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Response of adrenomedullin system to cytokine in cardiac fibroblasts—role of adrenomedullin as an antifibrotic factor

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Abstract

Objective: The adrenomedullin system acts as an autocrine or paracrine factor (or both) in the development of cardiac hypertrophy and in the regulation of cardiac function. However, several aspects of the local action of adrenomedullin remain unclear. We studied the effects of interleukin 1-beta (IL-1β) on the adrenomedullin system in cardiac fibroblasts and also examined the pathophysiological significance of such effects.

Methods: We cultured rat neonatal cardiac fibroblasts with or without IL-1β and measured (1) two molecular forms of adrenomedullin in culture medium by specific immunoradiometric assay; (2) gene expression of adrenomedullin, calcitonin receptor like receptor (CRLR), receptor activity modifying protein2 (RAMP2), and RAMP3, components of the adrenomedullin receptor, by Northern blot analysis or RT-PCR analysis; (3) intracellular cAMP levels in response to exogenously administered adrenomedullin; and (4) ^{3}H-proline incorporation with and without a specific adrenomedullin antisense oligodeoxynucleotide.

Results: (1) IL-1β time-dependently increased the levels of two molecular forms of adrenomedullin, adrenomedullin-mature and adrenomedullin-glycine ($P<0.01$). In contrast to known levels in plasma (about 10%), adrenomedullin-mature was a major molecular form in the culture medium of cardiac fibroblasts and myocytes (65–80%). (2) IL-1β significantly increased gene expression of adrenomedullin and its receptor components (adrenomedullin: +46%, CRLR: +460%, RAMP2: +32%, RAMP3: +350%, all $P<0.01$). (3) Preincubated IL-1β elevated the intracellular cAMP response to exogenous adrenomedullin administered at a concentration of 10^{-7} M (+26%, $P<0.05$). (4) Adrenomedullin antisense oligodeoxynucleotide treatment significantly lowered adrenomedullin—mature levels in culture medium (−50%). Adrenomedullin nonsense oligodeoxynucleotide treatment did not change ^{3}H-proline incorporation or mRNA levels of collagen I and III, whereas adrenomedullin antisense oligodeoxynucleotide treatment significantly increased ^{3}H-proline incorporation and mRNA levels of collagen I and III in IL-1β-treated cardiac fibroblasts.

Conclusion: These results provide evidence that the adrenomedullin system acts as an autocrine antifibrotic factor in the regulation of collagen synthesis in cardiac fibroblasts exposed to higher cytokine levels. This may beneficially modulate the pathophysiology of certain cardiac diseases.

Keywords: Adrenomedullin; Heart failure; Hypertrophy; Cytokine; Fibrosis; Interleukin 1-beta

1. Introduction

Adrenomedullin (AM), a novel vasodilatory peptide, is widely distributed in various tissues and organs, including the heart [1]. High expression of the AM gene and specific

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binding sites for AM peptide are found in the heart [2]. The coincident expression of AM peptide, AM mRNA, and AM receptors in the heart suggests that AM peptide acts as an autocrine factor or a paracrine factor (or as both) involved in the development of cardiac hypertrophy and the regulation of cardiac function [3,4]. Indeed, we and other groups have demonstrated that AM tissue concentrations and AM mRNA expression are increased in rats with cardiac hypertrophy and heart failure [5,6]. Moreover, cardiac fibroblasts produce and secrete greater amounts of AM than do myocytes, [7] and AM inhibits collagen synthesis and proliferation in cardiac fibroblasts, possibly via a cAMP signaling mechanism [8,9]. These findings suggest that increased AM may play a role in cardiac remodeling by suppressing collagen synthesis in cardiac fibroblasts. Inflammation has recently received attention as a major factor in the development and progression of cardiac hypertrophy and heart failure [10, 11]. In fact, increased levels of cytokines such as interleukin 1-beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) are found in the failing heart [10, 11]. Interactions between AM and inflammatory cytokines have been reported. IL-1β and TNF-α have been shown to increase AM gene expression in vascular smooth muscle cells and endothelial cells [12,13]. We also demonstrated that IL-1β increases AM secretion and mRNA levels in cultured cardiac fibroblasts [14].

However, several aspects of the local action of AM remain unclear. First, a recent study of the AM receptor showed that a seven-transmembrane receptor, the calcitonin receptor-like receptor (CRLR), can function as an AM receptor with the coexpression of single transmembrane receptor activity modifying proteins (RAMP), RAMP2 or RAMP3 [15,16]. We recently reported that CRLR, RAMP2, and RAMP3 are upregulated in cardiac hypertrophy in vivo [17,18]. However, the mechanism of increased AM receptor in heart failure remains unknown. We hypothesized that increased cytokine levels may stimulate the gene expression levels of the AM system. Second, recent studies have revealed that two molecular forms of AM, an active mature form of AM (AM-m), and an intermediate inactive form of glycine-extended AM (AM-Gly), circulate in human plasma, AM-Gly being the dominant circulating form [19,20]. In addition, we have very recently shown that the major molecular form of AM in cardiac tissues is AM-m and that this form of AM is increased in left ventricular hypertrophy (LVH) and heart failure [21]. However, which molecular form of AM is predominantly secreted by cultured cardiac fibroblasts remains unknown. The effects of cytokine stimulation on the release of AM-m and AM-Gly also remain unclear. Third, although the levels of AM and its mRNA are increased by cytokine stimulation in cardiac fibroblasts [14] and AM has antifibrotic action in vitro and in vivo, [8,22] the pathophysiological significance of increased endogenous AM in cardiac fibroblasts remains to be established.

To address these questions, we used cultured cardiac fibroblasts obtained from neonatal rat heart. We investigated the effects of cytokine stimulation on the AM system in cultured cardiac fibroblasts.

2. Material and methods

All procedures were in accordance with our institutional guidelines for animal research. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Animals and materials

Neonatal Wistar rats (1–2 days old) were purchased from SLC (Shizuoka, Japan). Synthetic rat AM was purchased from Peptide Institute (Osaka, Japan). The cAMP RIA kit was purchased from Yamasa Shoyu (Chiba, Japan). ^{3}H-proline was purchased from Amersham Life Science. IL-1β was purchased from Genzyme Transgenics (Cambridge, MA) and TNF-α was purchased from Sigma. IL-1β, TNF-α, AM, and ^{3}H-proline were dissolved in Dulbecco's modified Eagle medium (DMEM).

2.2. Cell culture

Enriched cultures of neonatal (days 1–2) cardiac myocytes (MCs) and nonmyocytes (NMCs) were prepared from the hearts of Wistar rats as described previously [23], with minor modifications [8,24]. In brief, the apical halves of the ventricles were recovered and ventricular MCs were dispersed in a balanced salt solution containing 0.06% collagenase II (Worthington Biochemical, Freehold, NJ), agitated for 6 min at 37 °C, and pipetted approximately 20 times. MCs were differentiated from NMCs by the discontinuous Percoll gradient method.

The purified MCs were used to study the release of two molecular forms of AM, AM-m and AM-Gly, with or without stimulation by IL-1β or TNF-α. We used 10 ng/mL of IL-1beta and 20 ng/mL of TNF-α in this study, because we previously examined the dose–response curve of IL-1β and TNF-α on the secretion of AM in cardiac MCs and NMCs and found that 10 ng/mL of IL-1β and 20 ng/mL TNF-α are considered to be optimal dose [14]. The NMCs were allowed to grow to confluence and were then trypsinized and passaged 1:3. Subconfluent NMCs from the third passage were used to study the release of AM-m and AM-Gly, intracellular cAMP concentrations, and ^{3}H-Pro incorporation and were subjected to RT-PCR analysis and Northern blot analysis.

As the characterization of MCs and NMCs, immunocytochemical staining study shows that more than 96% of the MCs were stained with anti-sarcomeric actin antibody, and

little proportion of them shown positive staining with the other antibodies against smooth muscle specific actin (less than 0.2%), factor VIII (not significant) and vimentin (less than 3.5%). All the MCs stained with the anti-sarcomeric actin antibody were beating. Whereas the NMCs were stained only with anti-vimentin antibody, and none of the other three antibodies showed positive staining. Thus, the NMCs were confirmed to be exclusively composed of fibroblast.

2.3. Measurement of AM-m and AM-Gly levels

The primary cultured MCs and NMCs at three passages were grown to confluence in a 6-well plate. After incubation with fetal calf serum (FCS)-free medium, five hundred microliters of the conditioned medium of the cardiac MCs or NMCs was collected at 12 h, 24 h, and 48 h. Each medium sample for immunoradiometric assay was boiled in 10% volumes of 1 mol/l acetic acid for 10 min to inactivate intrinsic proteases. After cooling, the boiled medium was evaporated in a vacuum until dry. Immunoradiometric assay for rat AM-m and AM-total (AM-m+AM-Gly) was performed using a recently developed specific immunoradiometric assay kit (AM RIA SHIONOGI) with some modifications, as previously reported [21,25]. AM-Gly was calculated as following formula: (AM-Gly)=(AM-total)−(AM-m).

2.4. Quantification of messenger RNA (mRNA) by RT-PCR

After incubation with FCS-free medium, NMCs were further incubated with and without treatment of IL-1 β for 24 h. Total RNA from the NMCs was extracted using the acid guanidinium thiocyanate-phenol-chloroform method as previously reported [5,14]. First strand complementary DNA was synthesized from 5 μg total RNA with murine transcriptase (Ready to Go, Pharmacia Biotech), using oligo dT primers (Gibco, BRL). PCR and quantification of PCR products were performed as described in detail in our previous report [26]. The sets of primers used were as previously reported [21]. As an internal control, we measured glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in a similar manner. The numbers of PCR cycles for the three genes examined and control were as follows: CRLR, 25; RAMP2, 27; RAMP3, 32; and GAPDH, 21. For these numbers of PCR cycles, the RT-PCRs were all in the linear range of reaction. The quantity of each species of mRNA was expressed by the following formula: amount of original template of each molecule/amount of original template of GAPDH.

2.5. Northern blot analysis for AM

Total RNA was extracted from NMCs as described above. Northern blot analyses were performed with cDNA

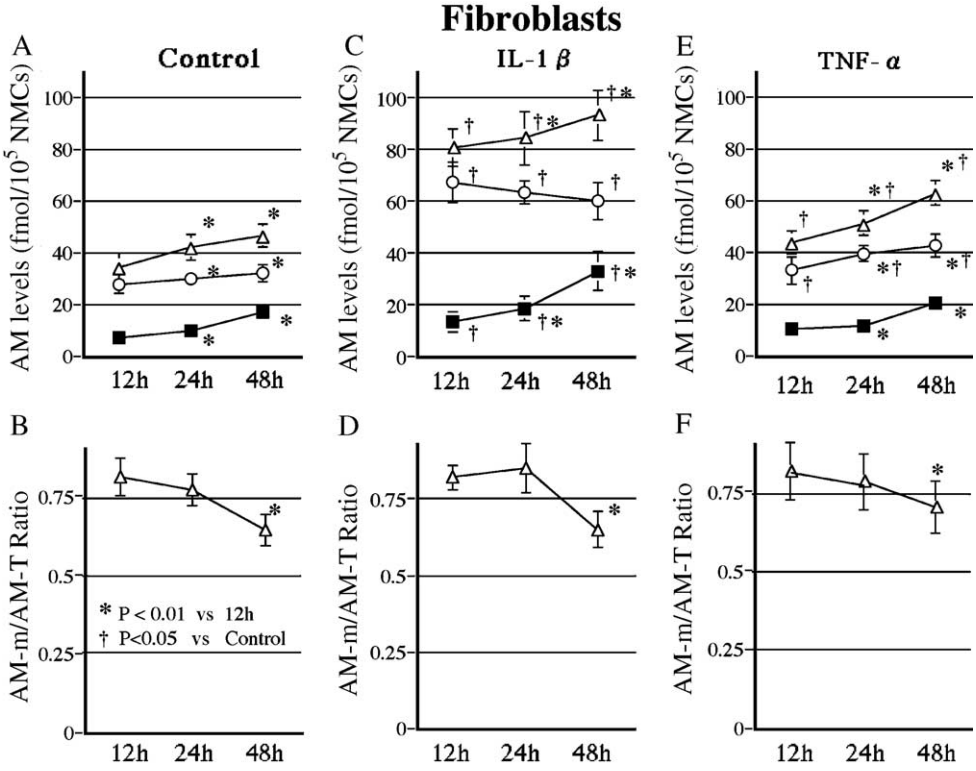

Fig. 1. Time courses of the control (basal secretion) and the effects of IL-1β (10 ng/ml) and TNF-α (20 ng/ml) on AM-m (AM-mature; open circle), AM-Gly levels (AM-glycine; closed square), and AM-total (AM-total; open triangle) (A, C, E) and AM-m/AM-T ratios (open triangle) (B, D, F) in cultured cardiac fibroblasts. Values are given as means±SD. *P<0.01 vs. 12 h; †P<0.05 vs. control.

probes for rat AM mRNA and GAPDH mRNA as previously reported [5,6]. Band intensity was estimated with a radioimage analyzer (BAS-5000, Fuji Photo Film, Tokyo, Japan).

2.6. Measurement of intracellular cAMP levels

The NMCs at three passages were grown to confluence in 24 well plates. After incubation with FCS-free medium, NMCs were further incubated with and without treatment of IL-1 β for 24 h. Then, NMCs were treated with various concentrations of AM for 10 min, the medium was removed, and the cellular extract was obtained with the use of cold 70% ethanol, as previously reported [24,26]. Each ethanol sample was evaporated in a vacuum until dry. The eluate was dissolved in RIA buffer. The RIA for cAMP was performed using an RIA kit (Yamasa Shouyu, Chiba, Japan).

2.7. Measurement of collagen synthesis

The effect of AM antisense on collagen synthesis in cardiac NMCs was evaluated on the basis of ^{3}H-proline incorporation into the cells as described previously [8]. The NMCs at three passages were grown to confluence in 24-well plates. After a preconditioning period with FCS-free medium for 24 h, the cultured cells were replaced with FCS-free DMEM with various concentrations of AM antisense or nonsense. To estimate collagen synthesis in NMCs, 0.5 μCi of ^{3}H-proline was added, and the plates were incubated for 24 h. The cells were rinsed twice with cold phosphate-buffered saline (PBS) 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 an aliquot was determined with the use of a liquid scintillation counter.

2.8. AM antisense experiments

Nonsense phosphorothioated oligodeoxynucleotide, 5′-G AT CG AT CG AT CG AT CG AT CG AT CG AT C-3′, and antisense phosphorothioated oligodeoxynucleotide of rat AM, 5′-CCTGCTGAAAGGGTGAGTTGTCAAGCAC-3′, were synthesized and purified by HPLC and were added into culture media at a concentration of 0.1 μmol/L. The fibroblasts at three passages were grown to confluence in 6-well plates and then incubated with FCS-free DMEM for 24 h. After 24-h preincubation in DMEM with or without antisense or nonsense oligodeoxynucleotide of AM, 500 μl of medium was collected, and AM-m and

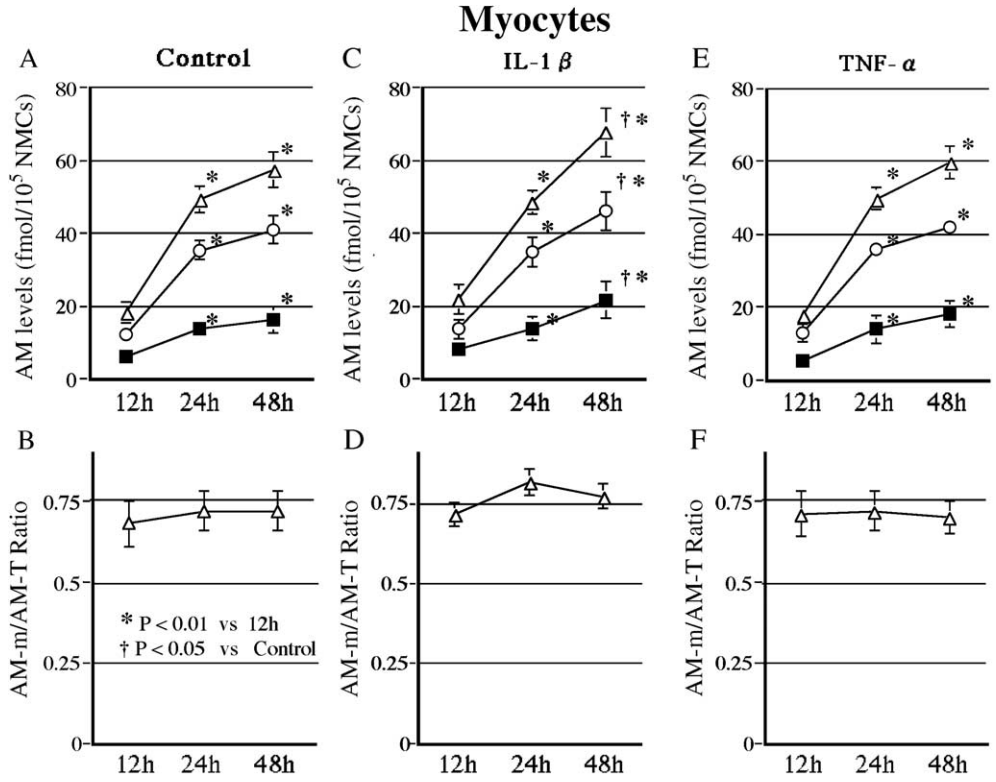

Fig. 2. Time courses of the control (basal secretion) and the effects of IL-1β (10 ng/ml) and TNF-α (20 ng/ml) on AM-m (AM-mature; open circle), AM-Gly levels (AM-glycine; closed square), and AM-total (AM-total; open triangle) (A, C, E) and AM-mature/AM-T ratios (open triangle) (B, D, F) in cultured cardiac myocytes. Values are given as means±SD. *P<0.01 vs. 12 h; †P<0.05 vs. control.

AM-Gly levels were measured as described above. In addition, the effect of nonsense and antisense oligodeoxynucleotide of rat AM on ^{3}H-proline incorporation was evaluated in 24-well plates as described above. Furthermore, the effects of nonsense and antisense oligodeoxynucleotide of rat AM on mRNA levels of collagen I and collagen III were also evaluated. Quantification of mRNA levels of collagen I and collagen III was performed by RT-PCR analysis described as above.

2.9. Statistical analysis

All data are expressed as means±SD. Multiple comparisons were performed by one-way analysis of variance followed by Scheffe's test. P values of less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. Basal release of two molecular forms of AM, AM-m and AM-Gly, in cardiac MCs and fibroblasts

To investigate which molecular form of AM is mainly secreted by cultured rat ventricular MCs and fibroblasts, AM-m, Gly, and AM-total concentrations in the medium of MCs and fibroblasts cultured without FCS were measured by specific immunoradiometric assay. Both MCs and fibroblasts secreted AM-m and AM-Gly into FCS-free medium (Figs. 1-A and 2-A). Interestingly, both types of cells mainly secreted AM-m (about 65–80%), an active form of AM. The basal release of AM from MCs (per 10^5 cells) was nearly equivalent to that of fibroblasts for 12–48 h of incubation.

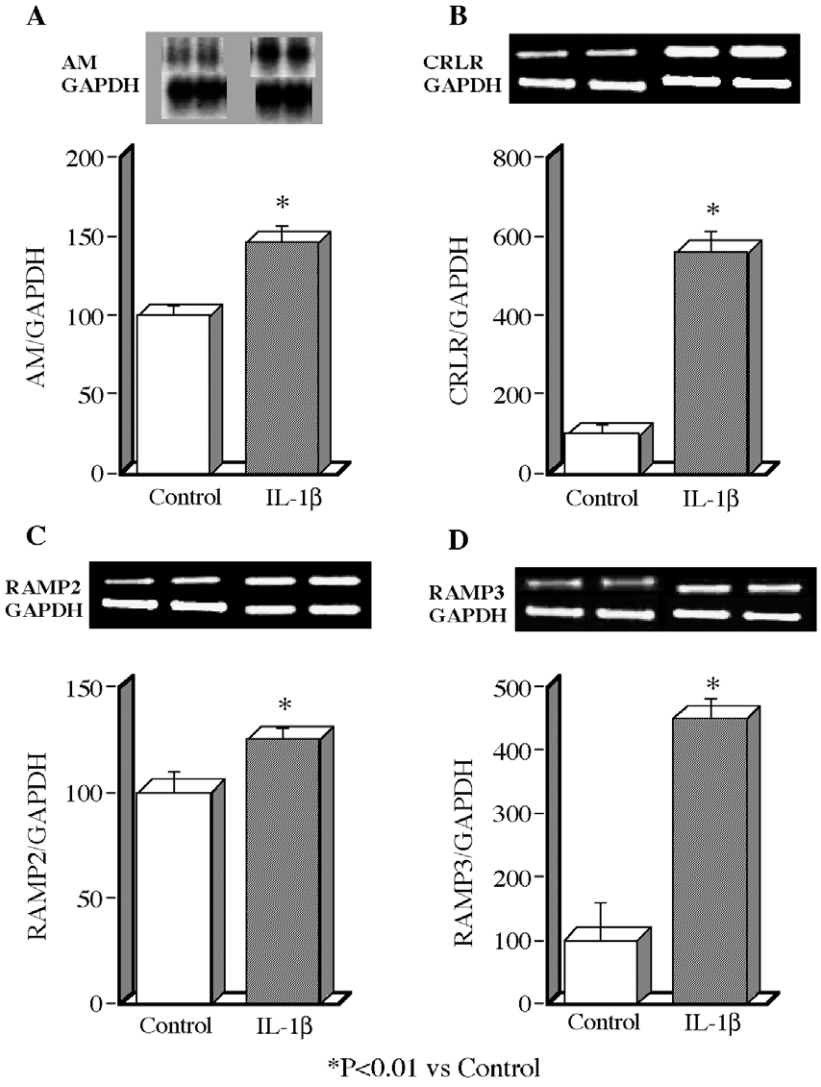

Fig. 3. Levels of the expression of rat AM, CRLR, RAMP2, and RAMP3 in cultured cardiac fibroblasts with or without treatment by IL-1β (10 ng/ml) are shown (A, B, C, D). Representative autoradiograms of rat AM and representative ethidum bromide stained agarose gels of RT-PCR products for CRLR, RAMP2, and RAMP3 and GAPDH mRNA bands at each PCR cycle (upper); AM, CRLR, RAMP2, and RAMP3 mRNA levels corrected for GAPDH mRNA levels (lower). Data are expressed as means±SD. *P<0.01 vs. control.

3.2. Effects of IL-1β and TNF-α on the secretion of two molecular forms of AM in cardiac MCs and fibroblasts

In cardiac MCs, secretion of AM-m, AM-Gly, and AM-total was slightly but significantly elevated only 48 h after stimulation with 10 ng/ml IL-1β (Fig. 2-C); secretion of neither AM-m, AM-Gly nor AM-total was stimulated by TNF-α. In contrast, AM-m, AM-Gly, and AM-total secretion from fibroblasts was markedly increased by IL-1β during the entire observation period (Fig. 1-C). TNF-α also increased AM-m and AM-total levels in cardiac fibroblasts during the entire observation period (Fig. 1-F). The stimulatory effect of IL-1β on the release of AM-m was greater than that of TNF-α (Fig. 1-C,E). The AM-m/AM-T ratio was significantly decreased at 48 h in cardiac fibroblasts. Thus, IL-1β remarkably increased AM-m levels in cardiac fibroblasts.

3.3. Expression of AM, CRLR, RAMP2, and RAMP3 mRNA in cardiac fibroblasts and effects of IL-1β on expression levels

Next, we examined the mRNA levels of AM and AM receptor components. The expression of rat AM mRNA in cultured rat cardiac fibroblasts was examined by Northern blot analysis. As shown in Fig. 3-A (upper), a single band hybridizing to the rat AM cDNA probe was found in fibroblasts. Fig. 3-A (lower) shows a quantitative analysis of these blots corrected for the levels of GAPDH mRNA as an internal standard. The expression of AM mRNA in fibroblasts was increased by IL-1β after 24 h (1.46-fold). The expression of rat AM receptor components, CRLR, RAMP2, and RAMP3 mRNA, in cultured rat cardiac fibroblasts was examined by quantitative RT-PCR analysis (Fig. 3-B,C,D). The expression of CRLR and RAMP3 mRNA in NMCs was remarkably increased by IL-1β after 24 h (5.6-fold and 4.5-fold, respectively), whereas RAMP2 was slightly but significantly increased (1.32-fold). Thus, gene expression levels of AM ligand and receptor were up-regulated in IL-1β-treated cardiac fibroblasts.

3.4. Effect of AM on cAMP levels in fibroblasts with and without stimulation by IL-1β

We then examined the intracellular cAMP response to AM in cardiac fibroblasts. AM increased the cAMP levels in the NMCs in a concentration-dependent manner (Fig. 4). An AM concentration of 10^{-7} mol/l or higher significantly increased intracellular cAMP levels in untreated fibroblasts, whereas an AM concentration of 10^{-8} mol/l or higher significantly increased intracellular cAMP levels in IL-1β-treated fibroblasts. Intracellular cAMP levels were significantly higher in IL-1β-treated fibroblasts than in IL-1β-untreated fibroblasts at AM concentrations of 10^{-8} mol/l or greater. Thus, increased expression of AM receptor components indeed augmented intracellular signaling in cardiac fibroblasts.

3.5. Effects of AM antisense on AM-m and AM-Gly levels in fibroblasts

To examine the role of endogenous AM, we synthesized AM specific antisense oligodeoxynucleotide. Fig. 5 shows the effects of AM antisense oligodeoxynucleotide on AM-m and AM-Gly levels in the medium of fibroblasts. Nonsense oligodeoxynucleotide did not change AM-m or AM-Gly levels in the medium of fibroblasts, whereas specific AM antisense oligodeoxynucleotide significantly reduced both AM-m and AM-Gly levels in the medium of fibroblasts.

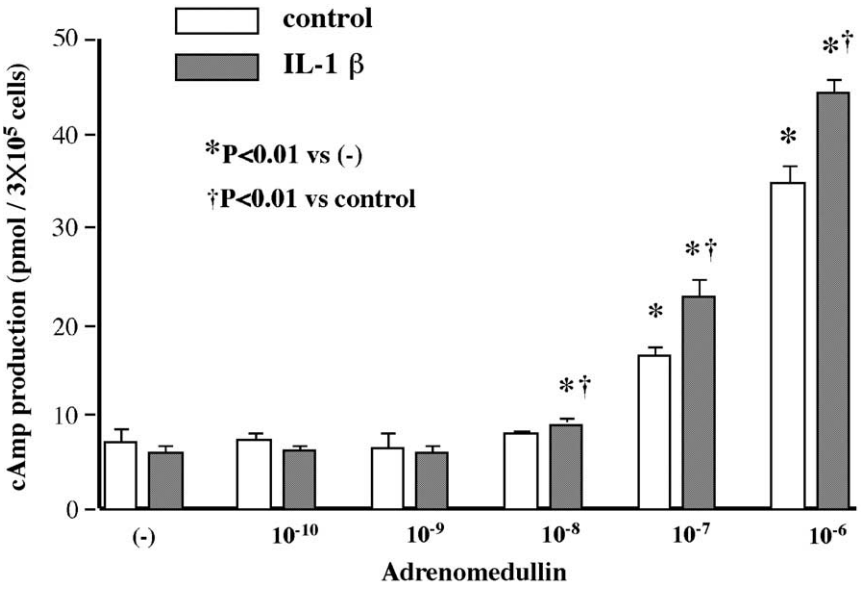

Fig. 4. Bar graph showing the effect of AM (10–10 to 10–6 M) on intracellular cAMP levels in cardiac fibroblasts with (hatched column) or without (open column) treatment by IL-1β (10 ng/ml). Means±SD, n=6. *P<0.01 vs. (−), †P<0.01 vs. control.

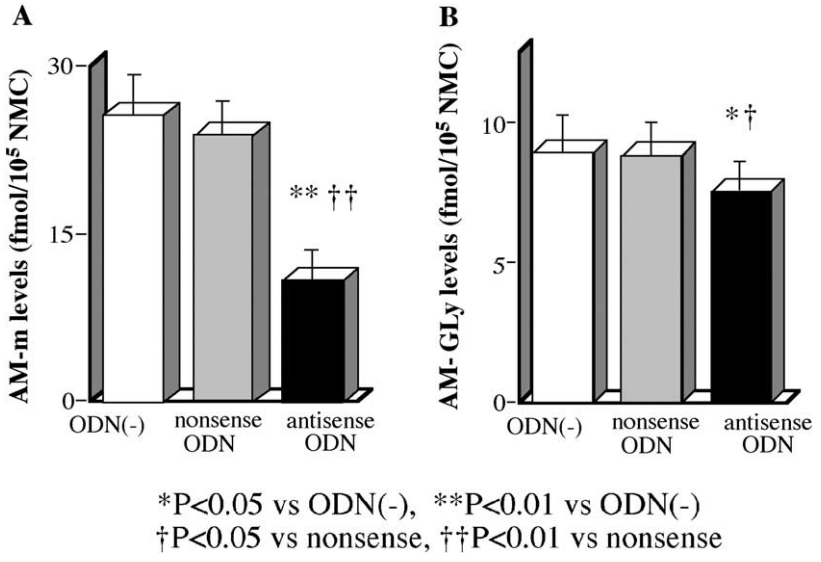

Fig. 5. Bar graph showing control (open column) and the effects of antisense (closed column) and nonsense (hatched column) oligodeoxynucleotides (ODN) for AM on AM-m (AM-mature) (A) and AM-Gly (B) levels in cardiac fibroblasts. Means±SD, n=6. *P<0.05 vs. ODN(−), **P<0.01 vs. ODN(−), $^{\dagger}P$<0.05 vs. nonsense, $^{\dagger\dagger}P$<0.01 vs. nonsense.

Thus, AM specific antisense treatment effectively inhibited the translation of AM.

3.6. Effects of AM antisense on collagen synthesis in fibroblasts

Next, we examined the effect of AM antisense on collagen synthesis in cardiac fibroblasts by measuring ^{3}H-proline incorporation. AM antisense or nonsense oligodeoxynucleotide did not change ^{3}H-proline incorporation in untreated fibroblasts (Fig. 6-A). In contrast, AM antisense, but not nonsense, oligodeoxynucleotide, significantly increased ^{3}H-proline incorporation in IL-1β-treated fibroblasts (Fig. 6-B), suggesting that endogenous AM inhibits collagen synthesis in IL-1β-treated fibroblasts. In addition, AM antisense, but not nonsense, oligodeoxynucleotide, slightly but significantly increased mRNA levels of collagen I and collagen III in IL-1β-treated fibroblasts (Fig. 7-A,-B).

4. Discussion

To investigate the pathophysiological significance of increased endogenous AM in cardiac hypertrophy or the

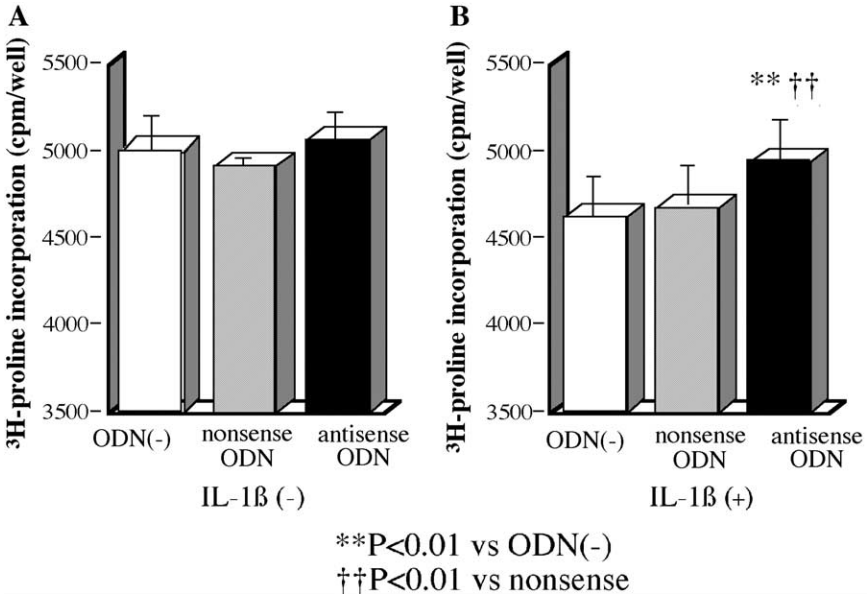

Fig. 6. Bar graph showing control (open column) and the effects of antisense (closed column) and nonsense (hatched column) oligodeoxynucleotides (ODN) for AM on ^{3}H-proline incorporation in cardiac fibroblasts with (B) or without (A) treatment by IL-1β (10 ng/ml). Means±SD, n=6. **P<0.01 vs. ODN(−), $^{\dagger\dagger}P$<0.01 vs. nonsense.

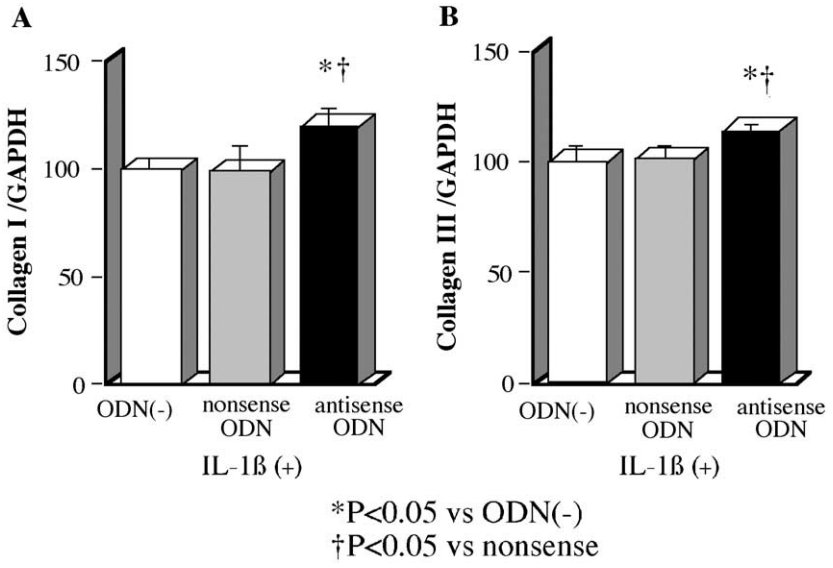

Fig. 7. Bar graph showing control (open column) and the effects of antisense (closed column) and nonsense (hatched column) oligodeoxynucleotides (ODN) for AM on mRNA levels of collagen I (A) and collagen III (B) in cardiac fibroblasts with treatment by IL-1β (10 ng/ml). Means±SD, n=4. *P<0.05 vs. ODN(−), †P<0.05 vs. nonsense.

failing heart, we used cultured fibroblasts. Previous studies have shown that IL-1β significantly increases immunoreactive AM levels in the medium of cardiac fibroblasts [7,14]. In this culture system, we found that AM-m was the major molecular form and that IL-1β increased both molecular forms of AM, i.e., AM-m and AM-Gly. We also found that IL-1β increased not only the gene expression of AM, but also the mRNA levels of its receptor components, CRLR, RAMP2, and RAMP3. In addition, preincubated IL-1β augments the intracellular cAMP response to exogenously administered AM. AM specific antisense oligodeoxynucleotide treatment significantly decreased AM-m and AM-Gly levels in cultured medium, and AM antisense oligodeoxynucleotide treatment significantly increased ^{3}H-proline incorporation and mRNA expressions of collagen I and III only in IL-1β-treated fibroblasts, but not control fibroblasts. These results support a pathophysiological role of AM as an autocrine antifibrotic factor acting to regulate collagen synthesis in cardiac fibroblasts in certain pathological conditions.

In the present study, we measured the two molecular forms of AM in the culture medium of rat cardiac fibroblasts. During the biosynthesis of AM, AM precursor is converted to C-terminal glycine-extended AM (AM-Gly), a 53-amino-acid peptide that is an inactive intermediate form of AM. Subsequently, inactive AM-Gly is converted to the active form of mature AM (AM-m), a 52-amino-acid peptide with a C-terminal amide structure, by enzymatic amidation [19]. Recent studies have shown that both plasma AM-m and AM-Gly levels are increased in parallel in patients with hypertension and heart failure [27,28]. In cardiac tissue, we recently analyzed the two molecular forms of AM in the normal heart, left ventricular hypertrophy, and failing heart. We showed that the major molecular form of AM in cardiac tissue is AM-m (50–70%) [17,18]. Interestingly, the AM-m/AM-T ratio is increased in LVH and is further increased in failing myocardium as compared with that in control rats [21]. Our study first showed that the major molecular form of AM was AM-m in cultured rat cardiac fibroblasts and MCs. IL-1β, but not TNF-α, slightly increased AM-m, AM-Gly, and AM-total levels in cardiac MCs at 48 h. In contrast, TNF-α slightly increased AM-m and AM-total levels, but not AM-Gly levels, whereas IL-1β remarkably increased AM-m, AM-Gly, and AM-total levels in cardiac fibroblasts. Thus, IL-1β appears to be a more important regulator of AM synthesis than TNF-α in cardiac fibroblasts. Furthermore, the AM-m/AM-T ratio did not change with time in cardiac MCs, but slightly but significantly decreased in cardiac fibroblasts at 48 h. Although the precise mechanism underlying the decreased AM-m/AM-T ratio at 48 h remains unknown, alteration of amidating enzyme activity may be involved. A previous study reported that amidating enzyme mRNA expression is detected in the heart [29]. Prepro AM production is increased in a time-dependent manner in cardiac fibroblasts; however, the rate of increase of amidating enzyme activity might be too slow to keep up with the increase in AM-Gly. Further study is necessary to elucidate the mechanism responsible for the regulation of AM synthesis and the roles of amidating enzyme in cardiac fibroblasts and MCs.

Identifying a distinct AM receptor has proved challenging [30]. However, McLatchie et al. [15] isolated and cloned a new family of single-transmembrane-domain proteins, which they called RAMP1, RAMP2, and RAMP3. They demonstrated that a seven-transmembrane receptor, the calcitonin receptor-like receptor (CRLR), can function as either a calcitonin gene-related peptide (CGRP) or as an AM

receptor, depending on the coexpression of RAMPs [15]. CRLR and RAMP1 generate a CGRP receptor, while CRLR and RAMP2 or CRLR and RAMP3 produce an AM receptor [15]. To date, few studies have examined the expression of CRLR and RAMP mRNAs in the heart [17,18]. Totsune et al. [31] reported that the mRNA levels of CRLR, RAMP2, and AM are increased in the atrium and ventricle of rats with heart failure. In addition, we very recently reported that the mRNA levels of CRLR, RAMP2, and RAMP3 are all upregulated in LVH and the failing heart [21]. To our knowledge, however, no previous study has investigated the expression of CRLR and RAMP mRNAs in cardiac fibroblasts with and without cytokine stimulation. Our study demonstrated that cardiac fibroblasts expressed mRNA of CRLR, RAMP2, and RAMP3, all of which were up-regulated by IL-1β in association with up-regulation of AM mRNA levels. Autelitano et al. [32] recently reported that cardiac myocytes express CRLR and RAMPs and that overexpression of either CRLR or RAMP2 potentiates the AM-signaling response determined by a cAMP-responsive element-luciferase reporter gene. Our study showed that precincubation of IL-1β significantly augmented the intracellular cAMP response to exogenously administered AM. Thus, overexpression of AM receptor component by IL-1β may augment the intracellular signaling response of AM.

We also investigated the role of augmented intracellular signaling response to AM in cardiac fibroblasts. Previous studies showed that AM inhibits collagen synthesis in cardiac fibroblasts in vitro [8,33] and in vivo [22]. In addition, we and others previously reported that AM is secreted in fibroblasts and that its secretion is augmented by cytokines [8,14]. We therefore used an AM-specific antisense method to test the hypothesis that an augmented signaling response to AM affects collagen synthesis. Recent studies have examined the physiological role of AM by using AM specific antisense oligodeoxynucleotide to suppress AM gene translation [34,35]. Available evidence indicates that this specific antisense technique is a powerful tool for clarifying the endogenous role of AM. Our results showed that treatment with AM specific antisense oligodeoxynucleotide significantly decreased AM peptide levels, as well as increased ^{3}H-proline incorporation and mRNA levels of collagen I and III, suggesting that endogenous AM may protect against collagen production.

Taken together, these findings suggest that the AM system, including ligand, AM mRNA, and mRNAs of receptor components in cardiac fibroblasts, is activated by cytokine stimulation, augmenting the intracellular signaling response to AM. The effect of AM is mediated by binding to its putative receptor, CRLR/RAMP2 or CRLR/RAMP3, and up-regulated expression of CRLR, RAMP, and RAMP^{3} has an important part in this process. Thus, an autocrine mode of interaction between AM and AM receptor is associated with augmentation of the intracellular signaling response. A protective role of AM against various cardiac insults, such as heart failure, myocardial infarction, and left ventricular hypertrophy has been demonstrated in animal models, and long-term infusion of AM or adenovirus-mediated AM gene transfer suppresses cardiac fibrosis and thereby improves cardiac function [22,36,37]. Thus, up-regulated cardiac expression of AM, CRLR/RAPM2, and CRLR/RAPM3 may be adaptive and protective responses to cytokine-stimulated conditions such as ischemia and heart failure. This hypothesis is supported by the findings of recent studies using AM knockout mice [33]. Up-regulation of the AM system may have a pathophysiological role in the modulation of specific processes in certain pathological conditions.

We have limitation of the study. As the results of the present study were obtained from neonatal fibroblasts, we cannot deny the possibility that neonatal fibroblasts may not represent the behavior of adult cells. However, previous studies demonstrated that AM gene delivery in vivo significantly suppresses cardiac fibrosis in hypertensive rat model and that IL-1β stimulates AM production in various cells. Therefore, it is possible that antifibrotic effect of AM and the stimulatory effect of IL-1β on the AM production are common phenomena in neonatal and adult fibroblasts. Further study is necessary to answer this question.

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