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Endogenous Cardiac Vasoactive Factors Modulate Endothelin Production by Cardiac Fibroblasts in Culture

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Endothelin, a potent vasoconstrictor, is produced by cardiac fibroblasts in culture and induces hypertrophy in cardiac myoctes. The purpose of this study was to determine whether vasoactive factors endogenous to the heart affect the production of endothelin by cultured cardiac fibroblasts. Vasoactive factors have been shown to play multiple roles in the adaptation of the heart to chronic overload, affecting both vascular tone and cell growth. Both atrial (ANP) and brain (BNP) natriuretic peptides are endogenous cardiac vasodilators and are produced by cultured myocytes in response to stimulation with endothelin. Treatment of cardiac fibroblasts with these peptides decreased endothelin production. Nitroprusside, an activator of guanylyl cyclase, decreased endothelin production indicating the involvement of cGMP in the response. Carbaprostacyclin, a stable derivative of prostacyclin, another endogenous cardiac vasodilator, also decreased endothelin production by fibroblasts. The combination of BNP and carbaprostacyclin was additive in decreasing endothelin production. In contrast, PGF2α and angiotensin II, both endogenous cardiac vasoconstrictors, increased endothelin production and overcame the inhibition induced by BNP and carbaprostacyclin. In summary, endothelin production by cardiac fibroblasts was decreased by the endogenous cardiac vasodilators ANP, BNP, and prostacyclin and increased by the endogenous vasoconstrictors PGF2α and angiotensin II.

Key Words: Endothelin; cardiac fibroblasts; ANP; BNP; prostacyclin; PGF2α; angiotensin.

Introduction

Overload in the adult heart leads to activation of local and systemic compensatory mechanisms to alleviate the fall in cardiac output. Activation of the renin-angiotensin and sympathetic systems effects vasoconstriction and sodium retention. In chronic overload this is accompanied by structural remodeling in the myocardium including myocyte hypertrophy, fibroblast hyperplasia and modulation of extracellular matrix. There is evidence from studies with cultured cells that vasoactive factors may play multiple roles in these adaptive processes in the heart. Vasoconstrictors, such as endothelin, angiotensin II, and PGF2α, are produced in the myocardium and have been shown to induce hypertrophy of rat neonatal myocytes (Wei et al., 1994; Dostal et al., 1992; Sawa et al., 1992; Nowak et al., 1980; Shubieta et al., 1990; Ito et al., 1991; Suzuki et al., 1991; Sadoshima et al., 1993; Lai et al., 1996). Endothelin and angiotensin II also have growth-promoting effects on smooth muscle cells and induce mitogenesis and increased collagen formation in adult rat cardiac fibroblasts (Campbell-Boswell and Robertson, 1981; Geisterfer et al., 1988; Villareal et al., 1993; Crabos et al., 1994; Wei et al., 1994). In contrast, the nitric oxide-producing vasodilators and ANP, a vasodilator produced primarily in the atria of the heart, have been shown to inhibit hypertrophy and mitogenesis of cultured vascular smooth muscle cells in culture (Garg and Hassid, 1989; Itoh et al., 1990). These observations have led to the hypothesis that endogenous vasoconstrictors can act as growth-promoting factors and endogenous vasodilators as growth-inhibiting factors (Dzau and Gibbons, 1991). It has been proposed that vasoactive substances regulate vascular function in the short-term by controlling vascular tone and in the long-term by influencing vascular structure through remodelling (Dzau and Gibbons, 1991).

The role that locally produced vasoactive substances may play in the development of the pathological hypertrophy of severe, long-lasting overload has not been defined. The clinical efficacy of angiotensin-converting enzyme inhibitors in decreasing myocyte hypertrophy in patients with heart failure implicates the activation of the renin-angiotensin system (SOLVD Investigators, 1991), but factors other than angiotensin II may also be involved. Studies in which cardiac hypertrophy was induced in rats by left ventricular overload with aortic banding showed that treatment with an endothelin receptor blocker blocked myocyte hypertrophy for the first two weeks after treatment (Ito et al., 1994). It has been suggested that endothelin may act as an initiating hypertrophy factor during the early phase of

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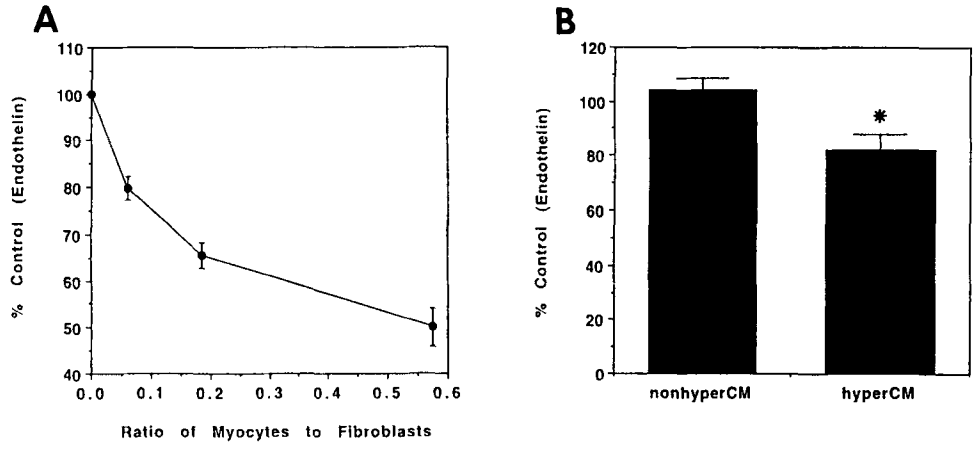

Fig. 1. (A) Effect of addition of rat neonatal cardiac myocytes to cultured cardiac fibroblasts on the production of endothelin by the fibroblasts. Passage one fibroblasts were cultured for 3 d, then freshly isolated myocytes added at varying concentrations. After 48 h of coculture, fresh medium was added and 24 h later the endothelin concentration was measured in the medium by radioimmunassay. Under these conditions the average endothelin concentration with no myocytes present is 40 pmol/L. **(B)** Effect of the addition of medium conditioned by cultured rat neonatal cardiac myocytes on the production of endothelin by cardiac fibroblasts. Myocytes in culture for 24 h were treated with 1 nmol/L LIF and endothelin (hyperCM) or diluent (nonhyperCM) for 48 h, then fresh medium without factors was added for 24 h. This conditioned medium (70% in fresh medium) was added to confluent passage one cardiac fibroblasts and at 24 h the endothelin concentration measured in the medium by radioimmunassay. $*P = 0.01$ vs nonhyperCM.

pressure overload, and that other factors, such as the local renin-angiotensin system, might take over as maintaining factors during the late phase of pressure overload (Ito et al., 1994).

Cardiac fibroblasts are a major constituent of the nonmyocyte component of the myocardium (Nag, 1980). Not only do they contribute to the pathology of heart failure from pressure overload through excess cell division and collagen production, but their numbers and location in the heart make them ideally suited to modulate myocyte growth through the production of paracrine factors. In culture, they produce factors which induce hypertrophy in myocytes (Long et al., 1991). We recently identified these factors as endothelin and leukemia inhibitory factor (LIF) (King et al., 1996). The purpose of this study was to determine whether vasoactive factors produced in the heart can modulate the production of endothelin, and thus hypertrophy activity, by cardiac fibroblasts in culture.

Results

This study was initiated with the observation that, when neonatal rat cardiac myocytes are cocultured with cardiac fibroblasts, endothelin production by the fibroblasts decreases over a 24-h period (Fig. 1A). This occurred even at relatively low concentrations of myocytes. To insure that this was not because of a difference in fibroblast cell growth, all cultures were treated with 0.1 m*M* bromodeoxyuridine (BRDU) in the absence of serum at the time the myocytes were added to the fibroblasts. This treatment suppressed fibroblast proliferation by 100% as measured by cell counts (data not shown), but did not decrease the inhibition of endothelin production. In coculture, cardiac myocytes are induced to hypertrophy by the secretion of LIF and endothelin from cardiac fibroblasts (King et al., 1996). To determine whether the myocytes were producing soluble factors that inhibit fibroblast endothelin production, we tested conditioned medium from endothelin and LIF-treated myocytes cultured without fibroblasts. Medium conditioned by hypertrophied myocytes significantly ($P < 0.01$) decreased production of endothelin by fibroblasts (Fig. 1B). In contrast, medium conditioned by untreated myocytes had no effect.

We next asked what vasoactive factors produced by hypertrophied myocytes could be responsible for this inhibition of endothelin production by the fibroblasts. We chose the atrial, brain, and c-type natriuretic peptides (ANP, BNP, and CNP) to test initially. ANP, BNP, and CNP each decreased endothelin production by cardiac fibroblasts in a dose-dependent manner (Fig. 2A). The order of potency is CNP > BNP = ANP.

Three types of receptors for the natriuretic peptides have been described: NPR-A and NPR-B which are guanylate cyclase-linked, and the unlinked type C (NPR-C), or clearance receptor. At submicromolar concentrations, ANP and BNP bind preferentially to NPR-A and CNP to NPR-B (Koller et al., 1991). In order to determine whether the clearance receptor was involved in the regulation of endothelin production by the fibroblasts, they were treated with two synthetic peptides, cANF and sub133, which bind specifically to NPR-C (Scarborough et al., 1989). At a dose of 500 nmol/L, neither peptide had an effect on endothelin production by the fibroblasts, suggesting that the clearance receptor did not mediate the decrease in endothelin production (Fig. 2B).

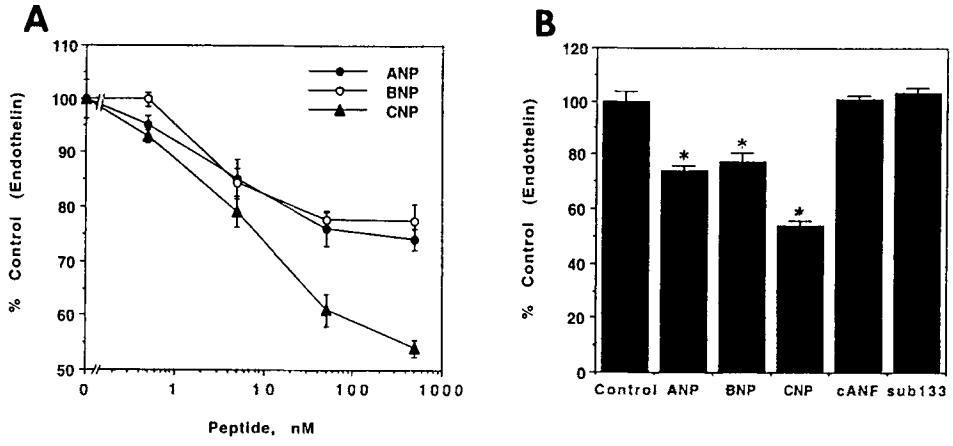

Fig. 2. (A) Effect of the natriuretic peptides on endothelin production by cardiac fibroblasts. Confluent passage one cardiac fibroblasts were treated with varying concentrations of ANP, BNP, and CNP. At 24 h the endothelin concentration in the medium was measured by radioimmunassay. **(B)** Effect of the C-receptor-specific peptides, cANF and sub133, on the production of endothelin by cardiac fibroblasts. Confluent passage one cardiac fibroblasts were treated with natriuretic peptides at 500 nmol/L. At 24 h the concentration of endothelin in the medium was measured by radioimmunassay. The endothelin concentration in the control medium was 154 pmol/L.*$P < 0.001$ vs Control.

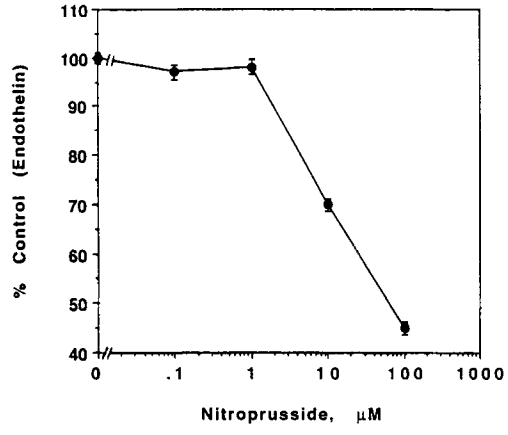

Fig. 3. Effect of sodium nitroprusside on endothelin production by cardiac fibrobasts. Confluent passage one cardiac fibroblasts were treated with varying concentrations of sodium nitroprusside. At 24 h the concentration of endothelin in the medium was measured by radioimmunoassay. The endothelin concentration in the control medium was 153 pmol/L.

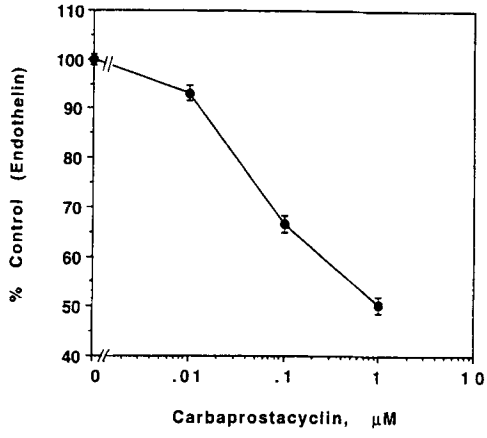

Fig. 4. Effect of carbaprostacyclin, a stable derivative of prostacyclin (PGI_2), on endothelin production by cardiac fibrobasts. Confluent passage one cardiac fibroblasts were treated with varying concentrations of carbaprostacyclin. At 24 h the concentration of endothelin in the medium was measured by radioimmunoassay. The endothelin concentration in the control medium was 149 pmol/L.

To confirm that the regulation of endothelin production was mediated through one or both of the other two receptors, and, therefore, was guanylate cyclase-mediated, the fibroblasts were treated with sodium nitroprusside, a reducing agent that activates soluble guanylate cyclase independent of cell surface receptor occupation. Sodium nitroprusside decreased endothelin production by the fibroblasts in a dose-dependent manner, achieving a 55% reduction of endothelin at 100 μmol/L (Fig. 3). While nitroprusside treatment also decreased cell number over the 24-h treatment period at 100 and 10 μmol/L, the 15% reduction in cell number was of a lessor magnitude than the reduction in endothelin production.

We next examined the effects of prostaglandin I_2 (prostacyclin) on endothelin production by cardiac fibroblasts. Prostacyclin was chosen because, unlike most of the other prostanoids, its activity on the vasculature is purely vasodilatory, it acts directly on vascular muscle cells and it is the most abundant prostaglandin in the heart (De Deckere et al., 1977; Nowak et al., 1980; Wennmalm et al., 1982). Since prostacyclin is unstable with a half-life in vitro of about 3 min, the stable derivative carbaprostacyclin was used in these studies (Cho and Allen, 1978). Treatment with carbaprostacyclin decreased the production of endothelin by cardiac fibroblasts in a dose-dependent manner (Fig. 4).

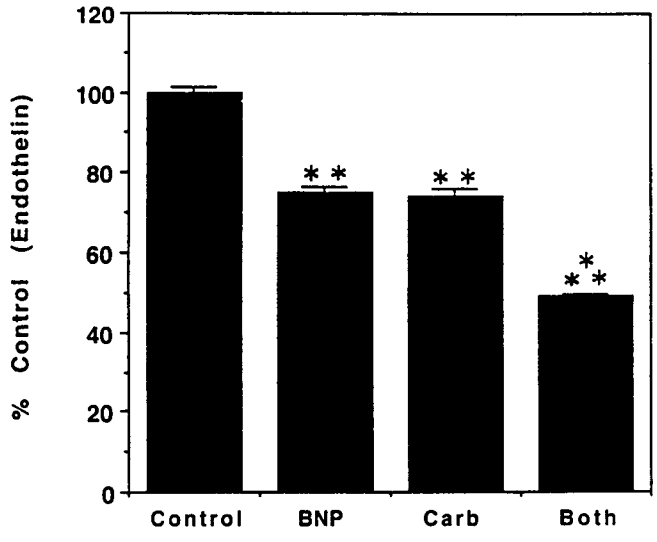

Fig. 5. Effect of the combination of carbaprostacyclin and BNP on endothelin production by cardiac fibrobasts. Confluent passage one cardiac fibroblasts were treated with 1 μmol/L carbaprostacyclin and 50 nmol/L BNP. At 24 h the concentration of endothelin in the medium was measured by radioimmunoassay. The endothelin concentration in the control medium was 170 pmol/L. $*P < .001$ vs. carbaprostacyclin alone and BNP alone. $**P < .001$ vs. Control.

When cardiac fibroblasts were treated with a combination of both carbaprostacyclin at 1 μmol/L and BNP at 50 nmol/L, there was an additive decrease in endothelin production to 50% of control vs about 75% for each separately (Fig. 5).

We next examined whether endogenous cardiac vasoconstrictors, such as PGF2α and angiotensin II, could modulate endothelin production by cardiac fibroblasts. PGF2α increased endothelin production by cardiac fibroblasts in a dose-dependent manner (Fig. 6). Angiotensin II also increased endothelin production by the cardiac fibroblasts in a dose-dependent manner (Fig. 7).

We next asked whether the vasoconstrictors PGF2α and angiotensin II would affect endothelin production by fibroblasts in the presence of the vasodilators BNP and carbaprostacyclin. Figure 8 shows that both vasoconstrictors can overcome the inhibition induced by both vasodilators.

Discussion

Paracrine regulation of cardiac myocyte and fibroblast function may play a significant role in maintaining normal homeostasis in the heart. Failure to maintain this regulation may, in part, lead to the pathology of heart failure. Cardiac fibroblasts in culture produce endothelin and LIF, which are positive regulators of myocyte hypertrophy in vitro (King et al., 1996). This study demonstrates that vasoactive substances endogenous to the heart can modulate the production of endothelin by cardiac fibroblasts in culture. The vasodilators carbaprostacyclin (a stable analog of PGI_2), ANP, BNP, and CNP all decrease endothelin production.

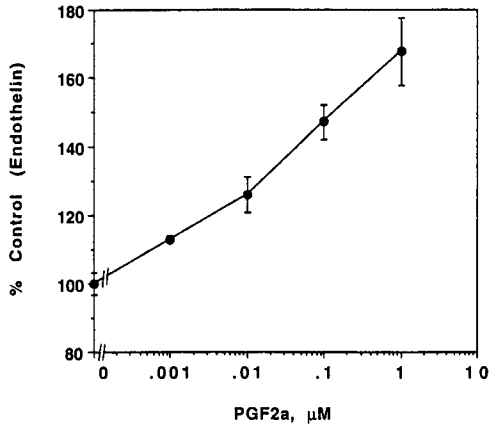

Fig. 6. Effect of PGF2α on endothelin production by cardiac fibrobasts. Confluent passage one cardiac fibroblasts were treated with varying concentrations of PGF2α. At 24 h the concentration of endothelin in the medium was measured by radioimmunoassay. The endothelin concentration in the control medium was 170 pmol/L.

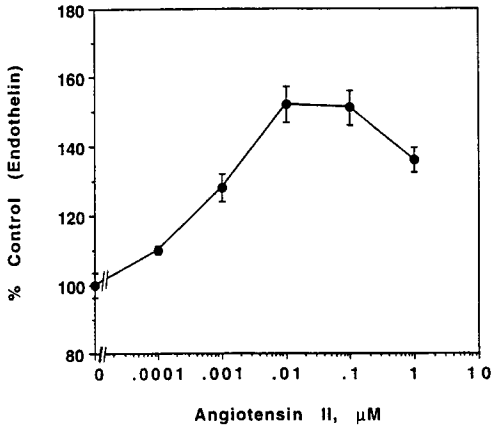

Fig. 7. Effect of angiotensin II on the production of endothelin by cardiac fibroblasts. Confluent passage one cardiac fibroblasts were treated with varying concentrations of angiotensin II. At 24 h, the concentration of endothelin in the medium was measured by radioimmunoassay. The endothelin concentration in the control medium was 177 pmol/L.

The vasoconstrictors prostaglandin F2α and angiotensin II increase endothelin production.

Since myocytes induced to hypertrophy in vitro by endothelin exhibit increased production of ANP and BNP (Horio et al., 1993; Shubieta, et al., 1990), these natriuretic peptides are good candidates for paracrine regulators. In vivo ANP and BNP are produced by myocytes of the adult atria and ventricles, respectively under normal conditions (Ogawa et al., 1991). When the ventricles hypertrophy, ANP is also produced by the myocytes of the ventricles (Arai et al., 1988). Both peptides have vasodilator activity (Sudoh et al., 1988). CNP is synthesized by endothelial cells in culture (Suga et al., 1992), and also has vasorelaxant properties.

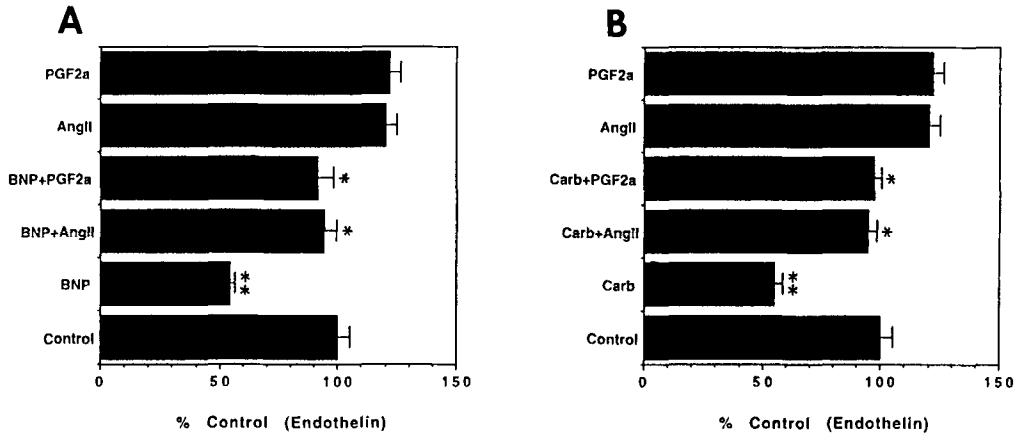

Fig. 8. Effect of PGF2α and angiotensin II on the inhibition of cardiac fibroblast endothelin production by BNP **(A)** and carbaprostacyclin **(B)**. Confluent passage one cardiac fibroblasts were treated with either 50 nmol/L BNP or 100 nmol/L carbaprostacyclin (Carb) followed immediately by the addition of either 100 nmol/L PGF2α or 100 nmol/L angiotensin II (AngII). At 24 h the concentration of endothelin was measured in the medium by immunoassay. The endothelin concentration in the control medium was 217 pmol/L. $*P < 0.01$ vs BNP **(A)** and Carb **(B)**. $**P < 0.001$ vs Control.

There is precedence for the control of endothelin production by the natriuretic peptides. Cultured rat endothelial and mesangial cells respond to ANP and BNP with a decrease in endothelin secretion induced by various agonists (Saijonmaa et al., 1990; Kohno et al., 1991; Emori et al., 1993; Kohno et al., 1993a,b; Kohno et al., 1995). According to the studies referenced above, this response in both cell types probably involves a cGMP-dependent process, although it has been reported that inhibition of the basal production and secretion of endothelin by cultured bovine aortic endothelial cells is mediated through the C receptor, which is not guanylate-cyclase-linked (Hu et al., 1992). Our results with neonatal rat cardiac fibroblasts in culture support the involvement of a cGMP-dependent mechanism in the control of endothelin production by the natriuretic peptides. Sodium nitroprusside treatment, which raises intracellular cGMP levels, decreased endothelin production. In contrast, the C receptor-specific peptides, cANF and sub133, had no activity at a relatively high dose. It has been shown by binding analysis with labeled ANP that cultured rat cardiac fibroblasts have a single class of high-affinity binding sites, 80% of which appear to be of the natriuretic peptide C receptor subtype and the remainder being A and B receptor subtypes (Cao and Gardner, 1995; Lin et al., 1995). The high proportion of clearance receptors may explain the need to use relatively high concentrations of the natriuretic peptides, since these receptors will compete for all three peptides. Although the clearance receptor does not mediate modulation of endothelin production by cultured cardiac fibroblasts, all three natriuretic receptors appear to be involved in the negative regulation of mitogenesis in these cells (Cao and Gardner, 1995). It has recently been reported that angiotensin II and endothelin stimulate thymidine incorporation by cardiac fibroblasts (Fujisaki et al., 1995). These two factors also increased mRNA for preproendothelin-1 indicating that endothelin may be an autocrine factor for these cells. Both effects were inhibited by ANP and BNP through a cGMP-mediated process.

Our results indicate that treatment with the prostacyclin derivative carbaprostacyclin decreases the production of endothelin by cardiac fibroblasts. Prostacyclin has also been shown to inhibit the secretion and production of endothelin from endothelial cells (Prins et al., 1994). The heart possesses the capability to produce several different prostanoids, all metabolites of arachidonic acid (Karmazyn and Dhalla, 1983). Recent studies have shown that cultured adult rat cardiac myocytes release prostacyclin after treatment with a calcium ionophore and arachidonic acid (Linssen et al., 1993).

If prostacyclin is secreted from the myocytes in response to endothelin stimulation, this would indicate at least two components, the natriuretic peptides and prostacyclin, both endogenous cardiac vasodilators, that may participate in a negative feedback loop between hypertrophied myocytes and fibroblasts. We have found that treatment of cardiac fibroblasts with a combination of BNP and carbaprostacyclin results in a greater decrease in endothelin production than treatment with either factor alone. The concept of a cardiac myocyte-endothelium feedback loop has been suggested, and our results extend this concept to include fibroblasts (Kohno et al., 1991; Emori et al., 1993). Thus both cell types which are capable of producing endothelin, endothelial cells and fibroblasts, are susceptible to negative control by myocytes that have been induced to hypertrophy by endothelin.

In contrast with prostacyclin, treatment with PGF2α increased endothelin production by the fibroblasts. PGF2α is present in cardiac tissue (Karmazyn and Dhalla, 1983), and has been shown to have vasoconstricting properties in the rat in vivo and in isolated preparations of bovine and cat coronary artery and rat heart (Csepli and Csapo, 1975;

Kalsner, 1975; Ogletree et al., 1978; Karmazyn et al., 1979). PGF2α has also been shown to increase muscle growth. PGF2α induced hypertrophy of neonatal rat cardiac myocytes in culture, concomitant with an increase in ANP production, and stimulated protein synthesis in isolated rabbit fore-limb and rat soleus muscle (Rodemann and Goldberg, 1982; Smith et al., 1983; Gardner and Schultz, 1990; Lai et al., 1996).

Angiotensin II is a potent direct vasoconstrictor (Baker et al., 1992). It is formed when angiotensin-converting enzyme cleaves angiotensin I. Angiotensin I is released by the action of renin on angiotensinogen. Although this usually occurs in plasma, there is evidence for a local renin-angiotensin system in the heart (Sawa et al., 1992). Functional angiotensin II receptors of the AT_1 subtype have been identified on both neonatal and adult rat cardiac fibroblasts in culture (Villareal et al., 1993; Crabos et al., 1994). Angiotensin II is mitogenic for neonatal rat cardiac fibroblasts and enhances extracellular matrix production in adult cells (Schorb et al., 1993; Crabos et al., 1994). Angiotensin II also induces a growth response in smooth muscle, that is mediated in part by endogenous transforming growth factor β1 (Gibbons et al., 1992). Although angiotensin II has been shown to induce hypertrophy of neonatal rat cardiac myocytes in culture (Sadoshoma and Izumo, 1993), this effect is probably mediated through contaminating cardiac fibroblasts. It has been observed that angiotensin II treatment of cultured cardiac fibroblasts potentiated their production of hypertrophy activity (Kim et al., 1995). This is in agreement with our results that treatment with angiotensin II increased endothelin production and thus hypertrophy activity. There is precedence for the stimulation of endothelin secretion by angiotensin II, in cultured mesangial cells, which is inhibited by ANP and BNP (Kohno et al., 1992).

Combination studies indicated that both PGF2α and angiotensin II can prevent the inhibition induced by BNP and carbaprostacyclin. The net concentration of endothelin produced by the fibroblasts, therefore, represents a balance of stimulatory and inhibitory factors. Further investigation is necessary to determine whether the same phenomenon would occur in endothelial cells. However, the regulation of the secretion of endothelin by a lung Clara cell line, RL-65 (Roberts et al., 1990), is distinctly different than that reported here for cardiac fibroblasts (Roberts, King, and Mather, unpublished data).

In conclusion, the results in this study support the hypothesis that vasoconstrictors can act as growth-promoting factors and vasodilators as growth-inhibiting factors. Whereas the original hypothesis includes both hyperplasia and hypertrophy as growth in the heart and vasculature, our observations are limited to the hypertrophy of cardiac myocytes. In vitro cardiac fibroblasts produce hypertrophic agents, including endothelin. We have shown that by regulating the production of endothelin by fibroblasts, endogenous cardiac vasoactive substances can indirectly affect the hypertrophy of cardiac myocytes. If these processes occur in the adult myocardium in vivo, the production of ANP, BNP, and prostacyclin may be a mechanism for local negative control of the structural adaptive response to overload. Angiotensin II and PGF2α may interfere with this process by stimulating endothelin production and thereby contribute to continuing myocyte hypertrophy and fibroblast proliferation, which are a part of the pathology in heart failure as a result of pressure overload.

Materials and Methods

Materials

The collagenase CLS 2 was purchased from Worthington (Freehold, NJ) and the Percoll from Pharmacia Biotech AB (Uppsala, Sweden). The culture media and supplements were purchased from Gibco-BRL (Grand Island, NY). The aprotinin and angiotensin II were purchased from Sigma (St. Louis, MO). The bromodeoxyuridine was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). The crystallized BSA was purchased from ICN Biomedicals (Aurora, IL). The T25 culture flasks were purchased from Corning Costar (Cambridge, MA) and the Lab Tek chamber slides from Nunc (Naperville, IL). Human/porcine endothelin-1 was purchased from American Peptides (Sunnyvale, CA). Atrial natriuretic peptide, rat (28 amino acids), brain natriuretic peptide, rat, and C-type natriuretic peptide (human, porcine, rat) were purchased from Bachem (Torrance, CA). C-ANF 4-23 (rat) was from Peninsula Laboratories (Belmont, CA). Carbaprostacyclin and PGF2α were purchased from Cayman Chemical (Ann Arbor, MI). The recombinant murine LIF and the sub133 were made at Genentech.

Myocyte Culture

Neonatal rat cardiac ventricular myocytes were cultured as previously described in King et al. (1996). Myocytes were isolated from 1-d-old Sprague-Dawley rats by a series of collagenase digestions followed by Percoll gradient purification (Iwaki et al., 1990). The myocytes, which band at the lower gradient interface, were collected, washed twice and resuspended in F12/DME medium with 15% (v/v) fetal calf serum to inactivate any toxic residuals from the gradient purification. After 10–30 min, the cells were washed once and diluted into serum-free F12/DME supplemented with 10 μg/mL transferrin, 1 μg/mL insulin, 1 μg/mL aprotinin, 2 mmol/L glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin ("assay medium"). Myocytes were plated at a concentration of 68×10^4 cells/mL in T25 flasks (5 mL/flask) precoated with 4% fetal calf serum in F12/DME for 4 h at 37°C.

Fibroblast Culture

The band at the upper gradient interface is enriched for fibroblasts in the procedure described in the Myocyte Culture section. They were collected, washed twice, and resus-

pended in F12/DME with 10% fetal calf serum (30 mL/50 hearts) and plated in T75 flasks (2/50 hearts). After one h at 37°C in 5% CO2, the flasks were gently swirled, unattached cells removed, and the medium replaced. After 4 d in culture, the cells were trypsinized and replated at 4×10^5 cells/mL in T25 flasks (5 mL/flask). At passage one, > 95% of the cells are fibroblasts. Ninety-nine percent of the contaminating myocytes are destroyed by the trypsinization procedure and < 1% of the cells were found to be endothelial cells (by immunocytochemical staining with an antibody to von Willebrand Factor to detect endothelial cells) (King, et al., 1996). After 5 d in culture, the cells were washed twice in serum-free F12/DME and conditioning medium (assay medium with 1 mg/mL BSA) was added with test substances. The conditioned medium was removed after 24 h, centrifuged at 500*g* for 6 min to remove cells and debris and stored at 4°C. Cell counts were done at the end of the conditioning period to determine whether the test substance had affected fibroblast proliferation. Only treatment with nitroprusside was found to change cell number vs the control (*see* Results).

Myocyte-Fibroblast Coculture

Passage one fibroblasts were cultured for 3 d. The cultures were washed twice with F12/DME and then freshly isolated myocytes were added in assay medium with 1 mg/ml BSA and 0.1 mmol/L BRDU to prevent cell division. After 48 h, the medium was removed, the cultures washed twice with F12/DME, and fresh assay medium with BSA and BRDU was added along with test substances. The conditioned medium was removed after 24 h, centrifuged to remove cells and debris and stored at 4°C. At the end of the conditioning period the number of fibroblasts/flask was determined to be approx 2×10^6. The ratio of myocytes to fibroblasts is based on the number of myocytes added to the cocultures. The percentage of myocytes attaching and surviving in the cocultures has not been determined.

Endothelin Measurements

Endothelin concentrations were determined with the Amersham Endothelin 1,2 (high sensitivity) assay system (Amersham, Arlington Heights, IL). This assay detects endothelin-1, -2 and big endothelin-1. All endothelin concentrations in this study should therefore be considered "total immunoreactive endothelin." The approximate average endothelin concentration in medium conditioned by fibroblasts alone for 24 h was 200 pmol/L. The average endothelin concentration in medium conditioned by fibroblasts for 24 h, but in the presence of 0.1 mmol/L BRDU and absence of serum for the previous 3 d was 40 pM.

Statistical Analyses

All experiments were done at least twice in duplicate with duplicate endothelin measurements. Data is expressed as percent of control to normalize endothelin concentrations which varied from experiment to experiment. Graphs are shown with means and standard error bars. Significance was determined by unpaired *t*-test or by one way analysis of variance followed by the Bonferroni multiple comparisons test.

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References

Arai, H., Nakao, Y., Saito, N., Sugawara, T., Yamada, H., Itoh, S., Shiono, M., Mukoyama, H., Ohkubo, H., Nakanishi, S., and Imura, H. (1988). *Circ. Res.* **62,** 926–930.

Baker, K. M., Booz, G. W., and Dostal, D. E. (1992). *Annu. Rev. Physiol.* **54,** 227–241.

Campbell–Boswell, M., and Robertson Jr., A. L. (1981). *Exp. Mol. Pathol.* **35,** 265–276.

Cao, L., and Gardner, D. G. (1995). *Hypertension* **25,** 227–234.

Cho, M. J., and Allen, M. A. (1978). *Prostaglandins* **15,** 943–954.

Crabos, M., Roth, M., Hahn, A. W. A., and Erne, P. (1994). *J. Clin. Invest.* **93,** 2372–2378.

Csepli, J., and Csapo A. I. (1975). *Prostaglandins* **10,** 689–697.

De Deckere, E. A. M., Nugteren, D. H., and Ten Hoor, F. (1977). *Nature* **268,** 160–163.

Dostal, D. E., Rothblum, K. C., Chernin, M. I., Cooper, G. R., and Baker, K. M. (1992). *Am. J. Physiol.* **263,** C838–C850.

Dzau, V. J., and Gibbons, G. H. (1991). *Hypertension* **18(suppl. III),** III115–III121.

Emori, T., Hirata, Y., Imai, T., Eguchi, S., Kanno, K., and Marumo, F. (1993). *Endocrinology* **133,** 2474–2480.

Fujisaki, H., Ito, H., Hirata, Y., Tanaka, M., Hata, M., Lin, M., Adachi, S., Akimoto, F., Marumo, F., and Hiroe, M. (1995). *J. Clin. Invest.* **96,** 1059–1065.

Gardner, D. G., and Schultz, H. D. (1990). *J. Clin. Invest.* **86,** 52–59.

Garg, U. C., and Hassid, A. (1989). *J. Clin. Invest.* **83,** 1774–1777.

Geisterfer, A. A. T., Peach, M. J., and Owens, G. K. (1988). *Circ. Res.* **62,** 749–756.

Gibbons, G. H., Pratt, R. E., and Dzau, V. J. (1992). *J. Clin. Invest.* **90,** 456–461.

Horio, T., Kohno, M., and Takeda, T. (1993). *Metabolism* **42,** 94–96.

Hu, R. M., Levin, E. R., Pedram, A., and Frank, H. J. L. (1992). *J. Biol. Chem.* **267,** 17,384–17,389.

Ito, H., Hirata, Y., Hiroe, M., Tsujino, M., Adachi, S., Takamoto, T., Nitta, M., Taniguchi, K., and Marumo, F. (1991). *Circ. Res.* **69,** 209–215.

Ito, H., Hiroe, M., Hirata, Y., Fujisaki, H., Adachi, S., Akimoto, H., Ohta, Y., and Marumo, F. (1994). *Circulation* **89,** 2198–2203.

Itoh, H., Pratt, R. E., and Dzau, V. J. (1990). *J. Clin. Invest.* **86,** 1690–1697.

Iwaki, K., Sukhatme, V. P., Shubieta, H. E., and Chien, K. R. (1990). *J. Biol. Chem.* **265,** 13,809–13,817.

Kalsner, S. (1975). *Can. J. Physiol. Pharmacol.* **53,** 560–565.

Karmazyn, M., Leung, C. K. H., and Dhalla, N. S. (1979). *Can. J. Physiol. Pharmacol.* **57,** 1275–1282.

Karmazyn, M., and Dhalla, N. S. (1983). *Can. J. Physiol. Pharmacol.* **61,** 1207–1225.

Kim, N. N., Villarreal, F. J., Printz, M. P., Lee, A. A., and Dillman, W. H. (1995). *Am. J. Physiol.* **269,** E426–E437.

King, K. L., Lai, J., Winer,J., Luis,E., Yen, R., Hooley, J., Williams, M., and Mather, J. P. (1996). *Endocrine*, **5,** this issue.

Kohno, M., Yasunari, K., Yokokawa, K., Murakawa, K., Horio, T., and Takeda, T. (1991). *J. Clin. Invest.* **87,** 1999–2004.

Kohno, M., Horio, T., Ikeda, M., Yokokawa, K., Fukui, T., Yasunari, K., Kurihara, N., and Takeda, T. (1992). *Kidney Internat.* **42,** 860–866.

Kohno, M., Horio, T., Ikeda, M., Yokokawa, K., Fukui, T., Yasunari, K., Murakawa, K., Kurihara, N., and Takeda, T. (1993). *Am. J. Physiol.* **264,** F678–F683.

Kohno, M., Ikeda, M., Johchi, M., Horio, T., Yasunari, K., Kurihara, N., and Takeda, T. (1993). *Am. J. Physiol.* **265,** E673–E679.

Kohno, M., Yokohawa, K., Mandal, A. K., Horio, T., Yasunari, K., and Takeda, T. (1995). *Metabolism* **44,** 404–409.

Koller, K. J., Lowe, D. G., Bennett, G. L., Minamino, N., Kangawa, K., Matsuo, H., and Goeddel, D. V. (1991). *Science* **252,** 120–123.

Lai,J., Winer, J., Yen, R., Li, W., King, K. L., Jin, H., Yang, R., Ko, A., Zeigler, F., Cheng, J., Bunting, S., Paoni, N. F. (1996). *Am. J. Physiol.* in press.

Lin, X., Hänze, J., Heese, F., Sodman, R., and Lang, R. E. (1995). *Circ. Res.* **77,** 750–758.

Linssen, M. C. J. G., Engels, W., Lemmens, P. J. M. R., Heijnen, V. V. T., Bilson, M. V., Reneman, R. S., and van der Vusse, G. J. (1993). *Am. J. Physiol.* **264,** H973–H982.

Long, C. S., Henrich, C. J., and Simpson, P. C. (1991). *Cell Reg.* **2,** 1081–1095.

Nag, A. C. (1980). *Cytobios* **28,** 41–61.

Nowak, J., Kaijser, A., and Wennmalm, A. (1980). *Prostaglandins Med.* **4,** 205–214.

Ogawa, Y., Nakao, K., Mukoyama, M., Hosoda, K., Shirakami, G., Arai, H., Saito, Y., Suga, S., Jougasaki, M., and Imura, H. (1991). *Circ. Res.* **69,** 491–500.

Ogletree, M. L., Smith, J. B., and Lefer, A. M. (1978). *Am. J. Physiol.* **235,** H400–H406.

Prins, B. A., Hu, R. M., Nazzario, B., Pedram, A., Frank, H. J., Weber, M. A., and Levin, E. R. (1994). *J. Biol. Chem.* **269,** 11,938–11,944.

Roberts, P. E., Phillips, D. M., and Mather, J. P. (1990). *Am. J. Physiol. Lung Cell. Mol. Physiol.* **3,** 415–425.

Rodemann, H. P. and Goldberg, A. L. (1982). *J. Biol. Chem.* **257,** 1632–1638.

Sadoshima, J. and Izumo, S. (1993). *Circ. Res.* **73,** 413–423.

Saijonmaa, O., Ristimäki, A., and Fyhrquist, F. (1990). *Biochem. Biophys. Res. Commun.* **173,** 514–520.

Sawa, H., Tokuchi, F., Mochizuki, N., Endo, Y., Furuta, Y., Shinohara, T.,Takada, A., Kawaguchi, H., Yasuda, H., and Nagashima, K. (1992). *Circulation* **86,** 138–146.

Scarborough, R. M., Hsu, M. A., Kang, L.-L., McEnroe, G. A., Schwartz, K, Arfsten, A., and Lewicki, J. A. (1989). in *Progress in Atrial Peptide Research,* vol. III (Brenner, B. M. and Laragh, J. H.) (eds.). Raven: New York. pp. 23–29.

Schorb, W., Booz, G. W., Dostal, D. E., Conrad, K. M., Chang, K. C., and Baker, K. M. (1993). *Circ. Res.* **72,** 1245–1254.

Shubieta, H. E., McDonough, P. M., Harris, A. N., Knowlton. K. U., Glembotski, C. C., Brown, J. H., and Chien, K. R. (1990). *J. Biol. Chem.* **265,** 20,555–20,562.

Smith, R. H., Palmer, R. M., and Reeds, P. J. (1983). *Biochem. J.* **214,** 153–161.

SOLVD Investigators. (1991). *N. Engl. J. Med.* **325,** 293–302.

Sudoh, T., Kangawa, K., Minamino, N., and Matsuo, H. (1988). *Nature* **332,** 78–81.

Suga, S., Nakao, K., Itoh, H., Komatsu, Y., Ogawa, Y., Hama, N., and Imura, H. (1992). *J. Clin. Invest.* **90,** 1145–1149.

Suzuki, T., Hoshi, H., Sasaki, H., and Mitsui, Y. (1991). *J. Cardiovasc. Pharmacol.* **17(Suppl. 7),** S182–S186.

Villareal, F. J., Kim, N. N., Ungab, G. D., Printz, M. P., and Dillman, W. H. (1993). *Circulation* **88,** 2849–2861.

Wei, C–M., Lerman, A., Rodeheffer, R. J., McGregor, C. G. A., Brandt, R. R., Wright, S., Heublein, D. M., Kao, P. C., Edwards, W. D., and Burnett, J. C. (1994). *Circulation* **89,** 1580–1586.

Wennmalm, A. (1982). In: *Prostaglandins and the Cardiovascular System* (Oates, J. A.) (ed.). Raven: New York. pp. 303–331.