam et al., 2000). The mechanism by which PTHrP enters the nucleus is not exactly known, but PTHrP phosphorylation by cdc2, a cell cycle-linked enzyme, seems to be mandatory (Lam et al., 1999). Intracrine effects of PTHrP include the modulation of cell proliferation (Massfelder et al., 1997) or apoptosis (Henderson et al., 1995). In endothelial cells, these are key processes linked to angiogenesis and atherosclerosis.

The aim of our study was to analyze whether the stimulation of α-adrenoceptors or angiotensin II receptors increases the expression of PTHrP in coronary endothelial cells. Experiments were performed on growing (day 1) and nongrowing (day 2) cultures. We also investigated its cellular localization as another important aspect in regard to cellular expression of PTHrP. Finally, we used isolated coronary endothelial cells to study whether PTHrP exerts an intracrine effect that influences growth and/or resistance to apoptosis.

Materials and Methods

Cell Culture. Male Wistar rats (250 to 300 g) were used for the isolation of coronary endothelial cells. These were isolated as described previously (Piper et al., 1990) and grown for 1 or 2 days before use. As reported previously (Noll et al., 1995), the purity of these cultures was >95% endothelial cells, as determined by the uptake of acetylated low-density lipoprotein labeled with 1,1′-diotadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate, contrasted with <5% cells that were positive for α-smooth muscle actin.

Immunoblots. Supernatants from coronary endothelial cells and cell fractions were used as described previously (Schluter et al., 2000). Samples containing 60 μg of protein were loaded onto 12.5% SDS-polyacrylamide gel electrophoresis and blotted onto membranes. Blots were incubated first with an antibody directed against PTHrP (antibody GF08; Calbiochem, Bad Soden, Germany) and then with an anti-mouse Ig antibody coupled to alkaline phosphatase (Schluter et al., 2000). The specificity of the antibody was proofed by blocking the antigen with synthetic PTHrP(1–84). Where indicated, immunoblots were performed with an antibody directed against bcl-2 (BD Biosciences, Heidelberg, Germany) or actin (antibody PC612; Calbiochem). In separate experiments, nuclear extracts were collected as described previously (Schluter et al., 1995).

Immunoblots were densitometrically scanned and analyzed via ImageQuant (Amersham Biosciences Inc., Piscataway, NJ). Individual samples were analyzed in triplicate with an intra-assay variability of 17%. The detection limit was 1.1 ± 0.3 pg synthetic PTHrP(1–84) (n = 3).

[^{3}H]Prazosin-Binding Assay. Binding studies were performed on confluent monolayers of coronary endothelial cells, which were incubated in duplicate with [^{3}H]prazosin in modified Tyrode's solution for 2 h at room temperature. Supernatants were used to determine the unbound [^{3}H]prazosin. The cells were washed with ice-cold PBS and dissolved in 1 ml 0.1 mM NaOH/0.01% (w/v) SDS to quantify bound [^{3}H]prazosin. Radioactivity of the samples was determined by liquid scintillation spectrometry. Nonspecific binding was determined by the addition of excess unlabeled prazosin (10 μM) and subtracted.

Replacement curves were determined for 5-methyl-urapidil (5-MU). In these experiments, cultures were incubated first with various amounts of antagonists and second with 300 pmol/l [^{3}H]prazosin. To determine the amount of α_{1A}-adrenoceptors, cultures were pretreated for 30 min with chloroethylclonidine (30 μM) to alkylate α_{1B}- and α_{1D}-adrenoceptors. Thereafter, cells were washed with PBS and used as described above.

Immunofluorescence Microscopy. Immunofluorescence microscopy on endothelial cells was performed as described previously (Muhs et al., 1997). Endothelial cells, grown on coverslips, were washed, fixed with 100% methanol, and washed again. Cells were permeabilized, covered with 100 μl of anti-PTHrP antibody (diluted 1:100 in PBS), and incubated for 6 h at 37°C. Afterward, the coverslips were washed again, covered with 200 μl of anti-mouse IgG coupled to tetramethylrhodamine B isothiocyanate (TRITC; diluted 1:100 in PBS), and incubated for 6 h at 37°C. The coverslips were finally mounted onto glass slides with a drop of polyvinyl alcohol medium and were dried overnight at room temperature. Cells were further stained with HOE33258 (5 μg/ml) for 15 min for nuclear staining before they were fixed onto glass slides.

Transfection of Endothelial Cells with Phosphorothioated Oligonucleotides. Antisense oligonucleotides (5′-TGAACCAGCCTCCGCAGCAT-3′ and 5′-ATGCTGCGGAGGCTGGTTCA-3′) targeted to a continuous region of PTHrP mRNA in antisense and sense directions, respectively, were synthesized (Invitrogen, Carlsbad, CA) according to the method used by Akino et al. (1996). To increase the stability of these oligonucleotides in cells (exonuclease resistance), they placed four phosphorothioate-modified nucleotides at each end. Coronary endothelial cells were harvested by trypsination, incubated with 10 μg/ml of the oligonucleotides, and exposed in an electroporation apparatus (Gene Pulser II; Bio-Rad, Munich, Germany). Transfection was performed at 400 μF for 5 ms.

Proliferation. The proliferation rate of cultured coronary endothelial cells was determined as described previously (Taimor et al., 1999b). Briefly, cells were seeded on 96-well plates and incubated as indicated. To determine the amount of cell growth, medium was removed, and cells were fixed and stained with 1% (w/v) methylene blue dissolved in Tris-borate/EDTA buffer. Thereafter, cultures were washed five times, and 100 μl of ethanol/HCl (1:1) was added. The amount of protein was read as absorption at 630 nm.

Apoptosis. For induction of apoptosis, cultures were irradiated with 254 nm UV light at 80 J/m^2 as described previously (Taimor et al., 1999a). Then, cultures were incubated for an additional 20 h in the standard incubator. To quantify the amount of apoptosis, the medium was removed and replaced by 1 ml of PBS with the addition of HOE33258 (5 μg/ml) at 37°C for additional 30 min. Cultures were analyzed in a fluorescence microscope, and apoptotic cells were identified by clear nuclear-chromatin condensation.

Statistics. Data are given as means ± S.E. from n different culture preparations. Statistical comparisons between groups were performed by one-way analysis of variance and use of Student-Newman-Keuls test for post hoc analysis. A P value of less than 0.05 was considered to indicate statistical significance. Comparisons between two groups were performed by means of a t-test for independent samples with a critical P value equal to 0.05. Data analysis was computed using SAS software, version 6.11 (SAS Institute Inc., Cary, NC). Experimental data for prazosin-binding studies were analyzed by fitting sigmoid curves to the experimental data using Prism 3.0 (GraphPad Software Inc., San Diego, CA).

Results

Intracellular Localization of PTHrP. We investigated the intracellular localization of PTHrP in coronary endothelial cells by immunofluorescence microscopy. As long as cells were analyzed at day 1, PTHrP was found in the cytoplasm or near the plasma membrane. However, once coronary endothelial cells had reached higher density (near confluence, day 2), a significant amount of PTHrP was also found at the nucleus (Fig. 1A). This staining pattern is indicated in cells that were double-stained with anti-PTHrP and HOE33258, which stains the nuclei (Fig. 1B). We further harvested cells at day 1, 2, and 3, separated nuclear proteins, and analyzed these again via immunoblotting. As illustrated in Fig. 2A, PTHrP was constantly found in coronary endothelial cells, but not before the second day in the nuclei. The molecular weight of nuclear PTHrP was smaller than that in the cytoplasmic fraction. However, in both cases, the apparent molecular weight was higher than that predicted from the amino-acid composition. Using the DIG Glycan detection assay (Roche Diagnostics, Mannheim, Germany), both isoforms of PTHrP, cytoplasmic and nuclear, were positively stained, indicating posttranslational modification by glycosylation (Fig. 2B).

Influence of Phenylephrine on the Expression of PTHrP in Coronary Endothelial Cells. To study whether the stimulation of α-adrenoceptors induces the expression of PTHrP in coronary endothelial cells, we incubated these cells with phenylephrine (10 μM) or angiotensin II (1 μM). Treatment was started either at day 1 after seeding (subconfluent and growing culture) or at day 2 after seeding (near-confluent and nongrowing culture). A representative growth curve of coronary endothelial cells from day 1 to 3 is shown in Fig. 3A. Cells were harvested and analyzed for PTHrP expression the next day. The addition of phenylephrine caused an increase in PTHrP expression by 36 ± 16% when the cells were stimulated at day 1 (Fig. 3B) ($n = 4$ cultures, $P < 0.05$). This increase in PTHrP expression was mainly related to a significant nuclear staining for PTHrP in phenylephrine-treated cells compared with control cultures (Fig. 3C). Phenylephrine reduced PTHrP expression by 19 ± 8% when cells were stimulated at day 2 (Fig. 3B). In comparison to phenylephrine, angiotensin II did not alter the cellular expression of PTHrP at day 1 or 2 as illustrated in Fig. 4. On average, PTHrP expression in angiotensin II-treated cultures was +4 ± 26% and −6 ± 29% versus control values at day 1 or 2, respectively ($n = 4$, P was not significant compared with control values).

In the subsequent set of experiments, cells were generally stimulated with phenylephrine 1 day after seeding to increase the expression of PTHrP. Experiments were repeated in the presence of actinomycin D (5 μM), which inhibits the induction of PTHrP expression on the transcriptional level. Phenylephrine increased cellular PTHrP expression in the presence of actinomycin D (Fig. 5). On average, PTHrP expression was −12 ± 14% ($n = 4$, P value was not significant compared with control) in the presence of actinomycin D alone and +26 ± 3% ($n = 4$, $P < 0.05$ compared with actino-

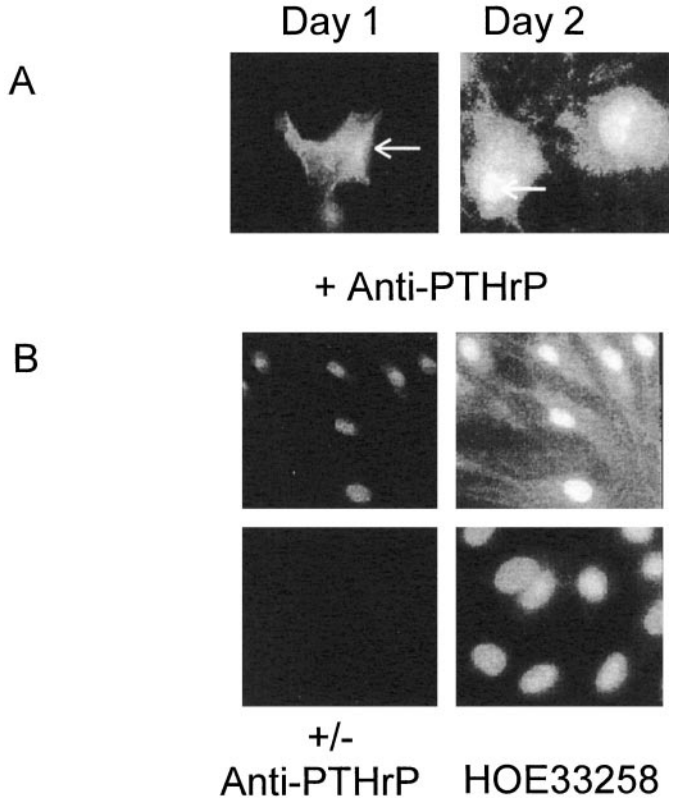

Fig. 1. Nuclear localization of PTHrP in coronary endothelial cells. A, immunofluorescence microscopy of cultures from days 1 and 2. Arrows, predominant staining of PTHrP in the cytoplasm at day 1 and additional staining in the nucleus at day 2. B, immunofluorescence microscopy of cultures from day 2. Left, cells incubated with anti-PTHrP and subsequently IgG-TRITC (top) or with IgG-TRITC only (bottom). Right, the same cells incubated with HOE33258.

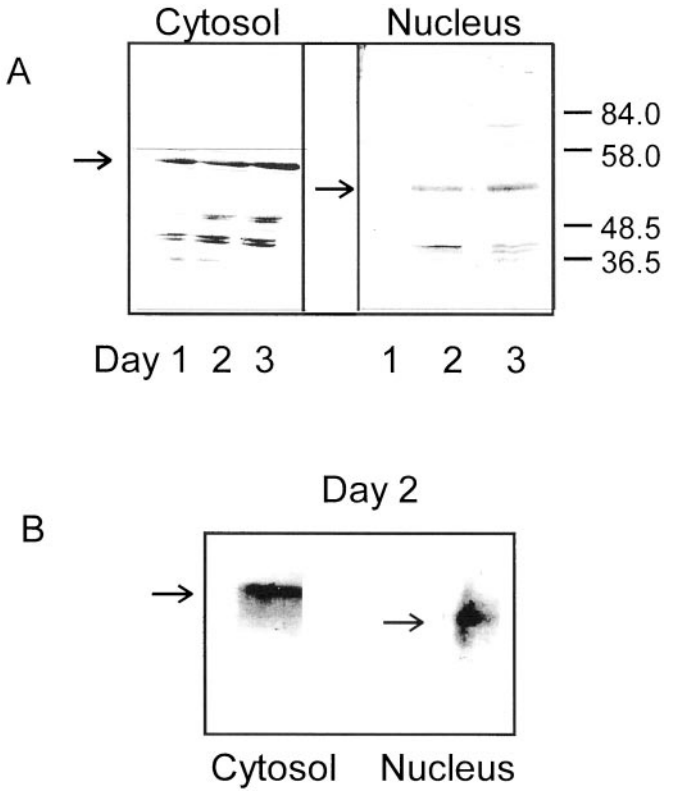

Fig. 2. A, representative immunoblot of cellular PTHrP expression in cultured coronary endothelial cells or purified nuclear extracts from these cultures harvested at days 1, 2, and 3, respectively. B, DIG Glycan detection assay of cytoplasmic and nuclear PTHrP.

mycin D alone) in presence of actinomycin D plus phenylephrine.

In the presence of cycloheximide, which was used to inhibit translational activity, we found a gradual loss of PTHrP with a $t_{1/2}$ of 16.40 ± 3.22 h (n = 4). The increased expression of PTHrP in coronary endothelial cells did not cause a release of the peptide to the supernatant. Neither in untreated control cultures nor in cells incubated with phenylephrine was PTHrP detectable in the supernatant within 1 h. However, ionomycin (10 μM) caused a significant release of PTHrP. Under these conditions, 5.5 ± 0.4% of total PTHrP were found in the supernatant within 1 h.

Receptor Subtype Analysis. In the second set of experiments we identified the α-adrenoceptor subtype that is involved in the induction of PTHrP expression evoked by phenylephrine. Experiments were performed in the absence or presence of yohimbine (10 μM), an α_2-adrenoceptor antagonist, or prazosin (10 μM), an α_1-adrenoceptor antagonist. Prazosin, but not yohimbine, antagonized the induction of PTHrP expression caused by phenylephrine (Fig. 6). Both antagonists did not change basal expression of PTHrP.

Through the use of [^{3}H]prazosin-binding studies, the amount of α_1-adrenoceptors in coronary endothelial cells was found to be 23.8 ± 4.8 fmol/mg of protein (n = 4; K_D = 210 pM). After pretreating the coronary endothelial cells with chloroethylclonidine (CEC, 30 μM), the amount of prazosin-binding decreased to 11.2 ± 1.8 fmol/mg of protein (n = 4; K_D = 191 pM). CEC causes a nonreversible alkylation of α_{1B}- and α_{1D}-adrenoceptors. Thus, approximately 53% of α_1-adrenoceptors present in rat coronary endothelial cells are of the α_{1A} subtype. It is consistent with this finding that 5-MU, an α_{1A}-adrenoceptor antagonist, replaced [^{3}H]prazosin in a biphasic manner, with a high- and low-affinity binding site (pK_B = 8.64 and 6.47, respectively). 5-MU concentrations greater than 100 nM were necessary to displace specifically bound [^{3}H]prazosin completely, whereas in concentrations up to 30 nM, approximately 45% of [^{3}H]prazosin was replaced. The α_{1D}-adrenoceptor antagonist BMY7378 replaced [^{3}H]prazosin in a monophasic manner with a pK_B of 7.55.

From these [^{3}H]prazosin-binding studies, 5-MU, CEC, and BMY7378 were used to investigate the α_1-adrenoceptor subtype involved in the phenylephrine-mediated effect on PTHrP expression. In the presence of 30 nM 5-MU, which represents a concentration 10-fold greater than the pK_B for its high-affinity binding site, phenylephrine failed to increase the cellular expression of PTHrP (Fig. 5). In contrast, 100 nM BMY7378, which is 5-fold greater than its apparent pK_B value, did not influence the expression of PTHrP (Fig. 5). Neither 5-MU nor BMY7378 changed basal expression of PTHrP. In cells pretreated with CEC (30 μM for 30 min), basal PTHrP expression was slightly elevated. However, phenylephrine, was still able to increase the expression of PTHrP in cells pretreated with CEC (Fig. 6).

Functional Relevance of Cellular PTHrP. To study the role of PTHrP in coronary endothelial cells, these cells were transfected with either sense or antisense oligonucleotides directed against PTHrP. Transfection of coronary endothelial cells with antisense oligonucleotides caused a significant loss of endogenous PTHrP expression compared with cells transfected with sense oligonucleotides by 53 ± 12% within 24 h. A representative immunoblot is shown in Fig. 7A.

Basal proliferation of coronary endothelial cells was not

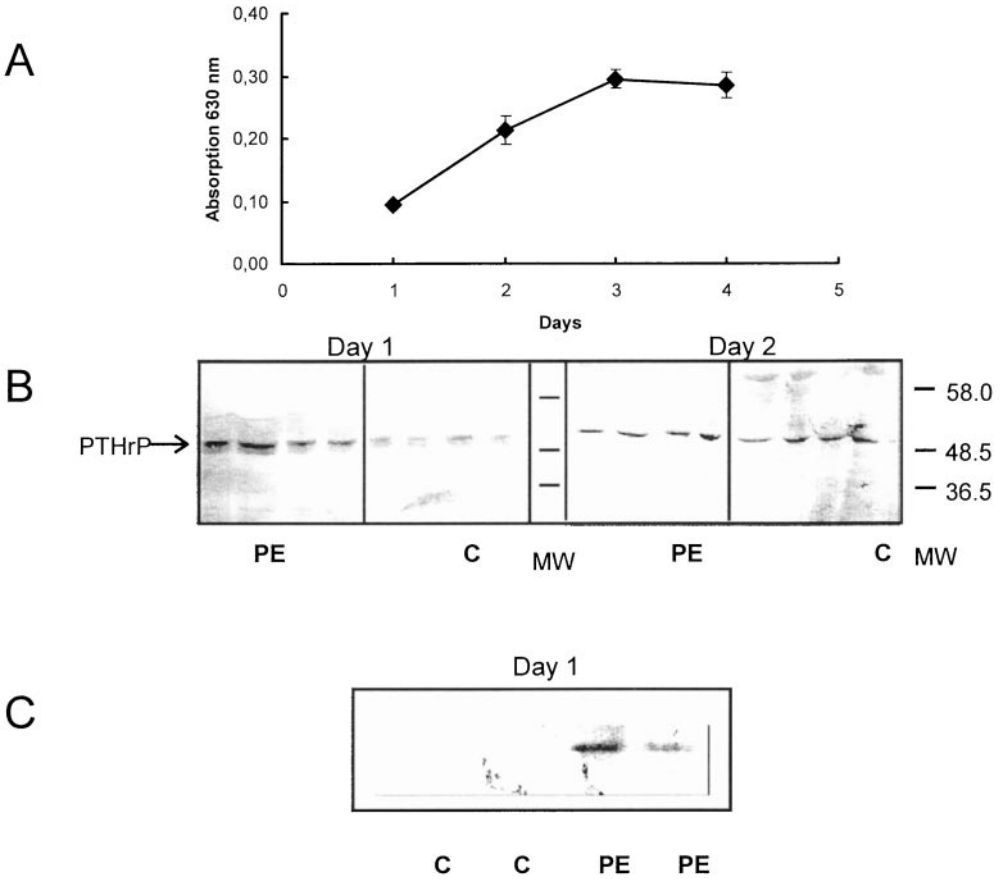

Fig. 3. Influence of phenylephrine on PTHrP expression in isolated coronary endothelial cells stimulated at day 1 or 2. A, growth curve for coronary endothelial cells seeded on culture dishes and grown for 1 to 3 days in the presence of 2% (v/v) fetal calf serum. B, representative immunoblot indicating the expression of PTHrP in these cultures stimulated after 1 or 2 days with phenylephrine (PE, 1 μM) or kept under control conditions (C). Arrow, PTHrP recognized by the antibody; MW, the position of the molecular weight markers. C, representative immunoblot of nuclear PTHrP in samples from day-1 cultures induced by phenylephrine (PE, 1 μM) or kept under nontreated control condition (C).

different between cells with high or low PTHrP expression (Fig. 7B). Cells treated with phenylephrine revealed an increased proliferation. Modification of endogenous PTHrP expression by transfection with either sense or antisense oligonucleotides did not modify this increase (Fig. 7B).

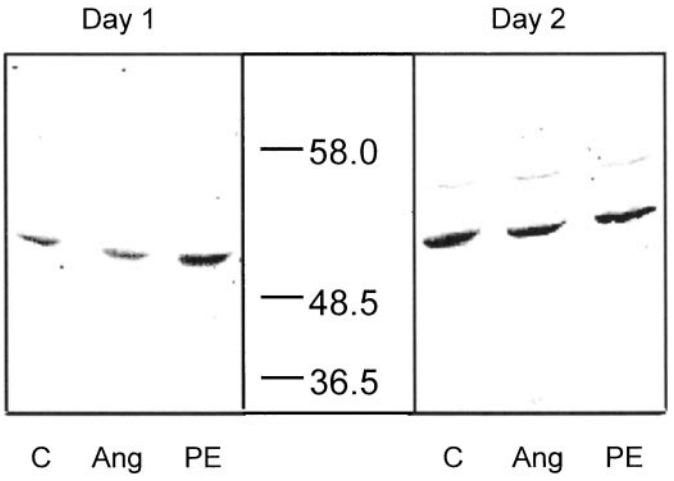

Fig. 4. Influence of angiotensin II on PTHrP expression in isolated coronary endothelial cells stimulated at day 1 or 2. The representative immunoblot indicates expression of PTHrP in coronary endothelial cells stimulated after 1 or 2 days with either angiotensin II (Ang, 1 μM), phenylephrine (PE, 10 μM), or kept under control conditions. MW, the position of the molecular weight markers.

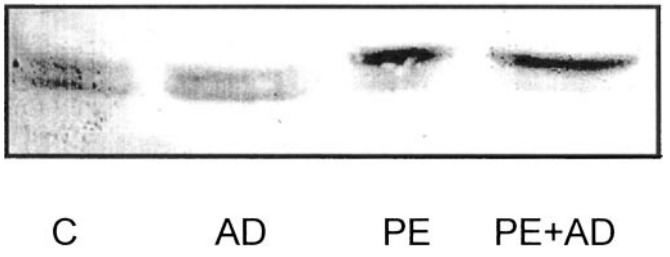

Fig. 5. Influence of actinomycin D on phenylephrine-induced PTHrP expression in isolated coronary endothelial cells. Cells were stimulated at day 1 with phenylephrine (PE, 1 μM) in the presence or absence of actinomycin (AD, 5 μM).

We finally investigated the influence of PTHrP expression on resistance against apoptosis. Cells exposed to UV at day 1 (no nuclear staining for PTHrP) were highly sensitive to apoptosis. Under these conditions, the number of apoptotic cells increased from basal 4.95 ± 1.65% to 8.90 ± 1.65% (n = 3, $P < 0.05$). Pretreatment of these cells with either sense or antisense oligonucleotides did not change UV-induced apoptosis (Fig. 8). Cells exposed to UV at day 2 (with nuclear staining of PTHrP) had significant lower rates of apoptosis (2.23 ± 0.19%). When PTHrP expression was down-regulated by transfection with antisense oligonucleotides, UV-induced apoptosis increased the number of apoptotic cells by 42 ± 8% to 3.17 ± 0.11% (Fig. 8). In contrast, transfection with sense oligonucleotides had no influence on UV-induced apoptosis.

In a final set of experiments, PTHrP expression was induced by phenylephrine. In these cells with significantly higher amounts of nuclear PTHrP staining (see Fig. 3C), basal rate of apoptosis decreased from 4.95 ± 1.95% to 1.32 ± 1.65%, and even under UV illumination, it increased to only 4.62 ± 2.31% and remained lower than in control cells with UV illumination (8.90 ± 1.65%). In a separate experiment, it was found that an increase in total expression of PTHrP in these cells by 43 ± 12% is accompanied by an increased expression of bcl-2 of 72 ± 15%, whereas expression of α-actin, which was used as loading control, was not different (+3 ± 12%) (Fig. 9).

Discussion

Our study investigated the role of α-adrenoceptor stimulation on PTHrP expression in coronary endothelial cells. The main findings of our study are first that phenylephrine induces the expression of PTHrP via stimulation of α_{1A}-adrenoceptors in proliferating coronary endothelial cells and second that increased expression of PTHrP in coronary endothelial cells improves their resistance against apoptosis. Thus, the novel finding of our study is that PTHrP expressed in coronary endothelial cells represents not only a source for

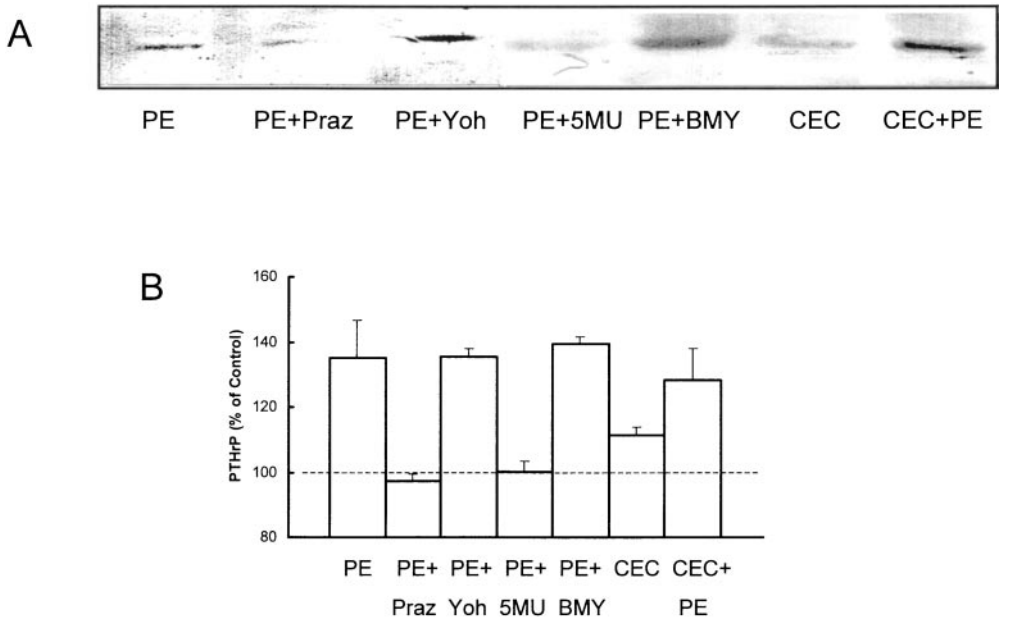

Fig. 6. Influence of α-adrenoceptor antagonists on phenylephrine-induced PTHrP expression in coronary endothelial cells. Cells were stimulated at day 1 with phenylephrine (PE, 10 μM) in the presence of prazosin (Praz, 10 μM), yohimbine (Yoh, 10 μM), 5-MU (30 nM), or BMY7378 (BMY, 100 nM). In case of chloroethylclonidine (CEC, 30 μM), cells were pretreated with the compound for 30 min before exposure to phenylephrine. Antagonists did not modify basal PTHrP expression except for CEC, which is shown in the figure. A, representative immunoblot. B, relative values compared with untreated control cultures. Data shown are means ± S.E. from n = 4 to 6 cell preparations. *, $P < 0.05$ versus control.

ventricular PTHrP which might act as a paracrine factor, but it also exerts an important intracrine effect in coronary endothelial cells.

Neither the regulation of PTHrP expression by adrenoceptor stimulation nor its role in coronary endothelial cells has been investigated before. We show here that α_{1A}-adrenoceptor stimulation increases the expression of PTHrP. Although this is consistent with a more general view suggested before (i.e., that the induction of PTHrP by vasoconstrictor agents represents a short feedback loop through which the local vasorelaxant actions of PTHrP function to oppose pressure activity of angiotensin II and other vasoconstrictor agents), such a general conclusion seems not to be justified. First, we found no strong effect of angiotensin II on PTHrP expression in coronary endothelial cells. Second, increased expression of PTHrP in coronary endothelial cells exposed to phenylephrine was not generally found and was limited to proliferating endothelial cells. In contrast, angiotensin II increases the expression of PTHrP in smooth muscle cells (Pirola et al., 1993; Massfelder et al., 1996). Thus, the expression of PTHrP in endothelial and smooth muscle cells are differentially regulated.

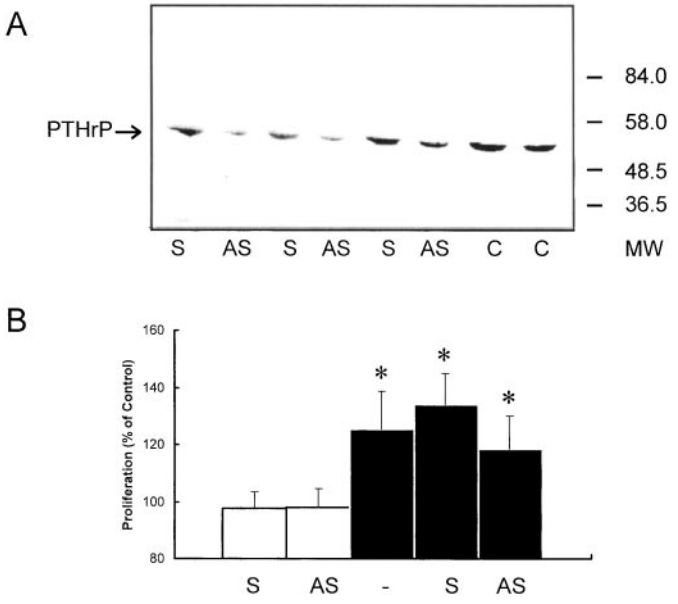

Fig. 7. Influence of transfection with antisense oligonucleotides on endothelial PTHrP expression. A, representative immunoblot indicating the expression of PTHrP in coronary endothelial cells 24 h after seeding and transfection with sense (S) or antisense (AS) oligonucleotides compared nontransfected cells (C). B, influence of the above-mentioned transfection on basal cell proliferation (□) or after induction with phenylephrine (PE, ■). Data given are means ± S.E. from $n = 10$ to 15 culture dishes. *, $P < 0.05$ versus control.

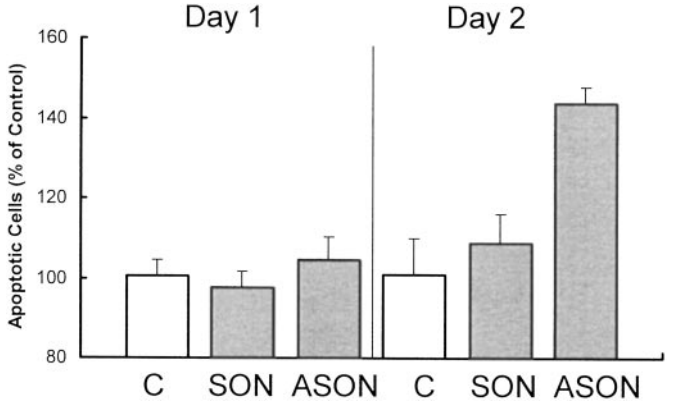

Fig. 8. Influence of transfection with antisense oligonucleotides on endothelial PTHrP apoptosis. Cells were transfected with sense (SON) or antisense (ASON) oligonucleotides and subsequently seeded for 1 or 2 days before they were exposed to UV (254 nm, 80 J/m^2). The number of apoptotic cells was calculated by counting cells with clear chromatin condensation compared with the total number of cells. The 100% value (UV-induced apoptosis) corresponds to 8.90 ± 1.65% and 2.23 ± 0.19% in day 1 or 2 cultures, respectively. Data are means ± S.E. from $n = 3$ preparations. *, $P < 0.05$ versus control.

It has been suggested that endothelial expression of PTHrP might play a role in angiogenesis, because phorbol esters stimulate differentiation and tube formation of cultured endothelial cells and increase their PTHrP expression (Rian et al., 1994). Studies in PTHrP-deficient mice showed an absence of a normal zone of vascular invasion in developing cartilage (Karaplis and Kronenberg, 1996). We have recently shown that TGF-β_1 down-regulates PTHrP in coronary endothelial cells and inhibits endothelial cell proliferation (Taimor et al., 1999b; Wenzel et al., 2001). In contrast, phenylephrine increased the proliferation of coronary endothelial cells and PTHrP expression (as shown in this study). All of these examples seem to suggest a causal role for endogenous PTHrP and proliferation in endothelial cells. However, when PTHrP expression was reduced in coronary endothelial cells by transfection with antisense oligonucleotides, it turned out that endothelial proliferation does not depend on cellular expression of PTHrP. In light of these new findings, it seems not to be justified to conclude that PTHrP expression in endothelial cells directly promotes angiogenesis, as was suggested before.

Another important issue of endothelial cell biology is the resistance of this cell type against apoptosis. The endothelial cell layer is exposed to blood flow and therefore is in direct contact with mechanical and biochemical factors contributing to apoptosis. Apoptotic cell damage of endothelial cells has been shown to contribute to the induction of atherosclerosis first by initiating the process and later on by favoring plaque rupture (Rossig et al., 2001). Nuclear localization of PTHrP has been shown to contribute to resistance against apoptosis in other cell types (Henderson et al., 1995). On endothelial cells, the growth inhibitor TGF-β_1 has been shown to induce endothelial apoptosis and down-regulate bcl-2 an antiapoptotic protein (Tsukada et al., 1995), and we could demonstrate that it decreased endothelial PTHrP expression (Wenzel et al., 2001). This correlation between the loss of PTHrP expression and the susceptibility to apoptosis suggests a causal relationship. Indeed, in our study, we pro-

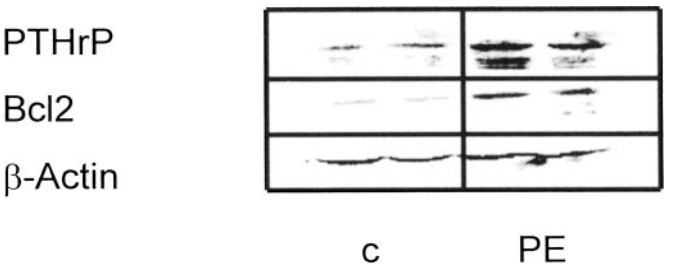

Fig. 9. Representative immunoblot of cellular PTHrP expression (first line), bcl-2 expression (second line), and β-actin expression (third line) in coronary endothelial cells stimulated at day 1 with phenylephrine (PE, 1 μM) or kept under control conditions (c).

vide evidence that nuclear-localized PTHrP contributes to apoptotic resistance of coronary endothelial cells. First, cells cultured at a low density had no nuclear staining for PTHrP but had high basal values of apoptosis in contrast to cells at high density with nuclear staining, which had low rates of basal apoptosis. Second, increasing the total expression of PTHrP in these cells by stimulation of α_{1A}-adrenoceptors, and in particular that of nuclear PTHrP, reduces the basal rate of apoptosis and reduces susceptibility to apoptosis. Third, in parallel, expression of bcl-2 was increased. Fourth, the transfection of coronary endothelial cells with antisense oligonucleotides directed against PTHrP reduces endogenous PTHrP expression and increased susceptibility to apoptosis.

Taken together, these data suggest an important role of PTHrP for endothelial differentiation as well as for α-adrenoceptor stimulation in mediating this effect. Expression and nuclear shuttle of PTHrP are both regulated in a cell-cycle–dependent way in other cell types as well (Okano et al., 1995, Lam et al., 1999). The exact mechanism by which endogenous PTHrP protects coronary endothelial cells against apoptosis remains to be elucidated. The observation that bcl-2 expression increases in parallel with nuclear staining for PTHrP might suggest that the up-regulation of anti-apoptotic genes is part of this mechanism. It is in line with the evidence that TGF-β_1 down-regulates both PTHrP and bcl-2 (Tsukada et al., 1995; Wenzel et al., 2001).

Taking in account the observed intracrine effect of PTHrP in coronary endothelial cells and its modification by α-adrenoceptor stimulation, we analyzed more precisely the receptor subtype by which phenylephrine induces these effects. Because of the different responsiveness to phenylephrine in presence of yohimbine and prazosin, we concluded that phenylephrine increases PTHrP expression by stimulation of α_1-adrenoceptors. Prazosin-binding experiments revealed a binding density of 23.8 pmol/mg of protein, which is in the same order of magnitude as that found on other endothelial preparations (Tabernero et al., 1996). Among these, approximately 53% belong to the α_{1A}-adrenoceptor subtype, because they were insensitive to inactivation by the alkylating agent CEC. It is consistent with this finding that 5-MU at low concentrations (10-fold excess compared with the pK_B of its high-affinity site) displaced approximately 45% of prazosin binding. Similar concentrations, however, were sufficient to inhibit completely the phenylephrine-induced increase in PTHrP expression. BMY7378, a selective α_{1D}-adrenoceptor antagonist, did not interfere with phenylephrine effects at 100 nM (5-fold excess to its apparent pK_B value). The inactivation of either α_{1B}- or α_{1D}-adrenoceptors with chloroethylclonidine caused a small increase in basal expression but did not inhibit the phenylephrine-induced increase. Taken together, these results suggest that the stimulation of α_{1A}-adrenoceptors induces cellular PTHrP expression in coronary endothelial cells. Our finding that phenylephrine increased the cellular content of PTHrP in the presence of actinomycin D suggests that this effect is at least in part regulated on the post-transcriptional level. This is in line with earlier findings on other PTHrP-expressing nonmalignant cell types, including smooth muscle cells (Pirola et al., 1993).

Our data reveal a novel role for PTHrP in vascular biology, because it shows an intracrine effect for PTHrP on coronary endothelial cells. The data found in this cell system support the idea that local expression of PTHrP protects the vascular bed. In addition, coronary endothelial cells can also release PTHrP in a calcium-dependent way, i.e., under hypoxic conditions, and thereby interact with neighboring cells such as cardiomyocytes or smooth muscle cells and improve cardiac function or regulate coronary resistance. However, the increased expression of PTHrP did not lead to a significant release of PTHrP under basal conditions. In other words, although coronary endothelial cells are able to release rapidly significant amounts of PTHrP under energy depletion (Schluter et al., 2000) in a mechanosensitive way (Degenhardt et al., 2002) or by treatment with ionomycin (as shown in this study), they did not release significant amounts of PTHrP under basal conditions. This does not rule out that they might release small amounts of PTHrP. However, even if this is the case, PTHrP released under basal conditions cannot interact in an autocrine way on endothelial cells, because these do not express a PTH/PTHrP receptor (Mok et al., 1989).

In regard to the novel findings of this study, future analysis must clarify important questions regarding the molecular mechanisms involved in these processes, such as determining the post-translational mechanisms by which phenylephrine increases endothelial PTHrP expression, the mechanism by which PTHrP is transported into the nucleus, how these mechanisms are coupled to cell-cycle control, and how PTHrP protects endothelial cells against apoptosis. These are important questions in regard to the specific role for PTHrP in the vascular bed as well as in other PTHrP-expressing cell types.

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