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Effect of neutral endopeptidase inhibitor on endogenous atrial natriuretic peptide as a paracrine factor in cultured cardiac fibroblasts

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1 Cardiac remodelling is a fundamental response to hypertension, myocardial infarction and chronic heart failure, and involves cardiac fibroblast proliferation and production of extracellular matrix components such as collagen. The present study was performed to examine the role of endogenous atrial natriuretic peptide (ANP) as a possible paracrine factor for cardiac fibroblasts, and to examine the effects of three neutral endopeptidase (NEP) inhibitors, thiorphan, phosphoramidon and ONO-BB-039-02 (ONO-BB) on endogenous ANP-induced changes in collagen synthesis by cultured neonatal rat cardiac fibroblasts.

2 Each NEP inhibitor singly had no significant effect on collagen synthesis by cardiac fibroblasts, except for maximum concentration (10^{-3} M) of thiorphan.

3 Exogenous ANP inhibited collagen synthesis in a concentration-dependent manner (10^{-8}–10^{-6} M). Thiorphan (10^{-4} and 10^{-3} M) and phosphoramidon (10^{-5} and 10^{-4} M) enhanced the ANP (10^{-7} M)-induced decrease in collagen synthesis. ONO-BB (10^{-5} and 10^{-4} M) slightly enhanced the ANP-induced decrease in collagen synthesis.

4 Myocyte-conditioned medium (MC-CM), as well as exogenous ANP, inhibited collagen synthesis dose-dependently. The decrease in collagen synthesis at 100% MC-CM was augmented by thiorphan (10^{-3} M), phosphoramidon (10^{-4} M) and ONO-BB (10^{-4} M).

5 HS-142-1, a natriuretic peptide receptor antagonist, significantly reduced the MC-CM plus thiorphan- and MC-CM plus ONO-BB-induced decrease in collagen synthesis, by 92 and 62%, respectively and showed a tendency to attenuate the MC-CM plus phosphoramidon-induced decrease in collagen synthesis by 40%.

6 Our observations suggested that endogenous ANP released from cardiomyocytes inhibited collagen synthesis as a paracrine factor and that NEP inhibitors enhanced the activity of this peptide in cardiac fibroblasts.

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Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; MC-CM, myocyte-conditioned medium; NEP, neutral endopeptidase

Introduction

Although cardiomyocytes occupy ~75% of the structural space of the heart, they constitute only one-third of the total cell population. The remaining non-myocytes consist mainly of cardiac fibroblasts in the interstitium. The cardiac hypertrophy or remodelling that occurs in response to chronic pressure overload or myocardial infarction is often accompanied with a disproportionate increase in collagen deposition. Thus, inhibition of collagen synthesis may exert a favourable effect on cardiac integrity.

Atrial natriuretic peptide (ANP), a cardiac hormone secreted from cardiomyocytes, is released into the circulation by pressure overload and has an important role in the regulation of body fluid homeostasis and systemic blood pressure. It has recently been shown that exogenous ANP is capable of inhibiting collagen synthesis in adult rat and human cardiac fibroblasts (Redondo *et al*., 1998). Therefore, in addition to acting as a circulating hormone, ANP may have some function as a paracrine factor. However, the local actions of ANP on the heart itself have not been fully elucidated. Although our recent study demonstrated the autocrine role of endogenous ANP in cardiomyocyte hypertrophy (Horio *et al*., 2000), the paracrine action of ANP on cardiac fibroblasts, located very close to myocytes that produce this peptide, remains to be studied. We conducted the present study to examine the direct effects of endogenous ANP on collagen synthesis in cultured cardiac fibroblasts of neonatal rats.

ANP is inactivated rapidly *in vivo*. One important metabolic pathway of its inactivation involves enzymatic degradation by neutral endopeptidase (NEP; also called enkephalinase, CALLA/CD10, EC3.4.24.11). NEP plays important roles in the modulation of peptide actions in various organs, including not only the kidney (Dussaule *et al*., 1993), lung (Ronco *et al*., 1988) and gastrointestinal tract (Bunnett *et al*., 1993) but also the heart (Dutriez *et al*., 1992).

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Since NEP inhibition appears to potentiate ANP activity in the heart, this inhibition may have a beneficial effect against cardiac impairment. In fact, it has been demonstrated that chronic administration of a NEP inhibitor, SCH 34826, reduces both cardiac mass and the amount of fibrotic tissue in the left ventricle in spontaneously hypertensive rats, suggesting that NEP inhibitors may regulate collagen synthesis in cardiac fibroblasts (Monopoli *et al*., 1992). However, there have been no reports concerning the action of NEP inhibitor during collagen synthesis in the heart. Therefore, we also investigated whether three NEP inhibitors, thiorphan, phosphoramidon and ONO-BB-039-02 (ONO-BB) influence the endogenous ANP-induced changes in collagen synthesis.

Methods

Cell culture

Primary cultures of neonatal ventricular myocytes and nonmyocytes were prepared as described previously (Horio *et al*., 1998). Briefly, apical halves of cardiac ventricles from 1- to 2-day-old Wistar rats were separated and minced in ice-cold balanced salt solution ((mM) NaCl 116, HEPES 20, NaH_2PO_4 12.5, glucose 5.6, KCl 5.4, and $MgSO_4$ 0.8, pH 7.35). Ventricular cardiocytes were dispersed in balanced salt solution containing 0.08% collagenase type II with agitation for 6 min at 37°C. The digestion steps were repeated five to seven times until the tissues were completely digested. The cells were combined, centrifuged, and resuspended in chilled foetal calf serum (FCS). To segregate myocytes from nonmyocytes, a discontinuous gradient of 40.5 and 58.5% Percoll was prepared in balanced salt solution, and ventricular cells were suspended in the 58.5% Percoll layer. After centrifugation at 3000 r.p.m. for 30 min, the upper layer consisted of a mixed population of nonmyocyte cell types, and the lower layer consisted almost exclusively of cardiac myocytes. Both myocytes and nonmyocytes were washed twice by centrifugation and resuspension to remove all traces of Percoll.

After myocytes were incubated twice on uncoated 10-cm culture dishes for 30 min to remove any remaining nonmyocyte, the nonattached viable cells (purified myocytes) were plated at a density of 3.0×10^6 cells onto gelatin-coated 10-cm culture dishes and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics (50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin) at 37°C for 48 h in humidified air with 5% CO_2.

Nonmyocyte cells were resuspended in DMEM with 10% FCS and plated onto uncoated 10-cm culture dishes for 30 min. Nonadherent cells and debris were then washed away, and fresh medium was added. Cells were allowed to grow to confluence, trypsinized, and passaged 1 : 3. This procedure yielded cultures of cells that were almost exclusively fibroblasts by first passage as described by Villarreal *et al*. (1993). Nonmyocytes at the second or third passage were plated at a density of 2.5×10^4 cells $well^{-1}$ onto 24-well plates and allowed to grow to confluence.

Preparation of myocyte-conditioned medium (MC-CM)

Myocytes were prepared and cultured as described above. After incubation in DMEM with FCS, the medium was changed to fresh serum-free DMEM and cells were incubated for 24 h. The medium was then collected as MC-CM.

Analysis of collagen synthesis

The effects of various agents on collagen synthesis in cardiac fibroblasts were evaluated by the incorporation of [^{3}H]-proline into cells as previously described (Horio *et al*., 1999). After incubation in DMEM with FCS, nonmyocytes were maintained in serum-free DMEM for 24 h. After the preconditioning period, the culture medium was replaced with fresh serum-free DMEM or MC-CM. Then, rat ANP, thiorphan, phosphoramidon, ONO-BB, and/or HS-142-1 were added. [^{3}H]-proline was also added at 1 μCi ml^{-1}, and then the plates were incubated for 24 h. After labelling, the cells were rinsed twice with cold phosphate buffered saline and incubated with 10% trichloroacetic acid at 4°C for 30 min. The precipitates were washed twice with cold 95% ethanol and solubilized in 1 M NaOH. The radioactivity of aliquots of the trichloroacetic acid-insoluble material was determined using a liquid scintillation counter.

Measurement of immunoreactive ANP and brain natriuretic peptide (BNP)

Cardiac fibroblasts were treated with or without ANP or MC-CM for 24 h, and the collected culture medium was acidified with acetic acid, boiled for 10 min to inactivate intrinsic proteases, and lyophilized. Radioimmunoassays for rat ANP or rat BNP were performed as previously reported (Horio *et al*., 2000). Antibodies against rat ANP and BNP did not cross-react with each other. These assays were performed in duplicate.

Drugs and reagents

The following agents were used in this study: thiorphan, phosphoramidon, Percoll (Sigma Chemical Co., Inc., St Louis, MO, U.S.A.), FCS, DMEM (Life Technologies, Grand Island, NY, U.S.A.), collagenase type II (Worthington Biochemical Corp., Freehold, NJ, U.S.A.), penicillin, streptomycin (ICN Biomedicals, Inc., Aurora, OH, U.S.A.) and rat ANP (Peptide Institute, Osaka, Japan). ONO-BB ((4S-4-[(2S)-Benzyl-3-mercaptopropionylamino]-4-(N-phenylcarbamoyl)-butyric acid) and HS-142-1 were kindly provided by Ono Pharmaceutical Co. Ltd. (Osaka, Japan) and Kyowa Hakko Kogyo (Tokyo, Japan), respectively.

Statistical analysis

The results are expressed as means ± s.e.mean. The statistical significance of differences between groups was estimated by one-way analysis of variance followed by Fisher's multiple comparison test. P values of less than 0.05 were considered statistically significant.

Results

Effects of NEP inhibitors on collagen synthesis in cardiac fibroblasts

To investigate whether NEP inhibitors directly modulate collagen synthesis, we examined the effects of three NEP inhibitors, thiorphan, phosphoramidon and ONO-BB, on [^{3}H]-proline incorporation into cardiac fibroblasts under

basal conditions. The [^{3}H]-proline incorporation was not altered by treatment with these inhibitors except at the maximum concentration (10^{-3} M) of thiorphan (Figure 1).

Effects of NEP inhibitors on exogenous ANP-induced changes in collagen synthesis in cardiac fibroblasts

As shown in Figure 2, exogenous ANP inhibited the [^{3}H]-proline incorporation in a concentration-dependent manner (10^{-8}–10^{-6} M). Additional effects of NEP inhibitors on collagen synthesis in cells treated with exogenous ANP are shown in Figure 3. Thiorphan (10^{-4} and 10^{-3} M) and phosphoramidon (10^{-5} and 10^{-4} M) significantly enhanced the ANP (10^{-7} M)-induced decrease in [^{3}H]-proline incorporation. ONO-BB (10^{-5} and 10^{-4} M) only slightly enhanced the ANP-induced decrease in [^{3}H]-proline incorporation, but this effect was not significant.

ANP and BNP contents in the culture medium

To investigate the levels of secretion of ANP and BNP into the medium from cultured cardiac myocytes or fibroblasts, we measured the amounts of immunoreactive ANP and BNP in the medium of cells after incubation for 24 h. As shown in Table 1, levels of basal release of ANP and BNP from cardiac fibroblasts for 24 h were <0.1 pmol well^{-1}. After a 24 h incubation with exogenous ANP (10^{-7} M), the content

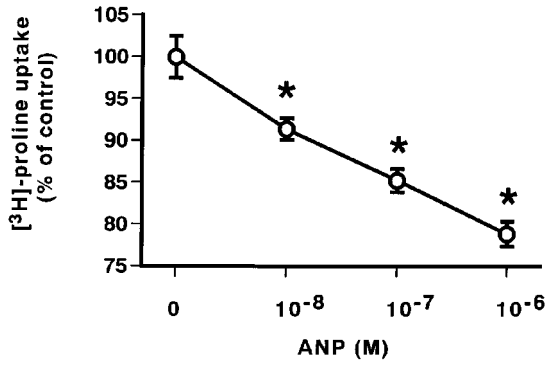

Figure 2 Inhibitory effect of exogenous ANP on collagen synthesis in cultured cardiac fibroblasts. Values are means±s.e.mean of 6–8 measurements. *$P<0.05$ vs control.

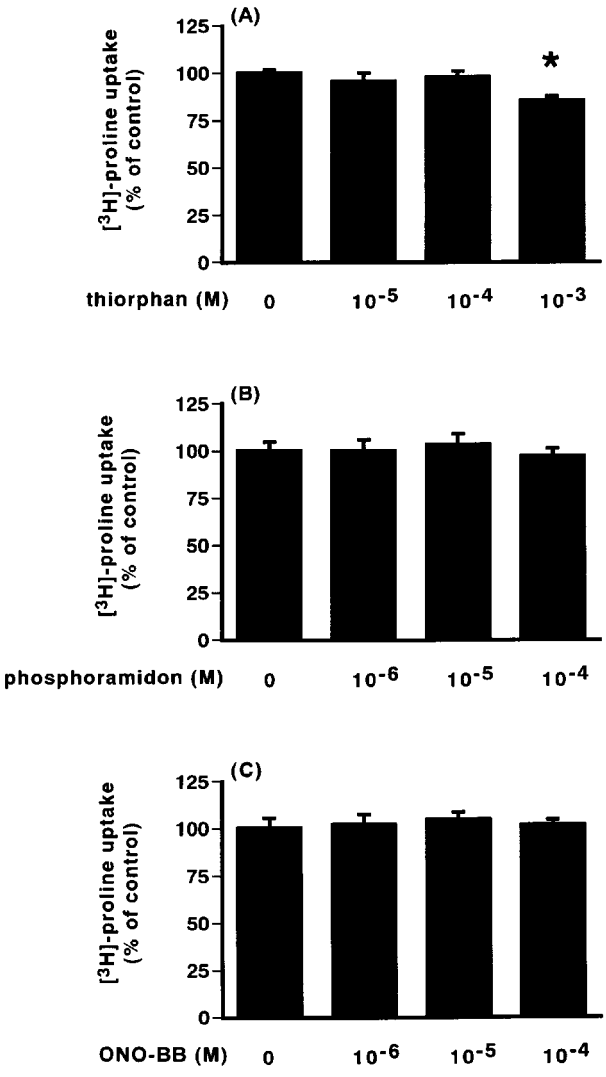

Figure 1 Effects of thiorphan (A), phosphoramidon (B) and ONO-BB (C) on collagen synthesis in cultured cardiac fibroblasts under basal conditions. Values are means±s.e.mean of 6–8 measurements. *$P<0.05$ vs control.

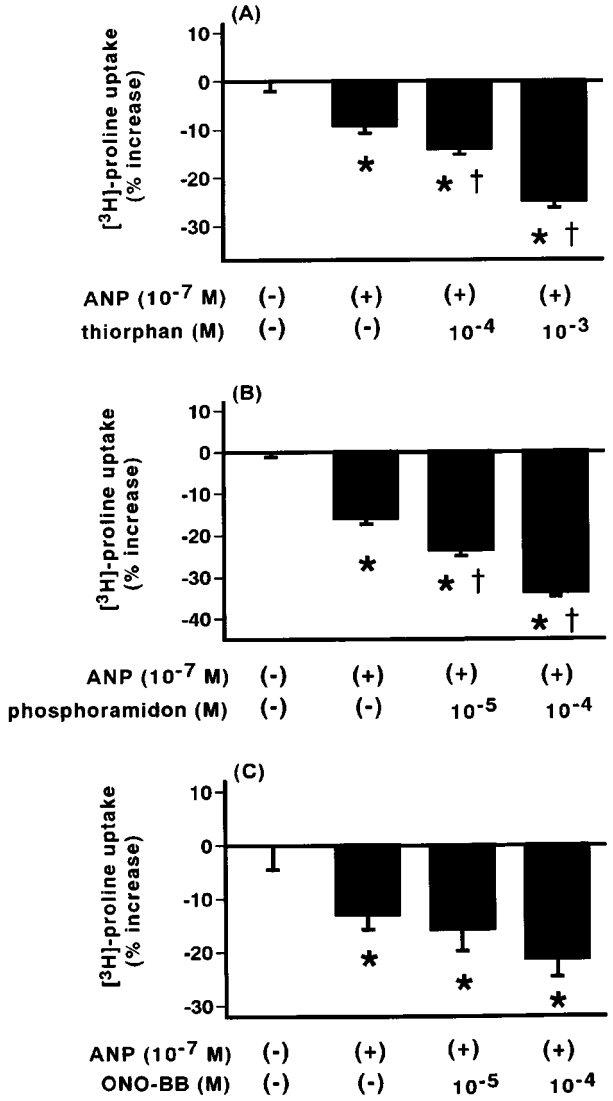

Figure 3 Effects of thiorphan (A), phosphoramidon (B) and ONO-BB (C) on exogenous ANP (10^{-7} M)-induced changes in collagen synthesis in cultured cardiac fibroblasts. Values are means±s.e.mean of 6–8 measurements. *$P<0.05$ vs control; †$P<0.05$ vs ANP alone.

of ANP in the medium was 22.4±1.9 pmol well^{-1}. No immunoreactive BNP was detected in this medium. The contents of ANP and BNP in the culture medium after 24 h incubation with 100% MC-CM were 6.0±0.3, 0.3±0.04 pmol well^{-1}, respectively.

Effects of MC-CM and NEP inhibitors on collagen synthesis in cardiac fibroblasts

To investigate the paracrine effects of cardiac myocytes on fibroblasts through humoral factors, the effects of MC-CM on collagen synthesis in cardiac fibroblasts were examined. The [^{3}H]-proline incorporation in these cells was decreased dose-dependently by treatment with 50–100% MC-CM (Figure 4). Compound effects of MC-CM and NEP inhibitors on collagen synthesis are shown in Figure 5. In the presence of 100% MC-CM, thiorphan (10^{-3} M), phosphoramidon (10^{-4} M) and ONO-BB (10^{-4} M) further decreased [^{3}H]-proline incorporation into the cells, by 14%, 11% and 7%, respectively.

Effects of HS-142-1 on MC-CM and NEP inhibitor-induced changes in collagen synthesis in cardiac fibroblasts

To elucidate whether the effects of MC-CM and NEP inhibitors were actually induced *via* ANP, we examined the effects of HS-142-1, a natriuretic peptide receptor antagonist, on collagen synthesis of cardiac fibroblasts treated with MC-CM plus NEP inhibitors. The decrease in [^{3}H]-proline incorporation by exogenous ANP or MC-CM was significantly blocked by 50 μg ml^{-1} HS-142-1, though this concentration of HS-142-1 did not affect the basal incorporation of [^{3}H]-proline (Figure 6A and B). HS-142-1 significantly attenuated the MC-CM plus thiorphan- and MC-CM plus ONO-BB-induced decreases in [^{3}H]-proline incorporation into the cells by 92 and 62%, respectively (Figure 7A and C) and showed a tendency to attenuate the MC-CM plus phosphoramidon-induced decrease in [^{3}H]-proline incorporation by 40% (Figure 7B).

Table 1 ANP and BNP contents in the medium of cultured cardiac fibroblasts

	Control	*ANP* (10^{-7} M)	*MC-CM* (100%)
ANP content (p mol well^{-1})	<0.1	22.4±1.9	6.0±0.3
(*n*)	(8)	(8)	(8)
BNP content (p mol well^{-1})	<0.1	<0.1	0.3±0.04
(*n*)	(8)	(8)	(8)

After the preconditioning period, the cultures were incubated in serum-free DMEM (control), DMEM with exogenous ANP (10^{-7} M) or MC-CM (100% replacement of culture medium with MC-CM). ANP and BNP contents of culture medium were measured after 24 h incubation. Values represent means± s.e.mean. Numbers in parentheses indicate the number of samples.

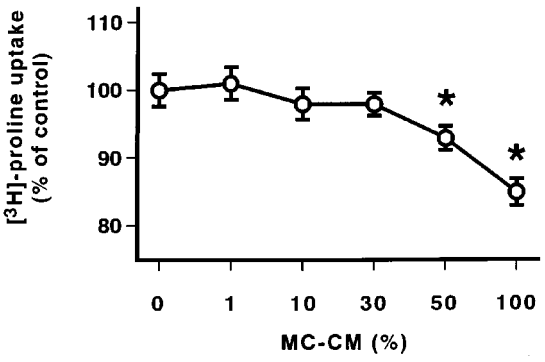

Figure 4 Inhibitory effect of MC-CM on collagen synthesis in cultured cardiac fibroblasts. Values are means±s.e.mean of 6–8 measurements. *P<0.05 vs control.

Discussion

First, we have demonstrated that endogenous ANP secreted from cardiomyocytes, as a paracrine factor, inhibits the collagen synthesis by cardiac fibroblasts. In the present study, MC-CM as well as exogenous ANP clearly decreased [^{3}H]-proline incorporation into fibroblasts. Although MC-CM is likely to contain many kinds of humoral factors in addition to ANP, the amount of ANP in the conditioned medium derived from cultured neonatal rat cardiomyocytes was extremely high and was much higher than that of BNP. Furthermore, our results concerning the effects of MC-CM

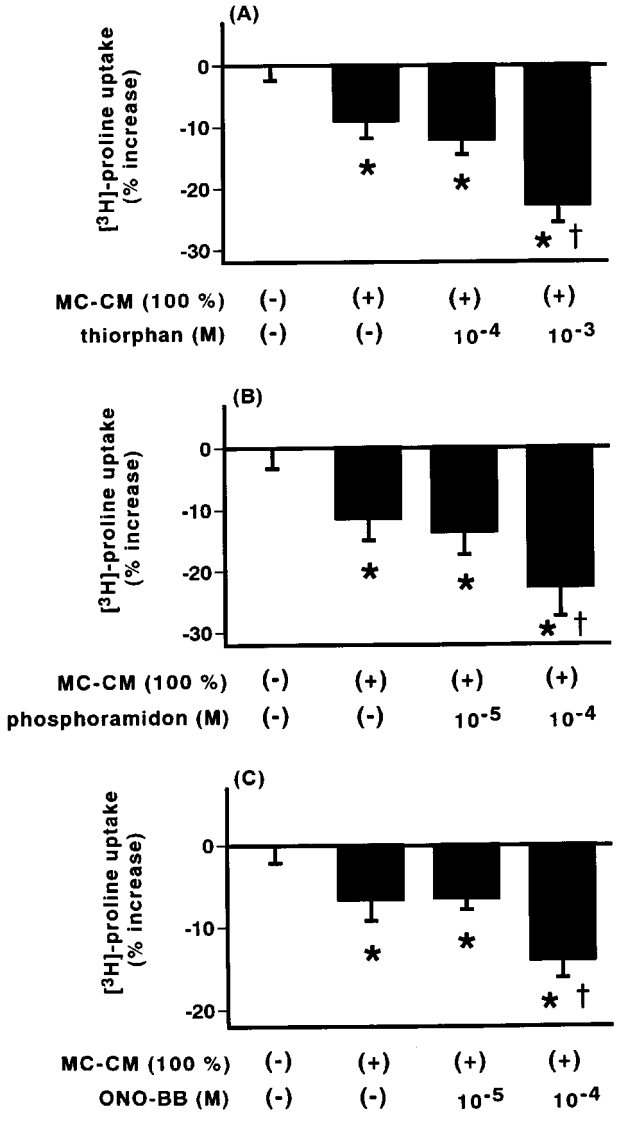

Figure 5 Effects of thiorphan (A), phosphoramidon (B) and ONO-BB (C) on MC-CM (100%)-induced changes in collagen synthesis in cultured cardiac fibroblasts. Values are means±s.e.mean of 6–8 measurements. *P<0.05 vs control; †P<0.05 vs MC-CM alone.

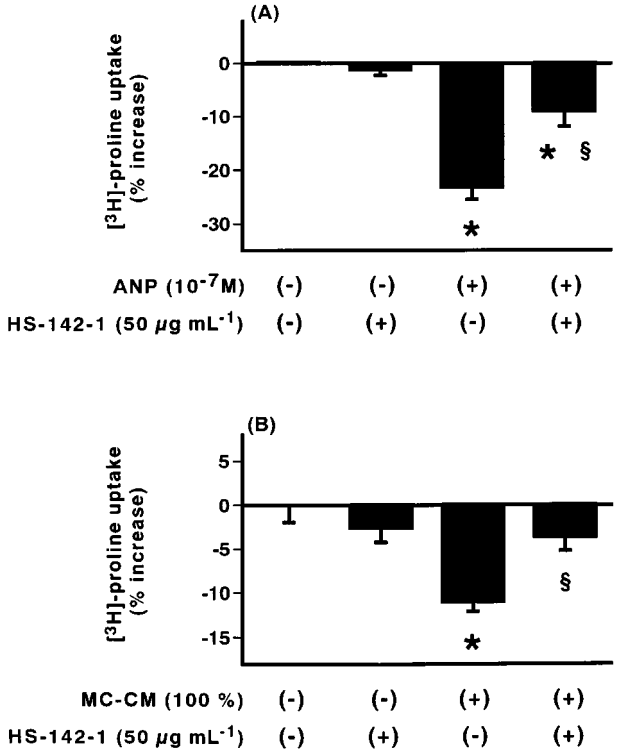

Figure 6 Effects of HS-142-1 on exogenous ANP (10^{-7} M)-induced (A) and MC-CM (100%)-induced (B) changes in collagen synthesis in cultured cardiac fibroblasts. Values are means±s.e.mean of 6–8 measurements. *$P<0.05$ vs control; §$P<0.05$ vs ANP or MC-CM alone.

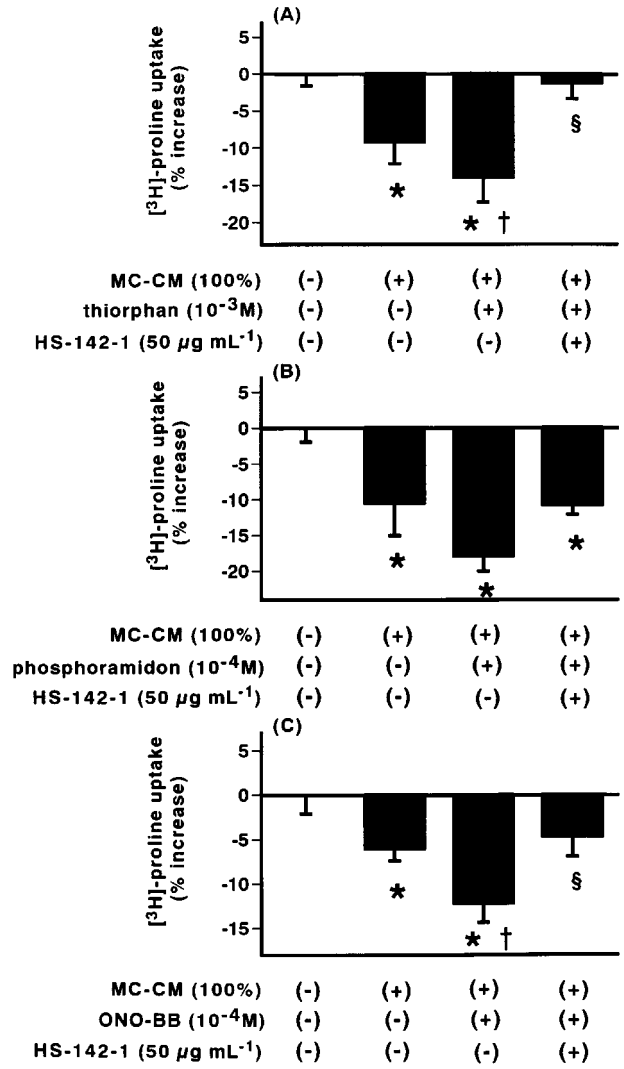

Figure 7 Effects of HS-142-1 on MC-CM and NEP inhibitor-induced changes in collagen synthesis in cultured cardiac fibroblasts. Cells were incubated with thiorphan (10^{-3} M) (A), phosphoramidon (10^{-4} M) (B) or ONO-BB (10^{-4} M) (C). Values are means±s.e.mean of 6–8 measurements. *$P<0.05$ vs control; †$P<0.05$ vs MC-CM alone; §$P<0.05$ vs MC-CM plus NEP inhibitor.

on collagen synthesis were consistent with those of a previous study using exogenous ANP (Redondo *et al*., 1998). Therefore, the inhibition by MC-CM observed in our study was attributable mostly to the action of endogenous ANP.

Recently, the cross-talk between cardiac fibroblasts and myocytes during the process of myocyte hypertrophy has attracted a great deal of attention, and some studies have suggested that cardiac fibroblasts may play a critical role in mediating the hypertrophic response to angiotensin II in the heart (Kim *et al*., 1995; Kuwahara *et al*., 1999). On the other hand, to our knowledge, it has not been reported whether cardiac myocytes influence the proliferation or extracellular matrix production by fibroblasts. The present study demonstrated a negative effect of myocytes on collagen synthesis by fibroblasts through ANP as a paracrine regulator, in contrast to the observation that cardiac fibroblasts have a positive hypertrophic effect on myocardial cells (Harada *et al*., 1997; Kuwahara *et al*., 1999).

Second, we have demonstrated for the first time that NEP inhibitors enhance the suppressive effect of endogenous ANP on collagen synthesis by cardiac fibroblasts. NEP is an ectoenzyme widely distributed in the lung and kidney. NEP has also been shown to be expressed in the heart and to have a role in degradation of ANP and BNP (Dutriez *et al*., 1992; Spencer-Dene *et al*., 1994). A recent study showed that inactivation of ANP was inhibited by the NEP inhibitors phosphoramidon and S-thiorphan in a cultured epithelial cell line (Ozaki *et al*., 1999). We found that a single administration of ONO-9902 (prodrug of ONO-BB-039-02) also inhibited degradation of plasma ANP and BNP in rats with myocardial infarction (unpublished observation). Therefore, the three NEP inhibitors used in our study have been demonstrated in previous studies to potentiate ANP activity. These inhibitors decreased collagen synthesis in cardiac fibroblasts treated with MC-CM or exogenous ANP, but not under basal conditions. In addition, the inhibitory effect of collagen synthesis on MC-CM plus NEP inhibitors was completely or partially blocked by HS-142-1, an ANP receptor antagonist. These results suggested that the inhibitory mechanism of collagen synthesis by NEP inhibitors is mainly mediated by the enhancement of the action of ANP secreted from myocytes.

Although HS-142-1 blocked thiorphan- and ONO-BB-induced decreases in collagen synthesis in the presence of MC-CM, the inhibitory effect of MC-CM plus phosphoramidon on collagen synthesis was not sufficiently blocked by HS-142-1. Therefore, we cannot exclude the possibility that phosphoramidon modulates the degradation of not only natriuretic peptides but also of other peptides. Xu *et al*. (1994) showed that endothelin converting enzyme exhibits 58% amino acid homology with NEP 24.11. Sawamura *et al*. (1993) partially purified the endothelin converting enzyme which is sensitive to phosphoramidon and is selective for the conversion of big endothelin-1 to endothelin-1 from the membrane fraction of porcine lung. Another study demonstrated that phosphoramidon blocked the vasoconstriction

caused by big endothelin-1, but was ineffective on the action of endothelin-1 in the vascular smooth muscle *in vitro* and *in vivo* (Fukuroda *et al*., 1990). These results suggest that phosphoramidon may inhibit not only natriuretic peptide degradation but also hydrolysis of big endothelin-1. Taken together with the finding that endothelin-1 increases the synthesis of both type I and III collagens in adult rat cardiac fibroblasts (Guarda *et al*., 1993), the reduction of endothelin production by phosphoramidon may be associated, at least in part, with its inhibitory effect on collagen synthesis.

A number of human and rat studies concerning the effects of NEP inhibitors on cardiovascular diseases and collagen deposition have been reported. Elsner *et al*. (1992) reported that acute and chronic treatment with a NEP inhibitor, candoxatril, decreased right and left ventricular filling pressures in patients with severe congestive heart failure. Marie *et al*. (1996) reported that 4-week treatment with a NEP inhibitor, SQ28,603, inhibited NEP activity and significantly attenuated the increases in right ventricular and left atrial weight in rats with ligation of the coronary artery, suggesting that it supressed cardiac changes after myocardial infarction. Monopoli *et al*. (1992) reported that chronic administration of another NEP inhibitor, SCH 34826, inhibited collagen deposition in the hypertrophied ventricle of spontaneously hypertensive rats. These findings indicated that NEP inhibitors may have inhibitory effects on cardiac hypertrophy and fibrosis. However, these effects of NEP inhibitors *in vivo* may be secondary to the reduction in haemodynamic stress. Our present *in vitro* study demonstrated that the NEP inhibitor-induced augmentation of the effect of endogenous ANP has a direct action on cardiac fibroblasts, independent of the haemodynamic changes.

Although ANP expression is minimal in the normal adult ventricular myocardium, cardiac overload and hypertrophy induce ANP production in the ventricles (Lee *et al*., 1988; Saito *et al*., 1989; Takemura *et al*., 1991; Tsuchimochi *et al*., 1988). In human failing or hypertrophied hearts, ANP synthesis in cardiac tissue was markedly increased and considerable levels of this peptide were detected in the ventricles (Saito *et al*., 1989; Takemura *et al*., 1991; Tsuchimochi *et al*., 1988). Under such conditions, NEP inhibitors may up-regulate ANP activity and thereby inhibit interstitial fibrosis in the heart. Consequently, the enhanced action of ANP by NEP inhibition on the heart may exert a favourable effect on cardiac integrity, suggesting that NEP inhibitors not only participate in the improvement of haemodynamics but also function as local regulators during the processes of cardiac remodelling and heart failure.

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References

BUNNETT, N.W., WU, V., STERNINI, C., KLINGER, J., SHIMOMAYA, E., PAYAN, D., KOBAYASHI, R. & WALSH, J.H. (1993). Distribution and abundance of neutral endopeptidase (EC 3.4.24.11) in the alimentary tract of the rat. *Am. J. Physiol.,* **264,** G497 – G508.

DUSSAULE, J.C., STEFANSKI, A., BEA, M.L., RONCO, P. & ARDAILLOU, R. (1993). Characterization of neutral endopeptidase in vascular smooth muscle cells of rabbit renal cortex. *Am. J. Physiol.,* **264,** F45 – F52.

DUTRIEZ, I., SALES, N., FOURNIE-ZALUSKI, M.C. & ROQUES, B.P. (1992). Pre- and post-natal ontogeny of neutral endopeptidase 24-11 ('enkephalinase') studied by in vitro autoradiography in the rat. *Experientia,* **48,** 290 – 300.

ELSNER, D., MUNTZE, A., KROMER, E.P. & RIEGGER, G.A.J. (1992). Effectiveness of endopeptidase inhibition (candoxatril) in congestive heart failure. *Am. J. Cardiol.,* **70,** 494 – 498.

FUKURODA, T., NOGUCHI, K., TSUCHIDA, S., NISHIKIBE, M., IKEMOTO, F., OKADA, K. & YANO, M. (1990). Inhibition of biological actions of big endothelin-1 by phosphoramidon. *Biochem. Biophys. Res. Commun.,* **172,** 390 – 395.

GUARDA, E., KATWA, L.C., MYERS, P.R., TYAGI, S.C. & WEBER, K.T. (1993). Effects of endothelins on collagen turnover in cardiac fibroblasts. *Cardiovasc. Res.,* **27,** 2130 – 2134.

HARADA, M., ITOH, H., NAKAGAWA, O., OGAWA, Y., MIYAMOTO, Y., KUWAHARA, K., OGAWA, E., IGAKI, T., YAMASHITA, J., MASUDA, I., YOSHIMASA, T., TANAKA, I., SAITO, Y. & NAKAO, K. (1997). Significance of ventricular myocytes and nonmyocytes interaction during cardiocyte hypertrophy: evidence for endothelin-1 as a paracrine hypertrophic factor from cardiac nonmyocytes. *Circulation,* **96,** 3737 – 3744.

HORIO, T., NISHIKIMI, T., YOSHIHARA, F., MATSUO, H., TAKISHITA, S. & KANGAWA, K. (1999). Effects of adrenomedullin on cultured rat cardiac myocytes and fibroblasts. *Eur. J. Pharmacol.,* **382,** 1 – 9.

HORIO, T., NISHIKIMI, T., YOSHIHARA, F., MATSUO, H., TAKISHITA, S. & KANGAWA, K. (2000). Inhibitory regulation of hypertrophy by endogenous atrial natriuretic peptide in cultured cardiac myocytes. *Hypertension,* **35,** 19 – 24.

HORIO, T., NISHIKIMI, T., YOSHIHARA, F., NAGAYA, N., MATSUO, H., TAKISHITA, S. & KANGAWA, K. (1998). Production and secretion of adrenomedullin in cultured rat cardiac myocytes and nonmyocytes: stimulation by interleukin-1β and tumor necrosis factor-α. *Endocrinology,* **139,** 4576 – 4580.

KIM, N.N., VILLARREAL, F.J., PRINTZ, M.P., LEE, A.A. & DILLMANN, W.H. (1995). Trophic effects of angiotensin II on neonatal rat cardiac myocytes are mediated by cardiac fibroblasts. *Am. J. Physiol.,* **269,** E426 – E437.

KUWAHARA, K., SAITO, Y., HARADA, M., ISHIKAWA, M., OGAWA, E., MIYAMOTO, Y., HAMANAKA, I., KAMITANI, S., KAJIYAMA, N., TAKAHASHI, N., NAKAGAWA, O., MASUDA, I. & NAKAO, K. (1999). Involvement of cardiotrophin-1 in cardiac myocyte-nonmyocyte interactions during hypertrophy of rat cardiac myocytes in vitro. *Circulation,* **100,** 1116 – 1124.

LEE, M.A., WEST, JR. R.E. & MOSS, J. (1988). Atrial natriuretic factor reduces cyclic adenosine monophosphate content of human fibroblasts by enhancing phosphodiesterase activity. *J. Clin. Invest.,* **82,** 388 – 393.

MARIE, C., MOSSIAT, C., GROS, C., MONTEIL, T. & BRALET, J. (1996). Effect on prolonged inhibition of neutral endopeptidase on cardiac hypertrophy in rats with myocardial infarction. *Cardiovasc. Drugs Ther.,* **10,** 593 – 598.

MONOPOLI, A., ONGINI, E., CIGOLA, E. & OLIVETTI, G. (1992). The neutral endopeptidase inhibitor, SCH 34826, reduces left ventricular hypertrophy in spontaneously hypertensive rats. *J. Cardiovasc. Pharmacol.,* **20,** 496 – 504.

OZAKI, J., SHIMIZU, H., HASHIMOTO, Y., ITOH, H., NAKAO, K. & INUI, K. (1999). Enzymatic inactivation of major circulating forms of atrial and brain natriuretic peptides. *Eur. J. Pharmacol.,* **370,** 307 – 312.

REDONDO, J., BISHOP, J.E. & WILKINS, M.R. (1998). Effect of atrial natriuretic peptide and cyclic GMP phosphodiesterase inhibition on collagen synthesis by adult cardiac fibroblasts. *Br. J. Pharmacol.,* **124,** 1455 – 1462.

RONCO, P., POLLARD, H., GALCERAN, M., DELAUCHE, M., SCHWARTZ, J.C. & VERROUST, P. (1988). Distribution of enkephalinase (membrane metalloendopeptidase, E.C. 3.4.24.11) in rat organs. Detection using a monoclonal antibody. *Lab. Invest.,* **58,** 210 – 217.

SAITO, Y., NAKAO, K., ARAI, H., NISHIMURA, K., OKUMURA, K., OBATA, K., TAKEMURA, G., FUJIWARA, H., SUGAWARA, A., YAMADA, T., ITOH, H., MUKOYAMA, M., HOSODA, K., KAWAI, C., BAN, T., YASUE, H. & IMURA, H. (1989). Augmented expression of atrial natriuretic polypeptide gene in ventricle of human failing heart. *J. Clin. Invest.,* **83,** 298 – 305.

SAWAMURA, T., SHINMI, O., KISHI, N., SUGITA, Y., YANAGISAWA, M., GOTO, K., MASAKI, T. & KIMURA, S. (1993). Characterization of phosphoramidon-sensitive metalloproteinases with endothelin-converting enzyme activity in porcine lung membrane. *Biochim. Biophys. Acta.,* **1161,** 295–302.

SPENCER-DENE, B., THOROGOOD, P., NAIR, S., KENNY, A.J., HARRIS, M. & HENDERSON, B. (1994). Distribution of, and a putative role for, the cell-surface neutral metallo-endopeptidases during mammalian craniofacial development. *Development,* **120,** 3213–3226.

TAKEMURA, G., FUJIWARA, H., MUKOYAMA, M., SAITO, Y., NAKAO, K., KAWAMURA, A., ISHIDA, M., KIDA, M., UEGAITO, T., TANAKA, M., MATSUMORI, A., FUJIWARA, T., IMURA, H. & KAWAI, C. (1991). Expression and distribution of atrial natriuretic peptide in human hypertrophic ventricle of hypertensive hearts and hearts with hypertrophic cardiomyopathy. *Circulation,* **83,** 181–190.

TSUCHIMOCHI, H., KURIMOTO, F., IEKI, K., KOYAMA, H., TAKAKU, F., KAWANA, M., KIMATA, S. & YAZAKI, Y. (1988). Atrial natriuretic peptide distribution in fetal and failed adult human hearts. *Circulation,* **78,** 920–927.

VILLARREAL, F.J., KIM, N.N., UNGAB, G.D., PRINTZ, M.P. & DILLMANN, W.H. (1993). Identification of functional angiotensin II receptors on rat cardiac fibroblasts. *Circulation,* **88,** 2849–2861.

XU, D., EMOTO, N., GIAID, A., SLAUGHTER, C., KAW, S., DEWIT, D. & YANAGISAWA, M. (1994). ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell,* **78,** 473–485.

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