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Taurine attenuates hypertrophy induced by angiotensin II in cultured neonatal rat cardiac myocytes

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Abstract

The effect of taurine on angiotensin II-induced changes in cell morphology and biochemistry of the cultured neonatal cardiomyocyte was examined. Angiotensin II (1–100 nM) alone caused a slow increase in the surface area of the myocyte accompanied by an induction of the expression of atrial natriuretic peptide (ANP) and an upregulation of transforming growth factor β_1 gene (TGF-β_1). The signaling pathway of angiotensin II (1–100 nM) was found to proceed through protein kinase C and the rapid activation of mitogen-activated protein (MAP) kinases. Pretreatment of the myocyte with taurine (20 mM) in the absence of angiotensin II had no visible effect on cell size or growth rate. However, the cells that were pretreated with taurine (20 mM) for 24 h exhibited reduced responsiveness to angiotensin II (100 nM) relative to surface cell area enlargement and the upregulation of the late and growth factor genes(ANP, TGF-β_1). Angiotensin II-mediated activation of the MAP kinases (extracellular signal-regulated protein kinase 1/2: ERK1/2) was not blocked by taurine. Taurine reduced the phosphorylation of a 29-kDa protein, a reaction which was enhanced by angiotensin II and appears to involve protein kinase C step. The results indicate that taurine is an effective inhibitor of certain aspects of angiotensin II action.

Keywords: Taurine; Angiotensin II; Cell size; Fetal gene expression; Growth factor gene expression; Protein kinase C

1. Introduction

The development of heart failure is thought to proceed through several phases, involving both compensatory and decompensatory steps. The initial phase induced by either hypertension or pressure overload is accompanied not only by morphological changes, such as an increase in myocyte size, but also by molecular changes, including a shift to the "fetal" phenotype of the cardiomyocyte (Childs et al., 1990; Schwartz et al., 1986; Izumo et al., 1987, 1988; Chassagne et al., 1993). These molecular changes in cardiac hypertrophy appear to participate in the modulation of cardiac systolic and diastolic functions (Kim et al., 1995).

One of the most important factors contributing to the development of heart failure is the neurohumoral peptide, angiotensin II (Baker et al., 1992; Lindpainter and Ganten, 1991; Sadoshima and Izumo, 1993a). This peptide hormone plays a major role in the regulation of fluid balance, blood pressure and cardiac function. Its direct cardiac effects include modulation of cation (Na^+, H^+ and Ca^{2+}) transport, increased heart rate, altered mechanical function, stimulation of protein synthesis and enhanced uptake and utilization of glucose (Baker et al., 1992; Lindpainter and Ganten, 1991). By acting through the angiotensin AT_1 receptor (AT_1 receptor), angiotensin II activates multiple phospholipid-derived second-messenger systems in cardiac myocytes. (Sadoshima and Izumo 1993b; Schmitz and Berk, 1997). Among these second-messenger systems, protein kinase C may play a critical role in angiotensin II-induced c-*fos* gene activation. Sadoshima et al. (1995)

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also have shown that an important step in angiotensin II action is the activation of the mitogen-activated protein (MAP) kinases in the cardiomyocyte. The MAP kinases are a family of serine/threonine kinases that are rapidly activated by phosphorylation following exposure of the myocyte to growth factors, such as epidermal growth factor, platelet-derived growth factor, insulin, thrombin and phorbol esters. The MAP kinases activated by angiotensin II are thought to contribute to cell hypertrophy in part by regulating the transcription of c-*fos* (Gille et al., 1992).

Another substance shown to be effective in modulating the severity of congestive heart failure is the amino acid, taurine (Azuma et al., 1983, 1992). This amino acid is found in very high concentration in the heart and is thought to regulate cellular volume, Ca^{2+} transport and contractile function (Huxtable, 1992; Rasmusson et al., 1993; Schaffer et al., 1994). We have previously reported that taurine suppresses angiotensin II-induced immediate early response gene expression, its stimulation of protein synthesis and its elevation in intracellular free calcium concentration {$[Ca^{2+}]_i$} of cultured neonatal cardiomyocytes (Takahashi et al., 1997). However, the mechanism underlying the taurine effect is poorly understood.

The present study was designed to test the hypothesis that taurine acts at the level of the MAP kinases and/or through protein kinase C to modulate angiotensin II activity and thus attenuates the hypertrophic response of the peptide. To examine this idea, the experiments focused on the interaction between taurine and angiotensin II, as evidenced by changes in cell size, expression of fetal and growth factor genes using Northern blot analysis and the phosphorylation of the MAP kinases (ERK1/2) and a protein kinase C substrate using Western blot analysis.

2. Materials and methods

2.1. Preparation of cardiomyocyte and noncardiomyocyte cultures

The preparation of primary cardiomyocyte and noncardiomyocyte cultures from 1-day old Wistar rats was performed as described by Sadoshima et al. (1992). For selective enrichment of the cardiomyocytes, the dissociated cells were preplated for 1 h, during which time the noncardiomyocytes attached readily to the bottom of the culture dish. Non-adherent cells, mostly myocytes, were plated at a density of $2–5 \times 10^6$ cells ml^{-1} $dish^{-1}$. Bromodeoxyuridine (0.1 mM) was added during the first 2 days to prevent proliferation of the noncardiomyocytes. This procedure yielded cultures with 90–95% myocytes, as assessed by microscopic observation of cell beating. The myocytes were kept in serum-containing culture medium, Dulbecco's modified Eagle's medium/F-12 (Dainippon Pharmaceutical, Osaka, Japan: 1:1 v/v) supplemented with newborn calf serum (5%; Dainippon), 3-mM pyruvic acid, 100-μM ascorbic acid, 5-μg/ml insulin, 5-μg/ml transferrin, 5-ng/ml selenium (Boehringer Mannheim, Germany) for 48 h followed by serum-free medium. Angiotensin II experiments were performed 24 h after transferring the cells to serum-free medium.

2.2. Evaluation of cell size

The morphological status of the cardiomyocytes was monitored with an inverted phase contrast microscope (IX70, Olympus, Tokyo, Japan) and videomonitor equipped with color video copy processor (SCT-CP710, Mitsubishi, Tokyo, Japan) Cell size was quantified by measuring cell surface area. The cell images were introduced into an intensified charged couple device camera (INT-11A; INTER DEC, Osaka, Japan) and videotaped by a VHS recoder (INTER DEC, Osaka, Japan). The shape and location of each myocyte was recorded before initiating the experiment by taking a photograph. An image processing software (NIH Image 1.59/Power Macintosh 7200; National Institutes of Health, Bethesda, MD, USA) was used to determine alterations in the size of cells. The surface area in the same cell was estimated by the ratio of before and after angiotensin II treatment by planimetry enlarged photographs using NIH image software.

2.3. Isolation and Northern blot analysis of mRNA

Total RNA of cardiac cells was isolated by using the guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987). The RNA concentration was determined spectrophotometrically by absorbance at 260 nm. Northern blot analysis was performed according to the procedure described by Kim et al. (1995). The cDNA probes used were as follows: rat atrial natriuretic peptide (ANP) cDNA, a 0.825-kb fragment; rat transforming growth factor β_1 (TGF-β_1) cDNA, a 1.0-kb fragment; and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a 1.3-kb fragment. The cDNA probes were labeled with [^{32}P]dCTP (specific activity, 3 mCi/mmol; New England Nuclear) by random primer extension using a Bca BEST Labeling Kit (Takara, Siga, Japan). Autoradiography was performed on a Kodak XAR-5 film with an intensifying screen at −80°C. Autoradiograms were quantified by an image analyzer (BAS1500, Fuji Film, Tokyo, Japan). Results were normalized to GAPDH gene expression.

2.4. Western blot analysis (Villalba et al., 1997)

After the appropriate treatment period, the cells were washed and harvested in ice cold phosphate-buffered saline (PBS) and then centrifuged for 5 min at 15 000 rpm at 4°C. Pellets were solubilized in buffer containing 2% sodium dodecyl sulfate (SDS), 62.5-mM Tris–HCl (pH 6.8) and 10% glycerol. The protein concentration of each sample

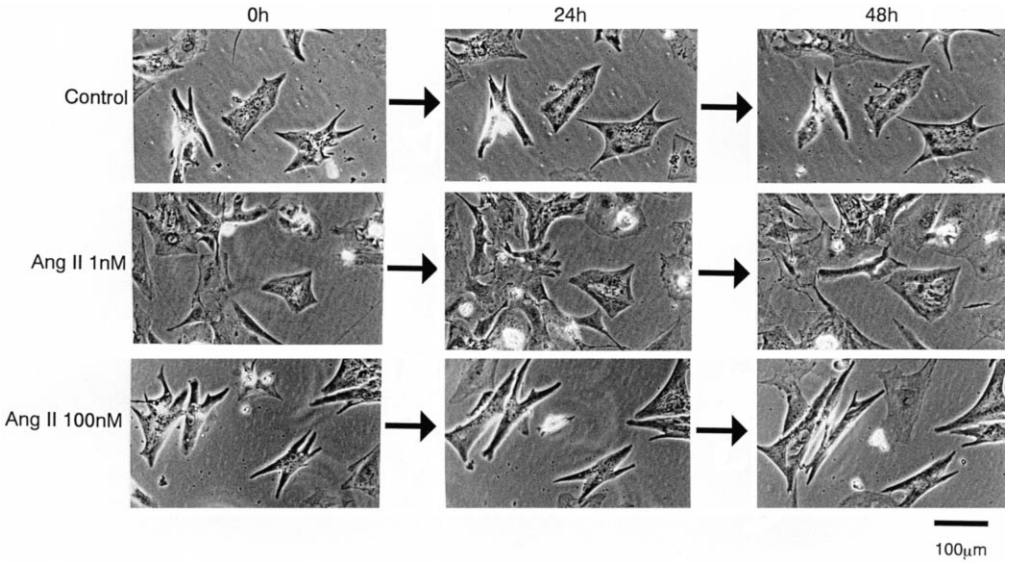

Fig. 1. Characteristic morphological changes induced by angiotensin II in neonatal rat cardiomyocytes. Cells were exposed to angiotensin II (1 and 100 nM) for 48 h. They were then examined for changes in cell size using phase contrast micrographs observed at a magnification of 100.

was determined by a Lowry assay (Lowry et al., 1951). Prior to electrophoresis, 0.1% (w/v) bromophenol blue and 0.05% (v/v) 2-mercaptoethanol were added to each sample, which was then boiled for 5 min and loaded (25 mg/lane) on a 12.5% gel. After the SDS–polyacrylamide gel electrophoresis run, the proteins were transferred to nitrocellulose membrane (0.2 mm pore size). For the protein binding analysis, the membranes were incubated with phospho-specific MAP kinase antibody (#9101S, 1:1000; New England Biolabs, USA) or protein kinase C (monoclonal antiphosphoserine; Research Biochemical International, USA), followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibody (1:2000). Signals were visualized with an enhanced chemiluminescence detection system (ECL;#PRPN2106B1 Amersham Life Science, Tokyo, Japan).

2.5. Statistics

Depending upon the design of the experiments, statistical significance was determined by either the Student's *t*-test or analysis of variance (ANOVA) combined with

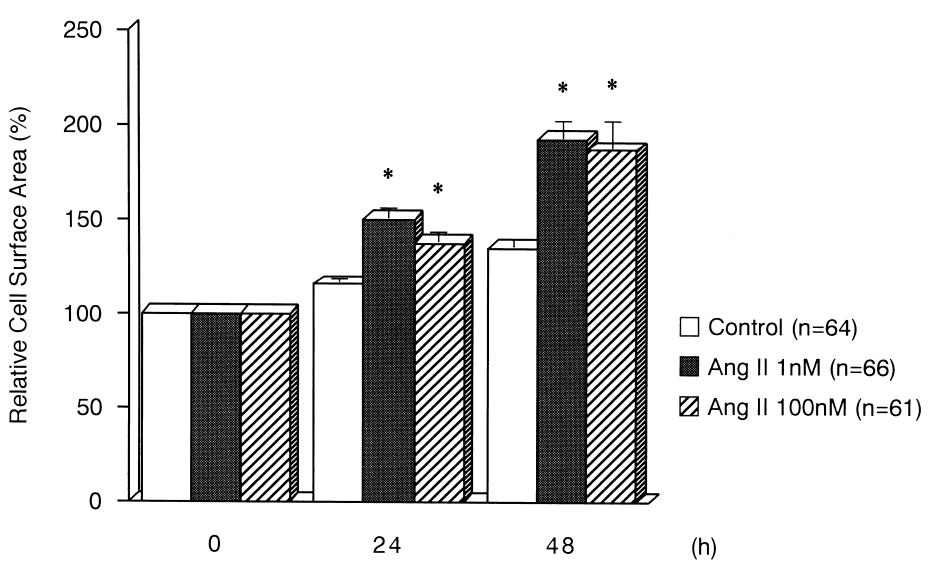

Fig. 2. Effect of angiotensin II on cell size of neonatal rat cardiomyocytes. Cells were incubated with standard serum-free medium containing 0 (control), 1 or 100 nM angiotensin II for a period of 24 or 48 h. Cell size was measured at each time point by measuring cell surface area by NIH image software. Relative cell surface area was calculated as follows: the cell size at 24 or 48 h per one of the same cell at 0 hr. Data shown represent means ± SEM of 64–66 cells obtained from three different primary cultures. $^{*}P < 0.05$ vs. control by ANOVA statistics.

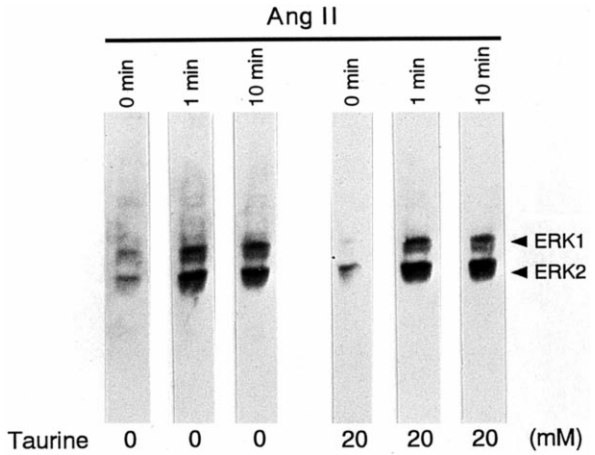

Fig. 3. Angiotensin II-induced phosphorylation of MAP kinase. Rat cardiomyocytes were incubated with buffer containing either 0- or 20-mM taurine for 24 h before being exposed to 100-nM angiotensin II for the indicated time. The cells were then lysed and the lysate containing equal amounts of protein (25 μg) were loaded on 12.5% gels and subjected to SDS–PAGE. MAP kinase activity was assessed by Western blot analysis using anti-phospho MAP kinase antibody. Shown is a representative Western blot from a total of three experiments.

Bonferroni's method to compare individual data points for significance. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated p value was less than 0.05.

3. Results

3.1. Angiotensin II-induced hypertrophic growth on cell size of the cardiomyocyte

Previously, we demonstrated that angiotensin II (1 nM) caused a significant increase in protein synthesis as measured by [^{3}H]-phenylalanine incorporation over 48 h (Takahashi et al., 1997). In this study, we examined the effects of angiotensin II on cardiomyocyte size. Fig. 1 is a pictorial representation of the hypertrophic response of the myocyte to angiotensin II stimulation. Cardiomyocytes treated for 24 h with angiotensin II (1 and 100 nM) showed enlarged cytoplasmic space compared to control cells prepared from the same hearts; this effect was more pronounced after a 48-h exposure to the peptide (Fig. 1). The surface area of the cardiomyocyte treated with 1- and 100-nM angiotensin II increased by 50% and 31% at 24 h and 93% and 65% at 48 h, respectively (Fig. 2). The control cells also increased in size by 15% at 24 h and 28% at 48 h, a rate expected for a normally growing cell.

3.2. Angiotensin II stimulation of MAP kinase, protein kinase C and induction of fetal genes

It has been proposed that serine/threonine kinases, such as the MAP kinases and protein kinase C, are phosphorylated in response to diverse growth stimuli. In agreement with Sadoshima et al. (1995), we found that angiotensin II (100 nM) rapidly activates one of the members of the MAP kinase family in the cardiomyocyte (Fig. 3). Both the 44- and 42-kDa MAP kinases (also called ERK1 and ERK2, respectively) were phosphorylated within 1 min of treatment with angiotensin II. The degree of phosphorylation reached a peak at about 10 min and then fell to near basal levels by 30 min (data not shown). Subsequently, angiotensin II (1 nM) also enhanced the phosphorylation of a 29-kDa protein by 43 ± 6%. As seen in Fig. 4, this protein is an in vivo substrate of protein kinase C. This effect was apparent even after 24 h of angiotensin II (1 nM) exposure when protein phosphatase 1 activity was reduced by incubating the cell medium with 25-mM glucose. The phosphorylation of this protein was prevented by the downregulation of protein kinase C, which was achieved through the incubation of the cells for 24 h with medium containing 100-nM myristate acetate (PMA) (Fig. 4.).

It is known that cardiac hypertrophy in vivo and in vitro are accompanied by changes in muscle phenotype, characterized by the expression of "fetal" type genes such as ANP. Therefore, as a marker of cardiac hypertrophy, we examined the upregulation of ANP by angiotensin II (Fig. 5). Although ANP mRNA was present under control con-

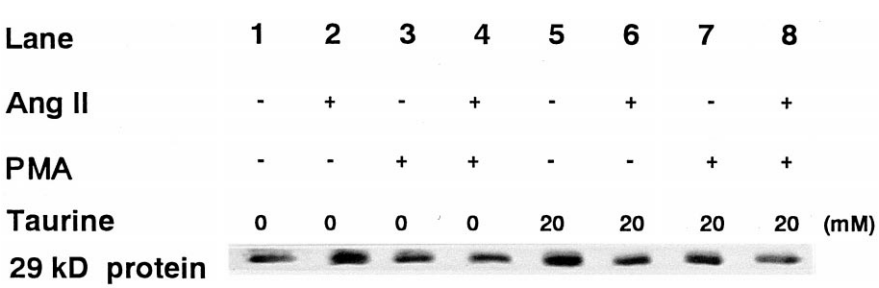

Fig. 4. Effect of taurine on the phosphorylation status of a 29-kDa protein kinase C substrate. Isolated cardiomyocytes were incubated for 3 days with medium containing either 0- or 20-mM taurine. On the last day, the taurine untreated and treated cells were exposed to either 1-nM angiotensin II (lanes 2 and 6), 100-nM phorbol myristate acetate (lanes 3 and 7), the combination of 1-nM angiotensin II and 100-nM phorbol myristate acetate (lanes 4 and 8) or no additions (lanes 1 and 5). The cells were harvested and examined for the phosphorylation status of a 29-kDa protein using Western blot analysis. Representative data are shown in the figure.

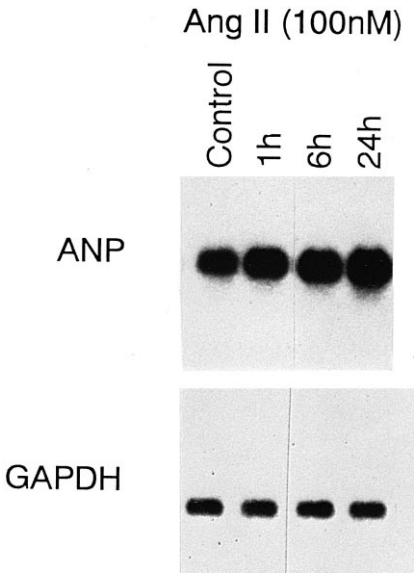

Fig. 5. Effect of angiotensin II on GAPDH and ANP expression of neonatal rat cardiomyocytes. Cardiomyocytes were incubated with medium containing 100-nM angiotensin II for the indicated period of time. RNA was isolated from the cells and subjected to Northern blot analysis using cDNA probes for both ANP and GAPDH mRNA. Shown is the autoradiogram, in which equal amounts of RNA were loaded onto the gel for each time point. The data shown are representative data for three experiments.

ditions in the neonatal cardiomyocyte, expression increased significantly following a 24-h incubation with angiotensin II (100 nM).

3.3. Inhibitory action of taurine on angiotensin II-induced hypertrophy of the cardiomyocyte

The effect of taurine treatment on angiotensin II-induced hypertrophy of the cardiomyocyte was examined in cells initially prepared in serum-free medium containing 20-mM taurine and then stimulated with 100-nM angiotensin II for a period of either 24 or 48 h in the presence of taurine. Fig. 6 summarizes the changes in the morphological status of cardiomyocytes treated with angiotensin II in the presence and absence of taurine. Whereas angiotensin II (100 nM) significantly increased cardiomyocyte cell size by 25% and 40% after 24 and 48 h of exposure, respectively, taurine prevented the size change (Fig. 6). Cell morphology and cell surface area were not significantly altered by the treatment with taurine alone (data not shown).

In addition to altering cell size, taurine treatment also suppressed the upregulation of the ANP gene by angiotensin II (Figs. 7 Figs. 8). This was not related to a direct effect of taurine since treatment with taurine alone exerted no influence on the basal levels of ANP gene expression.

Because TGF-β_1 is known to be a potent inducer of fetal genes in the neonatal rat cardiomyocyte, we also examined the influence of angiotensin II on TGF-β_1 mRNA levels. As shown in Fig. 8, a significant induction of TGF-β_1 mRNA was observed after angiotensin II treatment. Taurine inhibited the upregulation of TGF-β_1 but had no effect on TGF-β_1 expression in the absence of angiotensin II.

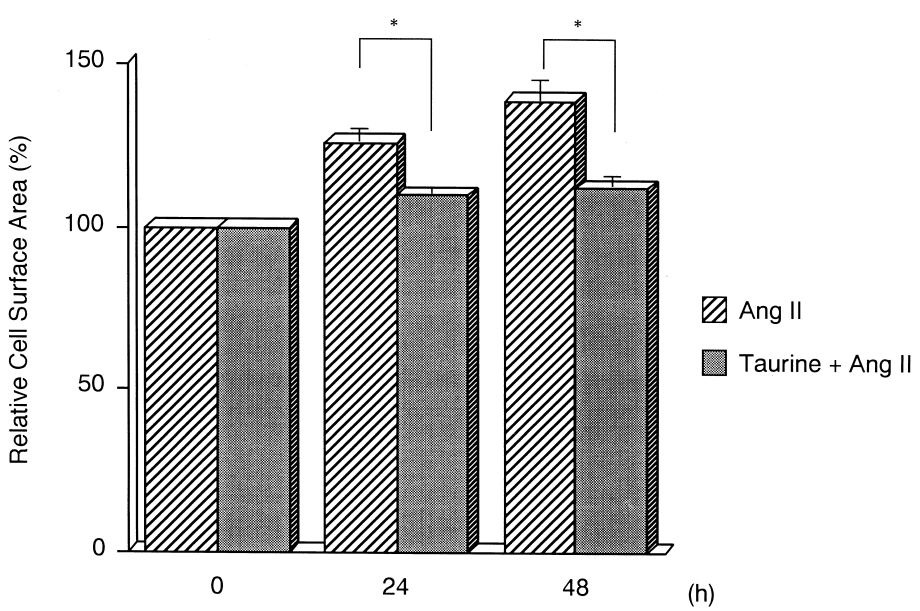

Fig. 6. Effect of taurine on angiotensin II-induced cellular hypertrophy. Rat neonatal cardiomyocytes were isolated in buffer containing 20-mM taurine and then exposed to buffer containing 20-mM taurine and 100-nM angiotensin II. Cell sizes of the taurine treated cells were compared with cells treated in the absence of taurine, according to the procedure described in Fig. 2. The change in cell surface area was estimated by planimetry. Data shown represent means ± SEM of 38–52 cells obtained from three different primary cultures. $^{*}P < 0.05$ vs. angiotensin II by *t*-test.

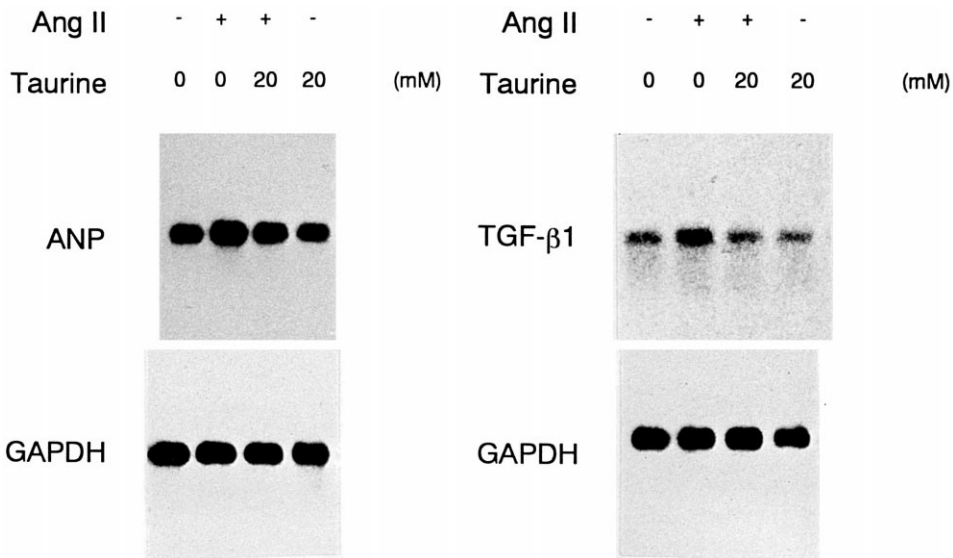

Fig. 7. Representative Northern blots of the effect of taurine on angiotensin II-induced upregulation of ANP and TGF-β_1. Cardiomyocytes were prepared in the presence or absence of taurine as described in Section 2.1. They were then exposed to 100-nM angiotensin II for 24 h in medium containing or lacking 20-mM taurine. Northern blots of RNA were prepared and analyzed as described in Fig. 5. The data shown are representative data for three experiments.

Several steps in the angiotensin II signal transduction pathway are potential sites of taurine action. Sadoshima and Izumo (1993b, 1995) have proposed that angiotensin II promotes cell hypertrophy though a protein kinase C and/or MAPK pathway. Since taurine blocks angiotensin II-induced stimulation of protein synthesis, we estimated the effects of taurine on angiotensin II-mediated stimulation of MAPK and protein kinase C activity, respectively. As seen in Fig. 3, angiotensin II activates the MAP kinases, ERK1 and ERK2 through phosphorylation mechanism. However, pretreatment of the cells with 20-mM taurine did not block angiotensin II-induced phosphorylation of the two MAP kinases (Fig. 3). On the other hand, taurine acted to limit the phosphorylation state of a 29-kDa protein kinase C substrate by angiotensin II; angiotensin II phosphorylation was reduced from $43 \pm 6\%$ to $10 \pm 5\%$ in the presence of taurine (Fig. 4). The effect of taurine on angiotensin II was incomplete, since PMA returned the

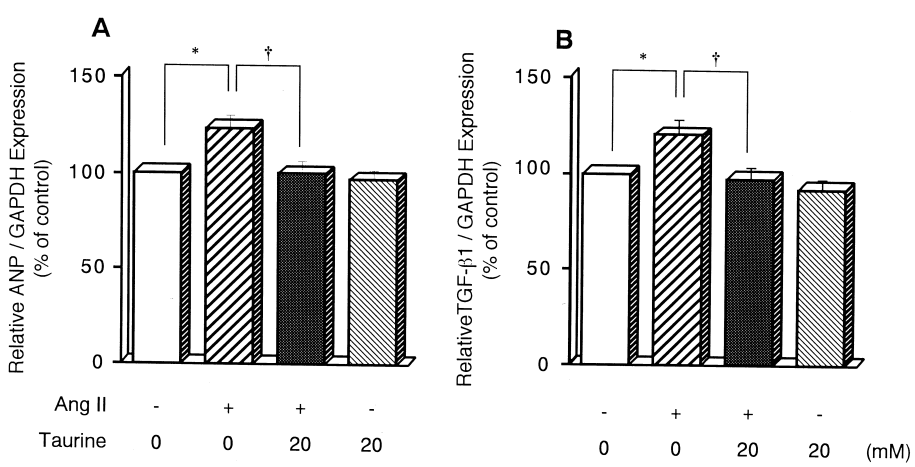

Fig. 8. Effect of taurine on angiotensin II-induced upregulation of ANP and TGF-β_1. Cardiomyocytes were prepared in the presence or absence of taurine as described in Section 2.1. They were then exposed to 100-nM angiotensin II for 24 h in medium containing or lacking 20-mM taurine. Northern blots of RNA were prepared and analyzed as described in Fig. 5. Data are expressed as %control, in which the control cells were exposed to neither taurine nor angiotensin II. All results represent means $\pm$ SEM of 8–13 samples obtained from three different primary cultures. $^{*}P < 0.05$ vs control; †$P <$ 0.05 vs 20-mM taurine plus 100-nM angiotensin II by *t*-test, respectively.

phosphorylation band to control levels. Taurine itself increased the phosphorylation of this protein, an effect reversed by PMA.

4. Discussion

Since cardiomyocytes are terminally differentiated and lose their ability to duplicate soon after birth, they respond to a rise in workload by increasing cell size (hypertrophy) rather than increasing cell number (hyperplasia) (Komuro and Yazaki, 1993). In agreement with this notion, we have previously shown that angiotensin II neither increases DNA synthesis nor cell number when added to cardiomyocytes in culture. However, angiotensin II significantly increases the rate of protein synthesis of the myocyte (Takahashi et al., 1997; Sadoshima et al., 1993). Moreover, as seen in the present study, angiotensin II induces hypertrophic growth of the cardiomyocyte, as reflected by changes in cell morphology and phenotype. Particularly noteworthy is the extent of the cell size increase noted 48 h after exposure to either 1 or 100 nM angiotensin II. Preceding this morphological change is a change in cell phenotype. We found that within 10 min of angiotensin II (100 nM) exposure, ERK1/2 is activated and there is an upregulation of ANP, the latter being a marker of the fetal phenotype.

The most important finding of this study is the observed interaction between taurine and angiotensin II in the cultured cardiomyocytes. Whereas exposure of the isolated myocyte to taurine (20 mM) in the absence of angiotensin II has no effect on cell size or cellular phenotype status, pretreatment with 20-mM taurine for 24 h reduces angiotensin II responsiveness relative to growth, ANP upregulation and TGF-β_1 expression. Accordingly, taurine exerts a beneficial effect relative to angiotensin II-induced changes in both the morphology and in the molecular status of the cardiomyocyte.

In this study, we also confirmed the work of Sadoshima et al. (1995), showing that angiotensin II activates the extracellular signal related kinases (ERK1/2) in the cardiomyocyte. ERK1/2 belongs to a family of serine/threonine kinases known as the MAP kinases. Also belonging to the MAP kinase family are stress-related kinases (c-*Jun* N-terminal protein kinase: JNK or stress-activated protein kinase: SAPK) and the p38 kinases. While the extracellular signal-related kinases are activated by oncogenes and growth factors, the stress-related kinases are activated in response to stress, cytokines and growth factors (Anderson et al., 1991; Nemenoff et al., 1993; Pulverer et al., 1991). The signaling pathway leading to the activation of p38 remains to be elucidated, although it is initiated by some cytokines, growth factors and stress. Previous studies have shown that angiotensin II is capable of activating both JNK (Kudoh et al., 1997) and the ERK1/2 MAP kinases (Booz et al., 1994; Sadoshima et al., 1995). Although not directly examined, there is every reason to believe that the p38 MAP kinase pathway will also be activated by angiotensin II since other Gq-linked hypertrophic agents activate p38 kinase (Nemoto et al., 1998).

The MAP kinase family of enzymes transduce signals from the cell membrane to the nucleus and therefore are thought to play central roles in the development of hypertrophic and transformed cardiomyocytes. ERK1/2 activates several protein kinases, including ribosomal S6 kinase and transcription factors, such as Elk. Although activation of the ERK pathway is necessary to induce cellular transformations in some tissues, ANP expression does not depend upon an active ERK pathway in the heart (Post et al., 1996). Moreover, rapamycin inhibits angiotensin II-induced stimulation of protein synthesis without affecting angiotensin II-induced activation of ERK1/2 (Sadoshima and Izumo, 1995). These data are consistent with the present finding that taurine inhibits the expression of ANP by angiotensin II without affecting the activation of ERK1/2. Thus, ERK1/2 activation does not appear to be required for many of the molecular actions of angiotensin II.

While the ERK1/2 pathway is proliferative, the JNK pathway is antiproliferative and proapoptotic (Schmitz and Berk, 1997). Interestingly, if taurine was capable of preventing the activation of the JNK pathway by angiotensin II, it would represent one mechanism by which the angiotensin II-induced upregulation of ANP could be blocked. However, since JNK is proapoptotic, it is unlikely that taurine acts through this mechanism because it would be inconsistent with the general properties and actions of taurine.

Many of taurine's actions are linked to the modulation of calcium homeostasis. In fact, taurine attenuates the angiotensin II-induced elevation in $[Ca^{2+}]_i$ (Takahashi et al., 1997). This is important because the rise in $[Ca^{2+}]_i$ activates pathways that contribute to the hypertrophic response of angiotensin II. It has previously been assumed that the activation of ERK1/2 is an important step in one of these calcium-dependent pathways. However, Booz et al. (1994) have argued that ERK1/2 can be activated by either a calcium- or a protein kinase C-dependent pathway. This observation explains the present finding that taurine blocks the angiotensin II-induced elevation in $[Ca^{2+}]_i$ but does not block the activation of ERK1/2.

In a previous study, we also reported that taurine suppresses the activation of c-*fos* by angiotensin II. This is presumably tied to the elevation in $[Ca^{2+}]_i$ since the introduction of the calcium chelator, 1,2-bis (2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis acetoxymethyl ester (BAPTA-AM), into the angiotensin II-stimulated cell, prevents both the rise in $[Ca^{2+}]_i$ and the upregulation of c-*fos* via a protein kinase C-dependent pathway (Sadoshima and Izumo, 1993b; Sadoshima et.al., 1995). In the present study, taurine acted to limit the phosphorylation of the protein kinase C substrate by an-

giotensin II. The effect of taurine on angiotensin II is incomplete, since PMA returns the phosphorylation band to control levels. Although taurine prevented the activation of protein kinase C by angiotensin II, this effect was not straightforward because taurine itself enhanced the phosphorylation of the band, in part through protein kinase C. Concerning the influence of taurine to the phosphorylation of 29-kDa protein, we could not provide an explanation for the data because the nature of the 29-kDa protein needs to be identified. The mechanism by which taurine-induced changes in $[Ca^{2+}]_i$ and protein kinase C activity affect c-*fos* expression, is an active area of research.

The present results indicate that taurine is an effective inhibitor of angiotensin II action. It is plausible that the beneficial effect of taurine in the treatment of heart failure could relate to its suppression of angiotensin II-mediated cellular responses.

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