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Expression of Procollagen C-Proteinase Enhancer in Cultured Rat Heart Fibroblasts: Evidence for Co-Regulation With Type I Collagen

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Abstract Procollagen processing by procollagen C-proteinase (PCP) is an important step in collagen deposition. This reaction is stimulated by another glycoprotein, known as PCP enhancer. The objective of this study was to identify factors that regulate the expression of PCP enhancer in cardiac fibroblasts and examine possible correlation with collagen expression. Rat heart fibroblasts were cultured in the presence or absence of three known stimulators of collagen synthesis: ascorbic acid, TGF-β, and aldosterone. The mRNA and protein levels of PCP enhancer and collagen type I were each assessed using Northern and Western blotting, respectively. Expression of PCP was assessed by RT-PCR and its activity in the culture media was determined using radioactive procollagen as the substrate. The levels of PCP enhancer mRNA increased 1.5- to 2-fold in response to ascorbate, TGF-β, or aldosterone. This increase was paralleled by an up to fourfold increase in the level of the pro α1(I) collagen chain transcript and was accompanied by a marked increase in the levels of the respective proteins in the culture media. PCP activity in the culture media was also increased, apparently, without effect on its expression. These results indicate that expression of PCP enhancer in cultured rat heart fibroblasts is coordinated with that of collagen. The observed augmentation of PCP activity may be a consequence of the increase in the levels of PCP enhancer in the culture media. J. Cell. Biochem. 90: 397–407, 2003.

Key words: fibrosis; ascorbic acid; TGF-β; aldosterone; gene expression

The progression of cardiac dysfunction and failure in hypertensive heart disease or following myocardial infarction depends greatly on the degree of cardiac fibrosis. Excessive collagen deposition, the hallmark of fibrosis, increases stiffness, impairs electric signal propagation, and reduces oxygen diffusion in the heart [reviewed by Weber et al., 1992]. Fibrillar collagens types I and III are the main collagenous components in both the normal and fibrotic heart tissue and both are produced by cardiac fibroblasts in culture [Eghbali et al., 1989]. Collagen expression in such cells is increased in response to mechanical load as well as growth factors such as TGF-β or hormones such as angiotensin II and aldosterone [Carver et al., 1991; Eghbali et al., 1991; Brilla et al., 1994].

Fibrillar collagens are secreted into the extracellular matrix as soluble precursors, procollagens, where they are processed to their mature form by specific procollagen C- and N-proteinases that remove the carboxyl- and amino-terminal propeptides, respectively [Prockop and Kivirikko, 1995]. Removal of the propeptides is crucial for

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collagen fibril-formation. The proteases involved are, therefore, key players in collagen deposition. Procollagen C-proteinase (PCP), the enzyme that cleaves the C-terminal propeptides from procollagens types I–III [Hojima et al., 1985; Kessler et al., 1986] as well as those of procollagens V [Kessler et al., 2001; Unsöld et al., 2002], and VII [Rattenholl et al., 2002], is identical to bone morphogenetic protein-1 (BMP-1) [Kessler et al., 1996; Li et al., 1996], a member of the tolloid family of Zn-dependent astacin-like metalloproteases, enzymes involved in developmental processes and tissue morphogenesis. Several PCPs have been identified, of which, BMP-1, mTld (mammalian tolloid) and BMP-1/His, are alternatively spliced products of the *BMP-1* gene [Takahara et al., 1994a]. BMP-1 and mTld can each process a number of additional extracellular matrix proteins that play important roles in matrix deposition, including the precursor of lysyl oxidase, the enzyme responsible for covalent cross-linking and stabilization of collagen fibrils, probiglycan, laminin 5, and chordin, a protein that inhibits the action of the TGF-β-like growth factor BMP-4 [reviewed in Kessler, 2003].

Cleavage of the C-terminal propeptides of procollagen type I by BMP-1/PCP is stimulated by PCP enhancer (PCPE), a glycoprotein that binds to the C-propeptides of procollagens I and III and increases the activity of PCP towards type I procollagen 10–20-fold [Adar et al., 1986; Kessler and Adar, 1989; Ricard-Blum et al., 2002]. PCPE consists of two N-terminal CUB domains that mediate binding to the procollagen C-propeptide [Takahara et al., 1994b] and a C-terminal netrin-like domain that shows homology to tissue inhibitor of matrix metalloproteinases [Banyai and Patthy, 1999] and might act as a weak inhibitor of matrix metalloproteinase-2 [Mott et al., 2000]. PCPE is most abundant in connective tissues rich in collagen I such as bones or tendons. It is also present in the heart, skeletal muscles, and kidney, but is barely detectable in the brain and liver [Kessler et al., 1990]. PCPE is expressed, however, in cirrhotic liver [Ogata et al., 1997], suggesting that it may be an important regulator of excessive collagen deposition in fibrosis. PCPE may also be involved in the control of cell growth and proliferation [Masuda et al., 1998; Kanaki et al., 2000]. Recently, a novel form of PCPE has been described and named PCPE2 [Xu et al., 2000; Steiglitz et al., 2002]. PCPE2 shows 43% homology to PCPE and possesses PCP-enhancing activity.

Despite its emerging role as a protein central to collagen deposition, little is known about the regulation of PCPE expression. TGF-β and ascorbic acid have been shown to increase the levels of PCP but not PCPE in a number of fibrogenic cell lines [Lee et al., 1997]. When applied in the presence of either serum or TGF-β, mechanical load has been shown to exert a similar effect on dermal fibroblasts, again, without effect on the levels of PCPE [Parsons et al., 1999]. Up-regulation of PCPE by TGF-β has been, however, demonstrated in liver stellate cells [Ogata et al., 1997] and in vascular smooth muscle cells [Kanaki et al., 2000] along with an increased expression of fibrillar collagens. While PCPE may play an important role in cardiac fibrosis, its expression by cardiac cells and possible correlation with collagen expression have not been addressed before.

Here we demonstrate that PCPE is expressed in cardiac fibroblasts and show that factors known to stimulate collagen synthesis in the heart, including ascorbate, TGF-β, and aldosterone, co-regulate the expression of PCPE and collagen in these cells.

MATERIALS AND METHODS

Cell Isolation and Culture

Neonatal rat heart fibroblasts were isolated from the ventricles using enzymatic dissociation as previously described [Shalitin et al., 1996]. The dissociated cells were suspended in DMEM:Ham's F12 (1:1), 10% fetal calf serum (FCS) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml; nystatin, 12.5 U/ml), plated onto 100 mm culture dishes (Corning, NY) and incubated for 60 min in a humidified incubator at 5% CO_2 and 37°C. Unsettled cells were then removed while the fibroblasts, which remained attached to the dishes, were grown further in fibroblast growth medium (FGM) consisting of DMEM, 10% FCS, 2 mM glutamine, and antibiotics. Such fibroblast cultures do not display non-fibroblast (e.g., myocytes or vascular cells) markers [Kessler-Icekson et al., 1984], indicating the homogeneity of this cell system. To obtain cultures containing both myocytes and fibroblasts, the pre-plating step was omitted and all of the cells, including myocytes, were allowed to attach and then grown in FGM. Adult heart fibroblasts were isolated from

the left ventricle and septum of male Wistar rats (200 g) as described [Eghbali et al., 1991]. Cells released in five to six digestion cycles were suspended in FGM and incubated for 2 h in 100-mm culture dishes. Unsettled cells were removed while the attached fibroblasts were incubated further in fresh FGM.

Fibroblasts from either neonatal or adult hearts were grown and handled using identical protocols and showed similar morphology and growth properties. Medium was replaced every 3–4 days. When confluent, the cells were sub-cultured at a 1:3 dilution for further proliferation. Confluent fibroblasts from these cultures were transferred into 60 mm dishes (1×10^6 cells/dish) and experiments were initiated 48 h later, at sub-confluency. Unless otherwise stated, the cells were preconditioned for 24 h with serum-free medium (DMEM, 10 mM HEPES pH 7.3, 0.1% BSA, 2.5 μg/ml insulin, 2.5 μg/ml transferrin, 2 mM glutamine, antibiotics) containing 75 μg/ml sodium ascorbate. In some experiments, the insulin, transferrin and BSA were replaced by 1% FCS, without effect on the results. The effectors studied were added in fresh serum-free medium and ascorbate was freshly added daily. Effector concentrations and exposure times were selected based on preliminary dose–response and time-course studies in which conditions required for maximal stimulation of PCPE and collagen mRNAs expression were defined. As a routine, each experiment was performed with fibroblasts from both, neonatal and adult fibroblasts, with essentially the same results. Culture media and supplements were purchased from Biological-Industries (Beit Haemek, Israel) and human recombinant TGF-β was from R&D Systems (Mineapolis, MN).

Unless otherwise stated, chemicals used in the study were from Sigma Chemical (St. Louis, MO).

In Situ Hybridization (ISH)

Cells were seeded on collagen-coated silane microscope slides, and grown as above. At sub-confluency, the cells were rinsed in PBS, fixed for 10 min with 4% formaldehyde in PBS and washed in PBS. The slides were immersed in 0.25% acetic anhydride for 10 min, rinsed in PBS and sterile water, air-dried, and stored at −20°C until processed. ISH was performed using digoxigenin-labeled antisense and sense cRNA probes [Pines et al., 1997]. PCPE probes were transcribed from a plasmid containing a 1.3 kb EcoR1 fragment of the mouse PCPE cDNA [Takahara et al., 1994b], using a commercial kit (Kenzo, Boehringer-Mannheim, Germany). The probes for the rat collagen α1(I) transcript were a courtesy of Dr. Mark Pines (Volkani Institute, Rehovot, Israel).

RNA Analysis

Total RNA was extracted and subjected to Northern blot hybridization as described [Shalitin et al., 1996]. A cDNA probe specific for the rat pro *α1(I)* gene (a kind gift from Dr. David Rowe) [Genovese et al., 1984], and a cDNA probe specific to mouse PCPE [Takahara et al., 1994b] served to detect the respective mRNAs. Hybridization signals were measured either by exposure to Kodak X-Omat AR film and soft-laser densitometry (Biomed Instruments, Fullerton, CA), or by phosphorimaging (Cyclone, Packard, UK). Loading variations were corrected by hybridization to 18S rRNA [Shalitin et al., 1996]. BMP-1 mRNA was assessed by RT-PCR using oligonucleotides 5′-TTGGGGGGTCCGGTTTCTTTTCTGC and 5′-CCCGAGGTCATCACCTCCTCCCAG as the down and upstream primers, respectively [Wozney et al., 1988]. Sequence analysis of the 179 bp amplicon, corresponding to nucleotides 2,040–2,218 in the human *BMP-1* gene, revealed 97% identity to the published human sequence (GI:179499). The RT-PCR results were normalized to GAPDH mRNA [Fort et al., 1985]. Twenty-four and 20 amplification cycles were performed for BMP-1 and GAPDH, respectively, both within their respective linear amplification range. Each reaction solution (20 μl) contained 0.08 μCi [α^{32}P]-dCTP and the amplification products were separated by electrophoresis in 2% agarose gels, cut out, and counted in a scintillation counter.

Immunoblotting Analysis

Culture media were centrifuged (3,000 rpm, 5 min, 4°C) and stored at −20°C. The corresponding cell-layers were rinsed with PBS and scraped into 250 μl of 1% SDS in PBS. Following sonication (20 s, Heat-System-Ultrasonics model W-375), each cell lysate was diluted 1:4 in the same buffer, heated 3 min at 100°C and the protein content was determined [Lowry et al., 1951]. Samples of culture media (30 μl of unconcentrated medium/lane; equivalent to 0.6% of the total volume) and cell-layer extracts

(30–35 μg protein/lane; 12% of the total cell extract fraction) were subjected to SDS–PAGE in 10 or 6% polyacrylamide gels, for detection of PCPE or collagenous species, respectively. Collagenous bands were analyzed after reduction with β-mercaptoethanol. Lanes with purified mouse PCPE or human procollagen preparations were included as standards in all analyses. Proteins were electroblotted to nitrocellulose membranes (Nitroplus, MSI) and detected with either a rabbit serum against a synthetic peptide within the C-telopeptide of the human collagen α1(I) chain (LF-67; a kind gift of Dr. L. Fisher) [Fisher et al., 1995] or an immunoaffinity-purified rabbit antibody against mouse PCPE [Lee et al., 1997]. Reactive bands were visualized with a goat anti-rabbit IgG antibody coupled to horseradish peroxidase and enhanced chemiluminiscence (ECL) and quantified by densitometry. In all samples, a single PCPE-reactive protein with mobility corresponding to that of the 55 kDa mouse PCPE [Kessler and Adar, 1989] was detected. The individual collagenous species were identified by electrophoretic migration as compared to bands in the standard purified human procollagen samples.

PCP Activity

Aliquots of the culture media were dialyzed against 0.05 M Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, pH 7.5 (buffer A) and assayed for PCP activity as described [Kessler and Adar, 1989]. Briefly, [^{3}H]-tryptophan labeled chick embryo procollagen type I (1.2 μg; 18,000 cpm; in 30 μl of buffer A containing 0.1 mg/ml BSA) was added to 170 μl of the dialyzed media samples and the reaction solutions (200 μl) were incubated for 16 to 18 h at 37°C. The reactions were stopped by adding EDTA (final concentration of 10 mM) and the amount of radioactivity in the free C-propeptide was determined after removal of undigested procollagen by selective ethanol precipitation.

Statistical Analysis

In each experiment, values obtained for mRNA, protein and enzyme activity in individual cultures were divided by the mean value derived for the corresponding fractions of the control plates (three to four replicates) in the same experiment. This provided a standard ratio called relative score. The relative scores obtained in this manner for identically treated cultures in several independent experiments were pooled into one group and analyzed statistically using the Student's t-test. $P < 0.05$ was considered statistically significant.

RESULTS

PCPE Is Expressed by Cultured Heart Fibroblasts

Cardiac fibroblasts from either neonatal or adult rat hearts were grown in conditions favorable for their proliferation. The cells displayed typical fibroblast morphology, with a few cells assuming a large stellate shape and some cells being binucleated. ISH analysis revealed PCPE (Fig. 1A,C,D) and pro α1(I) (not shown) transcripts in practically every cell in the culture. The uniform expression of pro α1(I) mRNAs in these cultures confirmed the fibrogenic nature and purity of the cells. In cultures containing cardiac fibroblasts and myocytes, the fibroblasts were readily distinguished from the myocytes by being positive for both PCPE and pro α1(I) mRNAs (Fig. 1E,F). These results indicate that both transcripts are produced exclusively by heart fibroblasts, suggesting that cardiac fibroblasts co-express type I collagen and PCPE.

Ascorbic Acid Stimulates PCPE Expression

Ascorbic acid is essential for hydroxylation of nascent pro α chains, a post-translational modification required for triple-helix formation and efficient secretion of procollagen [Prockop et al., 1976]. Ascorbate may also promote transcription of the procollagen genes [Lyons and Schwartz, 1984; Geesin et al., 1988; Nusgens et al., 2001]. To examine whether ascorbate affects the expression of PCPE, we incubated rat heart fibroblasts with or without ascorbate and measured the levels of PCPE mRNA and protein as compared to those of collagen. A Northern blot shown in Figure 2A demonstrates increased mRNA levels of both PCPE and pro α1(I) in fibroblasts from neonatal rats grown in the presence of ascorbate. The mRNA scores obtained in several such experiments show that on the average, the abundance of the PCPE and collagen mRNAs in fibroblasts incubated with ascorbate is approximately 1.5-fold higher than that seen in the controls (Fig. 2B). Essentially the same results were obtained with cardiac fibroblasts from adult rats (not shown).

The levels of PCPE and those of the collagen/procollagen species in the cell-layer extracts

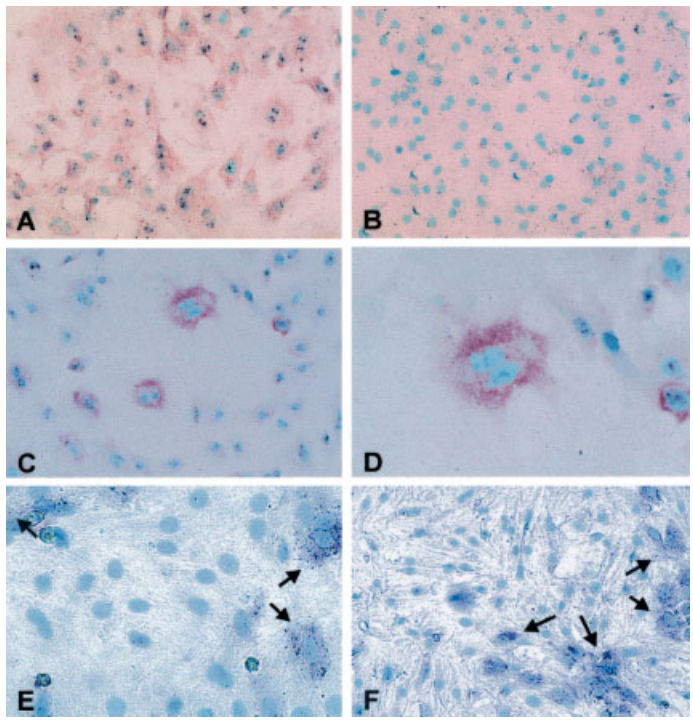

Fig. 1. Cardiac fibroblasts contain procollagen C-proteinase enhancer (PCPE) and type I collagen transcripts. In-situ hybridization of PCPE and type I collagen mRNA. **A**: PCPE transcripts (purple deposit) in neonatal fibroblasts. **B**: Neonatal fibroblasts probed with the sense PCPE probe (negative control). **C**, **D**: PCPE transcripts (purple deposit) in adult heart fibroblasts. **E**, **F**: Cultures containing a mixed population of myocytes and fibroblasts probed for either PCPE (E) or pro α1(I) (F) mRNA. With both probes, purple-blue deposits (arrows) are seen exclusively in the stellate-shaped fibroblasts. No such deposits are detected around the nuclei of the myocytes (light blue; myocyte cell limits are not obvious). Original magnifications: ×50 (A, B, C, E, F) and ×100 (D).

and media from fibroblasts incubated with or without ascorbate were compared by immunoblotting. Figure 2C shows that the amount of PCPE in the cell-layer fractions from cultures incubated without ascorbate was higher than that found in the cell-layer extracts from cultures incubated with ascorbate. By contrast, culture media from cells incubated with ascorbate contained at least twice as much PCPE as compared to those derived from cells grown without the vitamin. Since all cultures contained approximately the same number of cells per plate, and considering the fact that, on the same scale, the medium samples analyzed were 20-fold smaller than the corresponding cell lysates (0.6% as opposed to 12% of the respective fractions; see "Materials and Methods"), it appears that ascorbate increased both production and efficiency of secretion of PCPE. Immunoblots presented in Figure 2D show that the total amount and efficiency of secretion of the various (pro) collagen α1(I) species were both higher in cultures incubated with ascorbate than in those incubated in its absence. Also evident is that in the absence of ascorbate, processing was inefficient so that the band corresponding to the fully processed α1(I) chain was barely detectable and the main reactive band corresponded to the unprocessed pro α1(I) chain. In the presence of ascorbate, however, the band corresponding to the α1(I) chain predominated with little or no unprocessed pro α1(I) remaining. We conclude that in cardiac fibroblasts, stimulation by ascorbic acid of procollagen expression and secretion is paralleled by increased production and secretion of PCPE, an effect that could account for the increased rate of procollagen processing observed in these conditions.

PCPE Expression Is Up-Regulated by TGF-β

TGF-β is a universal stimulator of collagen synthesis [Eghbali et al., 1991]. The addition of TGF-β to cardiac fibroblasts from adult rats induced a 1.5- and 3-fold increase in PCPE and collagen mRNA, respectively (Fig. 3A). The observed increase in the level of PCPE mRNA was associated with a twofold increase in the amount of PCPE in the culture medium

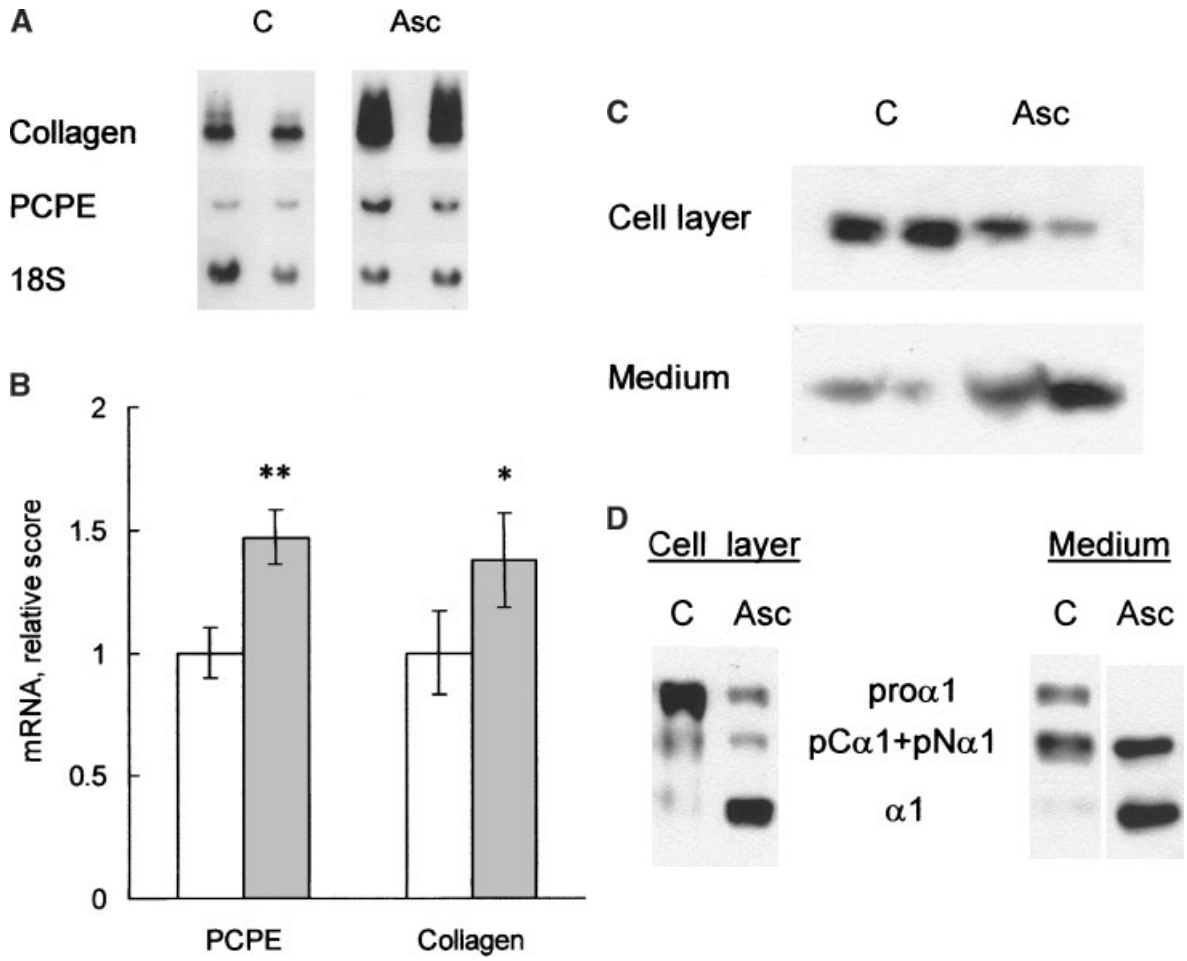

Fig. 2. Up-regulation of PCPE and type I collagen by ascorbic acid. Neonatal heart fibroblasts were incubated in serum-free medium with (Asc) or without (C) ascorbate for 48 h. **A**: A representative Northern blot of RNA from two replicate culture dishes probed for PCPE and pro α1(I) mRNAs, and for 18S rRNA. This blot shows a marked stimulation of PCPE and collagen I gene expression by ascorbate, higher than that of the pooled results shown in (**B**) below, to best exemplify the stimulatory effect of ascorbate. B: Bar graph presentation of the average mRNA scores. Open and solid bars, control and ascorbate containing cultures, respectively. Mean ± SE, $^{*}P < 0.05$, $^{**}P < 0.01$ versus control cultures; n = 5–10. **C**, **D**: Immunoblots of PCPE (duplicate cultures) and pro α1(I)/α1(I)-related antigens, respectively. Pro α1, pCα1, pNα1, and α1 designate intact pro α1(I) chain, partially processed procollagen chains lacking either the N- (pC) or the C- (pN) propeptides, and the fully processed α1(I) chain, respectively.

(Fig. 3B). Together these findings suggest a coordinated up-regulation of the genes for collagen I and PCPE by TGF-β, in these cells, further supporting the stimulatory effect of TGF-β on PCPE expression.

Aldosterone Stimulates PCPE Expression

The mineralocorticoid aldosterone has been shown to promote both accumulation of collagen in the heart [Brilla et al., 1993] and collagen synthesis in cardiac fibroblasts in culture [Brilla et al., 1994]. This urged us to examine whether aldosterone can also stimulate the expression of PCPE in such cells. As shown in Figure 4A, aldosterone induced an approximately 1.5-fold increase in the abundance of PCPE mRNA. This increase was paralleled by a similar rise in the levels of the pro α1(I) chain transcripts and was independent of the age of the animals from which the fibroblasts were derived since cells from neonatal and adult rat hearts displayed the same pattern (Fig. 4A). It also led to an approximately twofold increase in the levels of PCPE protein and those of the various collagenous species in the culture media (Fig. 4B). Addition of the aldosterone receptor antagonist spironolactone abolished the stimulatory effect of the hormone, indicating that the effect of aldosterone was mediated by its receptor in a specific manner.

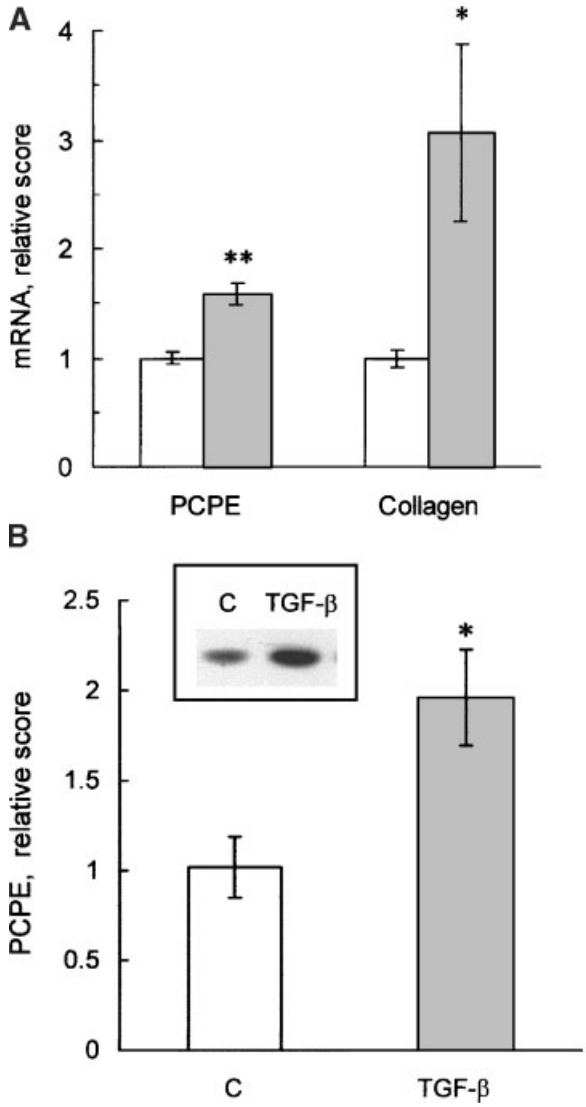

Fig. 3. TGF-β enhances PCPE expression in rat heart fibroblasts. Adult rat heart fibroblasts were preconditioned with ascorbate for 24 h prior to the addition of TGF-β (2.5 ng/ml) and incubated for another 24 h. **A**: Relative scores of PCPE and pro α1(I) mRNAs. Solid and open bars, cells incubated with or without TGF-β, respectively. Mean ± SE; $*P < 0.05$, $**P < 0.01$ versus control, n = 3–9. **B**: Relative scores of PCPE levels in the culture media assessed by immunoblotting and densitometry. Mean ± SE, $*P < 0.05$ versus control (n = 3–4). Inset, a representative immunoblot.

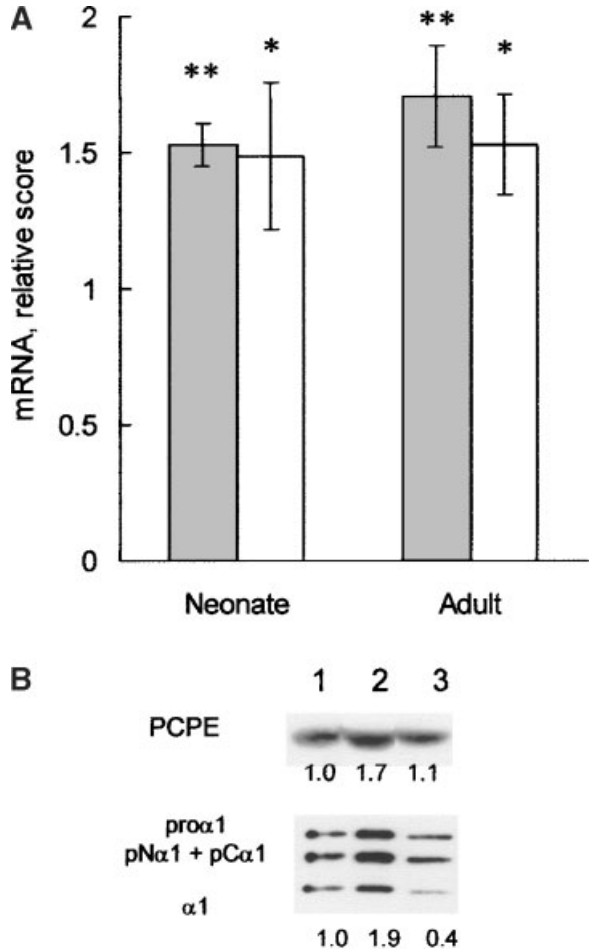

Fig. 4. Aldosterone increases PCPE expression in rat heart fibroblasts. Fibroblasts were preincubated with ascorbate for 24 h, then supplemented with aldosterone (10 nM) and incubated for additional 24 h. **A**: mRNA scores calculated relative to those of the respective controls. Solid and open bars, PCPE and pro α1(I), respectively. Mean ± SE, (n = 3–11), $*P < 0.05$, $**P < 0.01$ versus control. **B**: Representative immunoblots of PCPE and α1(I)-related collagen species in the culture media of adult heart fibroblasts: **lane 1**, control; **lane 2**, aldosterone (10 nM); **lane 3**, aldosterone + spironolactone (10 nM each). Pro α1, pCα1, pNα1, and α1 are as defined in the legend to Figure 2. The relative intensities of the PCPE and α1(I) collagen bands are presented below each panel.

The inhibitory effect of spironolactone was also evident at the mRNA levels (not shown).

PCP Activity

In view of the stimulatory effects of ascorbate, TGF-β, and aldosterone on PCPE expression, it was of interest to examine whether any of these compounds has also affected the expression of PCP. Figure 5 demonstrates that PCP activity in culture media from cells exposed to ascorbate increased twofold as compared to controls (cells grown without ascorbate). An increase in PCP activity was also observed in culture media from cells incubated in the presence of either TGF-β or aldosterone (Fig. 5). In these instances, however, PCP activity was only 20–30% higher than that of the respective controls (cells grown in the presence of ascorbate), a moderate increase that apparently represents incremental stimulatory effects of TGF-β and aldosterone over that of ascorbate alone. In any event, since

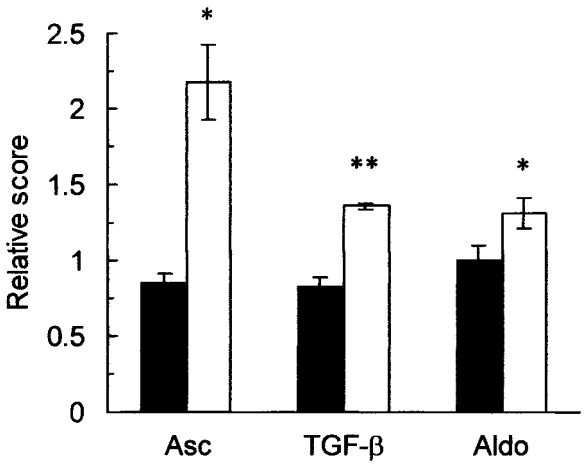

Fig. 5. PCP activity is increased in the presence of ascorbate, TGF-β, or aldosterone. Fibroblasts were incubated with ascorbate (Asc; neonate), ascorbate plus TGF-β (adult), or ascorbate plus aldosterone (Aldo; adult) as detailed in "Materials and Methods" and legends to Figures 2–4. The levels of BMP-1/PCP mRNA (solid bars) and PCP activity (open bars) are presented as ratios calculated relative to those of their respective controls (no additions or, as in the case of TGF-β and Aldo, addition of ascorbate alone). Mean ± SE, n = 3–6, $^{*}P < 0.05$, $^{**}P < 0.01$.

the media samples analyzed contained PCPE, the values obtained represent the combined action of both, the enzyme(s) and its enhancer protein. Because the amount of PCP produced by cardiac fibroblasts is very low, the band corresponding to the enzyme was undetectable by immunoblotting. Using RT-PCR for assessment of the BMP-1 mRNA, we found no increase in the levels of the BMP-1 transcripts following any of the treatments applied. In the absence of evidence for increased expression of BMP-1 in cells treated with ascorbate, TGF-β, or aldosterone, we attribute the increased PCP activity in the respective culture media to the increase in PCPE synthesis rather than that of PCP.

DISCUSSION

Expression of *PCOLCE* (the gene coding for PCPE) has been demonstrated in a variety of collagen producing cells, including several fibroblastic and osteoblastic cell lines [Lee et al., 1997], liver stellate cells [Ogata et al., 1997], human dermal fibroblasts [Parsons et al., 1999], and vascular smooth muscle cells [Kanaki et al., 2000]. A good correlation between type I collagen and PCPE expression has also been demonstrated in terms of their tissue distribution [Kessler et al., 1990]. We have, therefore, anticipated that cardiac fibroblasts, the cells responsible for collagen deposition in the heart, would also express PCPE. Confirming this prediction, we demonstrate here for the first time that cardiac fibroblasts express PCPE. The finding that practically every cell in cultures of cardiac fibroblasts exhibited transcripts of both PCPE and the pro α1(I) collagen chain favors the purity of these cultures and suggests that the genes coding for both proteins are co-expressed. This, along with the observation that myocytes are negative for both, the PCPE and COL1A1 transcripts, suggest that PCPE may serve as a specific marker of heart fibroblasts, additional to collagen type I.

Ascorbic acid controls collagen synthesis at several levels, including post-translational hydroxylation (which is required for triple-helix formation and efficient procollagen secretion), increased transcription and stabilization of the collagen transcripts [Prockop et al., 1976; Lyons and Schwartz, 1984; Geesin et al., 1988; Nusgens et al., 2001]. The correlation between collagen and PCPE expression in cultured heart fibroblasts and the well established roles of ascorbate in collagen biosynthesis and secretion, have led us to examine whether this vitamin is also required for maximal expression of PCPE. Our finding that ascorbic acid increases the level of PCPE mRNA and protein and, apparently, also promotes PCPE secretion, indicates a role for ascorbate in the regulation of PCPE expression which may be related to the stimulation by this vitamin of collagen synthesis, stability, and/or secretion. While these findings contradict those of Lee et al. [1997] who did not detect changes in the levels of PCPE in response to ascorbate in several cell lines, we have found recently that ascorbate stimulates PCPE expression in cultures of mouse NIH-3T3 and Rat2 fibroblasts (Gohar and Kessler, in preparation). In these cells, PCPE expression was also increased when grown in the presence of serum, and addition of ascorbate had little effect beyond that of the serum. Thus, the conflicting results might reflect differences in the nature of the cells studied (primary cultures as opposed to established cell lines), and perhaps also growth conditions.

TGF-β, an important stimulator of collagen deposition is expressed by a variety of cell types, including cardiac myocytes and fibroblasts [Flanders et al., 1995; Lee et al., 1997]. Our finding that, in cardiac fibroblasts, TGF-β in-

creases the levels of PCPE mRNA as well as those of PCPE is consistent with earlier observations, which documented stimulation of PCPE expression by TGF-β in cultured liver stellate cells, vascular smooth muscle cells, and dermal fibroblasts [Ogata et al., 1997; Parsons et al., 1999; Kanaki et al., 2000]. In the latter cell model, application of mechanical load in the presence of TGF-β did not induce further increase in the levels of PCPE [Parsons et al., 1999]. As was also observed by others [Ogata et al., 1997; Kanaki et al., 2000], the increase in PCPE mRNA in cardiac fibroblasts was paralleled by an increase in the steady state levels of the COL1A1 mRNA, suggesting a coordinated regulation of the two genes by TGF-β. Never the less, and as in the case of ascorbate, the effects of TGF-β may differ from one cell type to another since no enhancement of PCPE expression by TGF-β was detected in certain osteogenic cell lines despite the marked stimulation in the expression of collagen I and BMP-1 seen in these cells in response to this cytokine [Lee et al., 1997].

In addition to its endocrine action as a regulator of body electrolytes, aldosterone acts as a stimulator of cardiac fibrosis [Weber et al., 1992]. Evidence that aldosterone is produced in the heart [Silvestre et al., 1998] along with the demonstration that aldosterone receptors are expressed by heart cells [Lombes et al., 1995] suggest that the stimulatory action of aldosterone towards collagen synthesis in the heart may be mediated by paracrine as well as autocrine mechanisms. Consistent with this, aldosterone has been shown to stimulate collagen synthesis in cultured cardiac fibroblasts, an effect that was abolished in the presence of its receptor antagonist spironolactone [Brilla et al., 1994]. The same antagonist can repress cardiac fibrosis in vivo when administered into hypertensive rats [Brilla et al., 1993] and, as was recently found by us [Kessler-Icekson et al., 2002], it can also reduce the expression of both collagen and PCPE in the remodeling heart following acute myocardial infarction in rats. Unlike TGF-β and ascorbate, which are universal stimulators of collagen biosynthesis, stimulation of collagen synthesis by aldosterone appears to be restricted to the cardiovascular system. We show here that simultaneously with the increase in collagen expression, aldosterone stimulates the production of PCPE in cardiac fibroblasts. As in earlier studies, we have established the specificity of the effect by demonstrating that the action of aldosterone is blocked in the presence of the receptor antagonist, spironolactone. While aldosterone enhanced the expression of both PCPE and collagen, it did not increase the proportions of processed collagen species, as was the case with ascorbic acid (compare Fig. 4B with Fig. 2D). This difference is consistent with the limited increase in PCP activity in the respective culture media as compared to controls (Fig. 5) and may be attributed to the fact that in this set of experiments, all of the cells, including controls, were grown in the presence of ascorbate and thus produced saturating amounts of PCPE. It is, therefore, reasonable to assume that further increase in the level of PCPE in response to aldosterone will have little or no effect on the overall rate of procollagen processing in the culture media.

Previous studies [Lee et al., 1997; Parsons et al., 1999] have shown that stimulation of collagen gene expression by TGF-β and ascorbate was accompanied by a marked increase in the levels of PCP transcripts. Contrary to these reports, we did not detect such changes in cardiac fibroblasts in response to these effectors even though both stimulated the expression of COL1A1 in our cells. PCP activity in the culture media of the cardiac fibroblasts was, however, increased in response to both ascorbate and TGF-β, as was also observed with aldosterone (Fig. 5). We were unable to demonstrate changes in the amount of PCP protein in the culture media of any of the treated cells because its level in these media was below detection by immunoblotting. The possibility that the level of PCP was increased in response to the various effectors cannot, however, be ruled out. Furthermore, since we determined the level of the BMP-1 transcript at a single, relatively late, time point, we cannot exclude the possibility that a transient increase in the PCP transcript did occur at an earlier time, which could have led to an increase in the level of the enzyme in the culture media. In the absence of evidence for an increase in PCP expression, an alternative mechanism to account for the increase in the enzyme activity seen in culture media of fibroblasts stimulated by ascorbate, TGF-β, or aldosterone, could be that it resulted from the increase in the levels of PCPE in these media.

Excessive collagen deposition in cardiac fibrosis is a detrimental outcome of chronic

hypertension or myocardial infarction as it may lead to heart failure. The correlation between PCPE and collagen expression seen here in vitro is in accordance with the previously established correlation between the tissue distribution of PCPE and that of collagen type I [Kessler et al., 1990], further supporting the role of PCPE as a regulator of collagen maturation. PCPE may prove as a new target for intervention with excessive collagen deposition in the heart and other fibrotic tissues.

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