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Reduction of angiotensin II-induced activation of mitogen-activated protein kinase in cardiac hypertrophy

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Abstract. Mitogen-activated protein (MAP) kinases play a role in cell growth and are activated in the heart by cardiac stretch and various growth factors, but their role in signal transduction pathways once the heart has undergone hypertrophy is uncertain. To investigate the regulation of MAP kinases in the heart in response to angiotensin II (ang II), once cardiac hypertrophy has become established, ventricular and skeletal muscle explants were studied from Dahl S salt-sensitive and Dahl R salt-resistant rats that were on a high (6% NaCl) salt supplement in their diet. Cardiac hypertrophy was produced in the Dahl S but not R rat through NaCl-induced hypertension. MAP kinases were assayed by myelin protein phosphotransferase activity in MonoQ fractions of cell extracts. Ang II increased MAP kinases mainly in extracts from nonhypertrophic ventricles of Dahl R rats on a high-salt diet. Immunoblots revealed predominantly $p44^{ERK1}$ with lower amounts $p42^{MAPK}$ in rat ventricle, and no apparent changes with hypertrophy. In hypertrophied hearts, ang II-induced MAP kinase activity was less markedly increased and more rapdily fell to baseline levels in comparison to the response in nonhypertrophied hearts. Prolonged ang II exposure did not produce the same effect on MAP kinase activity in ventricles from Dahl S rats on a low-salt diet, or skeletal muscle from salt-fed Dahl R and S rats. The ability of phorbol myristate acetate to simulate MAP kinase and ang II to simulate translocation of protein kinase C from the cytosole to the membrane was similarly compromised in hypertrophied ventricles. These results are consistent with a disturbance in the regulation of cell-signalling pathways in cardiac hypertrophy in which the MAP kinase response to ang II is dramatically altered.

Key words. Mitogen-activated protein (MAP) kinase; cardiac hypertrophy; Dahl rat; angiotensin II.

Mitogen-activated protein (MAP) kinases function as key molecules in signalling processes stimulated by growth factors and in cell growth [1–4]. MAP kinases are subject to regulatory control by various processes including activation by phosphorylation on tyrosine and threonine residues through stimulation of MAP kinase activators which are also protein kinases [1, 2, 5, 6]. MAP kinase kinase is activated by various upstream kinases such as $p74raf_1$ [1, 5–7]. The function(s) of MAP kinases in the heart have not been well characterized. Cardiac hypertrophy is analogous to cell growth in various other cell types, as cardiomyocytes do not divide but respond to growth factors by increases in cell size or hypertrophy. A role for MAP kinases in cardiac hypertrophy is suggested by MAP kinase activation by cardiac stretch [8, 9], a potent stimulant of hypertrophic cardiac cell growth. MAP kinases can be activated by

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stimulation of cardiac cell surface receptors such as endothelin, fibroblast growth factors and α_1-adrenergic receptors [10–12] that also induce cardiac hypertrophy. Inhibition of MAP kinases prevent α_1-adrenergic receptor-induced gene expression in cardiomyocytes [11].

The octapeptide angiotensin II (ang II) exerts a spectrum of effects on the cardiovascular system that may include cardiac hypertrophy [13, 14]. A role for ang II in myocardial hypertrophy is supported by the data that ang II stimulates cardiac protein synthesis [14, 15]. Ang II activates MAP kinases in neontal rat cardiac cells [16, 17] and in vascular smooth muscle cells [18, 19]. Although MAP kinases are implicated in the process of cardiac hypertrophy, this is based on the study of normal cardiac cells. Inferences concerning the role of agonist signal transduction pathways from normal (cardiovascular) cells may be at variance with their actions in pathologic conditions where the pathways may no longer be relevant. Some agonists that stimulate cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent pathways can produce cardiac hypertrophy after acute exposure and induce downregulation of these same pathways once hypertrophy is established [20–22]. We have demonstrated that the effect of ang II to modulate cAMP signalling is markedly perturbed in hypertension-induced cardiac hypertrophy in the salt-sensitive Dahl S rat model of cardiac hypertrophy when compared with the genetically otherwise similar salt-resistant Dahl R rat that develops minimal cardiac hypertrophy on a high-salt diet [22, 23]. Cardiac hypertrophy is an important biological problem, as it is one of the most powerful indicators of morbidity and mortality in patients with hypertension [24]. We sought to test the hypothesis that the regulatory control of MAP kinase activation by ang II is altered by the process of cardiac hypertrophy.

Methods

Dahl rats. Sixteen inbred Dahl SR/Jr and 16 SR/Jr rats from Harlan Sprague Dawley (Indianapolis, IN, USA) were maintained on a diet of powdered Purina Labchow supplemented with an additional 6% NaCl for 9 to 10 weeks from weaning. The protocol was approved by the University Committee on animal care. Animals were permitted to drink tap water ad libitum. The degree of ventricular hypertrophy was assessed by determining the ventricle to total body weight ratio (mean $\pm$ 1 SEM), which was 2.9 ± 0.2 g/kg for 6% NaCl-fed Dahl R rats and 4.3 ± 0.4 g/kg for 6% NaCl-fed Dahl S rats. Two inbred Dahl SR/Jr and two inbred SR/Jr rats were maintained on the same diet without additional 6% NaCl and studied 9–10 weeks from weaning.

Stimulation of hearts. Rats were anaesthetized using 2.5% Halothane before sacrificing by cervical dislocation. Hearts were immediately removed and placed in oxygenated, 37 °C Dulbecco's Minimal Essential Medium (DMEM). Atria, major vessels and visible connective tissue were removed from the ventricles, the ventricles weighed before being minced into cubes approximately 1 mm^3 and the pieces rinsed $2\times$ in medium to remove blood. In some experiments up to three hearts from identical strain, sex and ages were mixed together. The pieces were divided up and incubated with 1 μM ang II or 100 nM phorbol myristate acetate (PMA) for 2 to 60 min at 37 °C in the presence of 95% O_2 and 5% CO_2.

Preparation of ventricular extract. After the period of stimulation, DMEM was aspirated from ventricular pieces. The pieces were rinsed with ice-cold homogenization buffer A (125 mM β-glycerol phosphate, pH 7.2, 12 mM EDTA, 5 mM EGTA, 2 mM sodium orthovanadate, 1 mM dithiothreitol and 1 mM phenylmethylsulphonyl fluoride) and then placed in 1 ml of buffer A. Samples were homogenized using a microtip-equipped Polytron (Brinkman Instruments) at setting 7 for three bursts of 10 s per burst, in an ice bath. The homogenates were centrifuged at 200,000*g* for 15 min in a Beckman TL-100 centrifuge, and the resultant supernatant fractions (cytosol) were either processed immediately or first stored frozen at -70 °C. The pellets were re-homogenized in 1 ml of buffer A that also contained 1% Nonidet, and the detergent-solubilized extract was obtained following centrifugation at 200,000*g* for 15 min and also stored frozen at -70 °C.

MonoQ chromatography. For MonoQ chromatography, $\sim$5 mg of cytosolic protein as loaded onto a MonoQ (1 ml) column equilibrated in column buffer B (25 mM β-glycerol phosphate, 10 mM MOPS, pH 7.2, 5 mM EGTA, 2 mM MgCl, 1 mM dithiothreitol and 2 mM sodium orthovanadate), at a flow rate of 1 ml/min. The column was developed with a 10-ml linear 0–0.8 M NaCl gradient in buffer B at 1 ml/min using a Pharmacia fast protein liquid chromatography system, and 250-μl fractions were collected.

Kinase and protein assays. All kinase assays were performed after MonoQ fractionation of the ventricular extracts. MAP kinase activity with myelin basic protein (MBP) as a substrate was assayed as described previously [25]. Protein kinase C (PKC) activity toward histone H1 (Sigma type III-S) was measured as reported [26]. Using bovine serum albumin (BSA) as a standard, the protein concentrations of the extracts were determined by the method of Bradford [27].

Electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on 1.5-mm thick gels using the buffer system described by Laemmli [28]. An 11% separating gel and a 4% stacking gel were used. Samples were boiled for 5 min in the presence of $5\times$ concen-

trated SDS-sample buffer (125 mM tris-HCl, pH 6.8, 4% SDS, 0.01% bromophenol blue, 10% mercaptoethanol and 20% glycerol) and electrophoresed for 17 h at 10 mA.

Immunoblotting. Affinity-purified rabbit polyclonal antibody developed against a synthetic peptide patterned after the C-terminus of rat extracellular regulated protein kinase 1 (ERK1) (ERK1-CT, CGG-P F T F D-M E L D D L P K E R L K E L I F Q U E T A R F O Q P G-APEAP). Rabbit polyconal anti-$p44^{MARK}$ antibodies, affinity-purified on a $p44^{MARK}$-agarose column were prepared as described [26]. Column chromatography fractions of ventricular cytosol were subjected to SDS-PAGE. Subsequently, the separating gel was soaked in transfer buffer (25 mM tris, 192 mM glycine, 20% methanol) for 5 min and then sandwiched with a nitrocellulose membrane. Proteins were transferred for 3 h at 300 mA. Subsequently, the nitrocellulose membrane was blocked with TBS (tris-buffered saline) containing 5% skim milk for 2 h at room temperature. The membrane was washed twice with TBS containing 0.05% Tween 20 (TTBS) for 5 min before incubation with rabbit polyclonal antibodies for MAP kinase (in 1% skim milk-TTBS; 1:1000 dilution) overnight at room temperature. The next day, the membrane was washed twice with TTBS before incubation with the second antibody [goat anti-rabbit immunoglobulin G (IgG) or goat anti-mouse IgG coupled to alkaline phosphatase in 1% skim milk-TTBS or 1% BSA-TTBS, respectively; 1:3000 dilution] for 2 h at room temperature. The membrane was rinsed with two washes of TTBS, followed by one wash with TBS before incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma) colour development solution (mixture of 3% nitro blue tetrazolium in 1 ml of 70% dimethylformamide and 1.5% 5-bromo-4-chloro-3-indolyl phosphate in 1 ml of 100% dimethylformamide before adding to 100 mg of 0.1 M $NaHCO_3$, 10 mM $MgCl_2$, pH 9.8). The colour was developed in 5 min to 4 h, and the reaction was stopped by rinsing the membrane in a large volume of water.

Results

Ang II stimulated MBP kinase activity within 5 min treatment of ventricular explants from the Dahl R rats. This activity was confirmed to be attributed to MAP kinases, since the peak of MBP phosphorylation was eluted from MonoQ columns (fraction numbers 29–31), with 0.3 M NaCl (fig. 1A), a salt concentration from which the MAP kinase isoforms $p42^{MAPK}$ and $p44^{ERK1}$ have previously been shown to be consistently released from this resin [25, 26]. Further evidence that the ang II-stimulated MBP phosphototransferase activity peak corresponded to MAP kinases was also provided by immunoblotting analysis of the MonoQ fractions with MAP kinase antibodies. The ERK1-CT antibody strongly reacted with proteins of 42 and 44 kDa that eluted in fraction numbers 29–31 from the MonoQ columns. The 42- and 44-kDa proteins were also visualized, to a lesser extent, with another antibody raised against the purified sea star MAP kinase $p44^{MAPK}$. In contrast, in ventricular explants from the Dahl S animal, on a high-salt diet, MBP kinase activity was minimally increased by ang II. The reduction in MAP kinase activity in ventricular explants from hypertrophic hearts of Dahl S rats (fig. 1B) was not due to decreases in the amount of $p42^{MAPK}$ and $p44^{ERK1}$, which were comparable in Dahl R and Dahl S rat ventricular explants (fig. 1C). The ERK1 band appears as a 'tight' band with slower mobility in the Dahl R rat samples, whereas in the Dahl S, it is a broad band with increased mobility. This indicates that in the Dahl R rats, the majority of the ERK1 was in phosphorylated form (indicated by the slower mobility) and activated form, whereas in the Dahl S rats, there was the presence of both phosphorylated and nonphosphorylated (inactive) forms.

Ang II produced an initial increase in MAP kinase activity that declined with prolonged ang II treatment, and after 1 h MAP kinase activity was slightly below control (untreated) levels (fig. 2). The concentration of 1 μM ang II was determined to be the optimal dose based on preliminary ang II concentration-response data for MAP kinase activation. In ventricular explants from hypertrophied hearts from the Dahl S rats, on high-salt diets, MBP kinase activity was less than in Dahl R rats on high-salt diets. In hearts from Dahl S rats, ang II for 5 min only partly stimulated MAP kinase activity, as it was less than one-half the activity compared with ang II-treated Dahl R rat hearts.

When skeletal muscle explants from high-salt-fed Dahl S and R rats were incubated with ang II for 20 min, MBP kinase activation was observed (fig. 3). In contrast to cardiac muscle, there was no blunting of the MBP kinase response in the Dahl S compared with the Dahl R rat. Interestingly, there appeared to be a greater increase in MBP phosphorylation on eluates 29–31 in Dahl S compared with Dahl R rats.

To determine whether the MAP kinase response was specific for ang II, ventricular explants from high-salt-fed animals were treated with phorbol ester, PMA (fig. 4). PMA, 100 nM, which directly activates PKC, produced an activation of MAP kinase in Dahl R ventricular explants comparable to what was achieved with ang II. Like ang II, PMA did not activate MAP kinase activation in the Dahl S ventricular explants. Indeed, a decrease in MAP kinase activity was observed.

To determine whether the effect of ang II was dependent on high-salt diet, Dahl S and R rats were kept on the same diet without supplementation with NaCl. The MAP kinase response to ang II was seen in ventricular explants of Dahl S and R rats (fig. 5), and the threefold

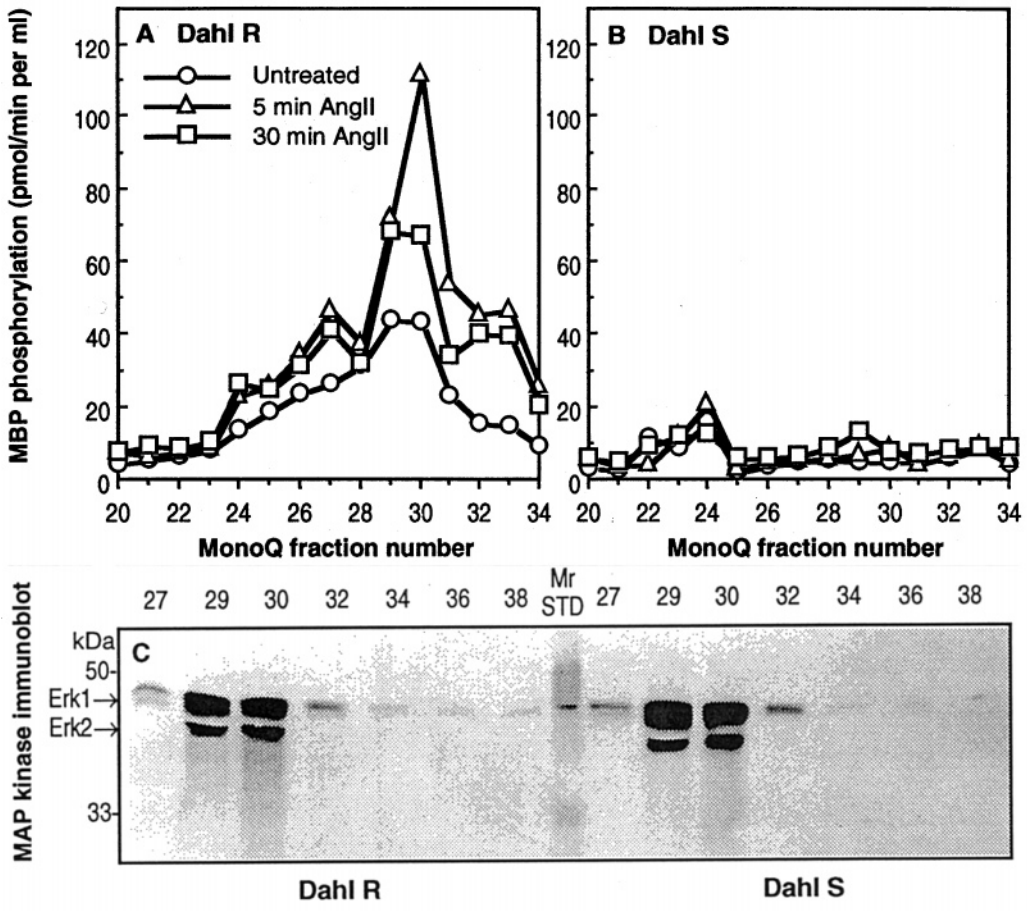

Figure 1. (*A*) MonoQ chromatography of extracts from high-salt-fed Dahl R and (*B*) results from Dahl S (right) ventricle explants. Cytosolic protein (5 mg) from control explants (0) and those treated with 1 μM ang II for 5 or 30 min. Column fractions were assayed for phosphotransferase activity toward 1 mg MBP/ml. (*C*) Immunoblotting analysis of high-salt-fed Dahl R (left) and Dahl S (right) rat ventricular extracts with MAP kinase antibody. MonoQ column fractions from ang II-treated explants (*A*, *B*) were subjected to Westrn blotting analysis with anti-ERK1-CT peptide antibodies. Panel C is from samples electrophoresed on the same slab. The electrophoretic migrations of the prestained marker proteins (STD) ovalbumin (50 K) and carbonic anhydrase (33 K) are shown. The data are from a pair of Dahl S and R rats with the S rat shown 140% the weight of the R rat heart. Similar results were obtained in three separate experiments.

response to ang II in Dahl S rats was similar to that in Dahl R rats. The lower response in Dahl S rats may be due to the mild cardiac hypertrophy in Dahl S rats even on a low-salt diet [22]. To determine whether the MBP kinase response was specific to ang II, ventricular explants, from high-salt-fed animals, were treated with ang II, and PKC activity was measured in cytosolic and membrane fractions. In ventricular extracts from Dahl R rats, ang II, for 20 min, produced a partial conversion of PKC from its cytosolic form to a membrane-bound form (fig. 6), which is thought to be indicative of activation of this kinase. In contrast, similar ang II treatment of ventricular explants from hypertrophied hearts from high-salt-fed Dahl S rats did not activate PKC and appeared to have the opposite effect on the subcellular distribution of PKC, i.e. less PKC was membrane-associated and more was cytosolic (fig. 7). There was a significant ($P < 0.05$, t test) difference between R and S rats in the change in membrane PKC activity in response to ang II.

Discussion

Ang II-induced activation of MAP kinase was demonstrated in ventricular extracts from adult rat heart. Although ventricular explants contain both myocyte and fibrocyte elements, each has been shown in culture to respond to ang II with an increase in MAP kinase activity in neonatal rat heart [16, 17]. In the Dahl rat ventricle at least two MAP kinases of 42 and 44 kDa were evident with MAP kinase antibodies. The 42-kDa

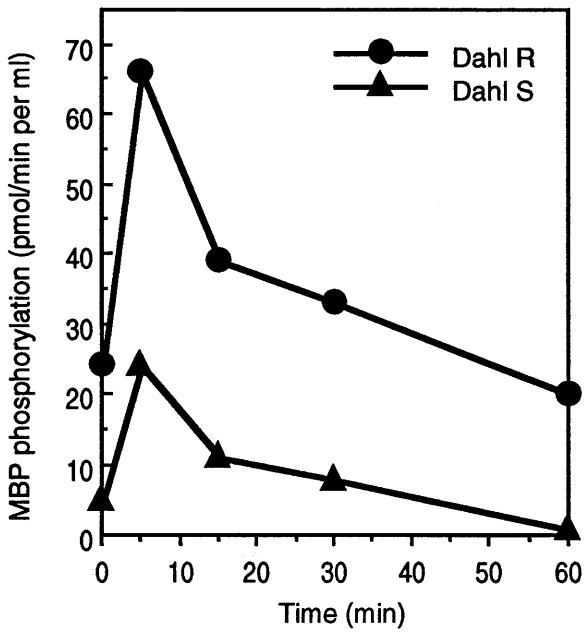

Figure 2. The time course of MAP kinase activation is shown by MonoQ chromatography assayed for phosphotransferase activity in extracts, treated with 1 μM ang II, from rat ventricle from another pair of high-salt-fed Dahl R and Dahl S rats.

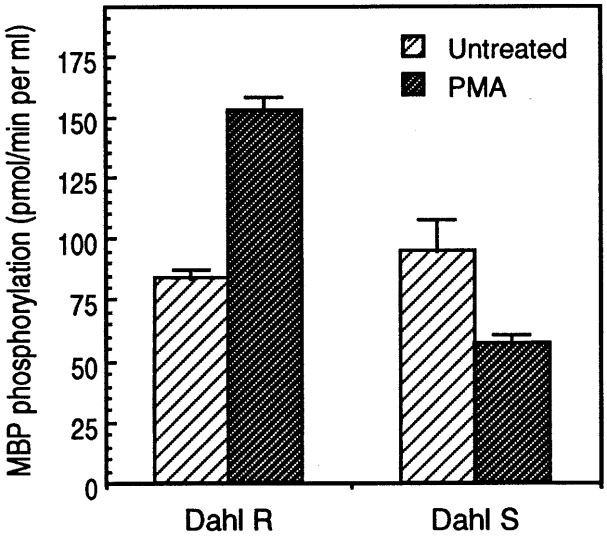

Figure 4. MBP phosphotransferase activities in the peak MonoQ fractions corresponding to the p42 and p44 MAP kinases were determined as described in the legend to figure 1. Ventricular explants were treated with PMA for 20 min ($n = 3$) or untreated ($n = 3$). The data are presented as the mean $\pm$ 1 SEM.

species can be almost unequivocally identified as $p42^{MAPK}$ or ERK2 based upon its size [26]. The doublet of ~44 kDa most likely corresponded to the phosphorylated and dephosphorylated forms of $p44^{ERK1}$. The latter isoform was the predominate MAP kinase in the ventricular extracts, since the two MAP kinase antibodies employed in this study display similar degrees of immunoreactivity to these kinases. In other systems such as human A431 cells [25] and rat B lymphocytes [29], these same antibodies revealed higher levels of

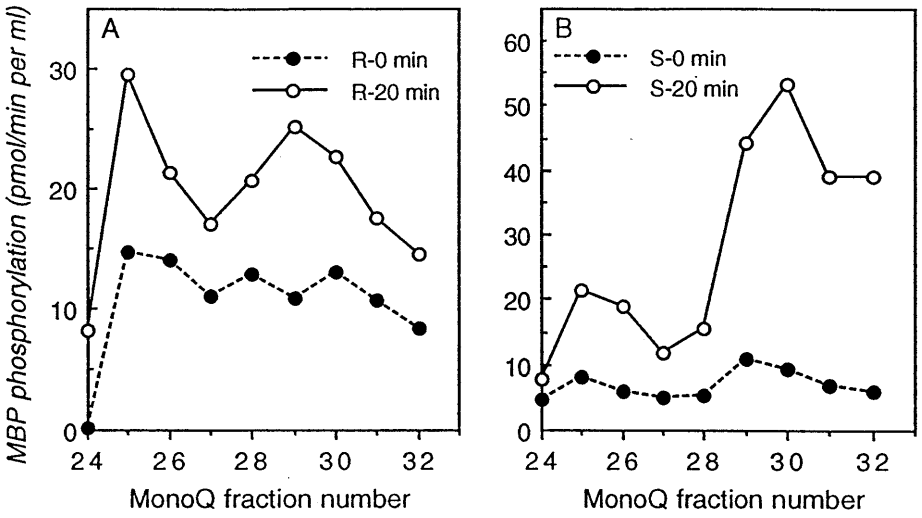

Figure 3. The MAP kinase response in skeletal muscle to ang II is shown in high-salt-fed Dahl R (*A*) and Dahl S (*B*). The MonoQ chromatography of skeletal muscle extracts from high-salt-fed Dahl R and Dahl S rats. Cytosolic protein (5 mg) from control explants (0) and those treated with 1 μM ang II for 20 min were loaded on the columns.

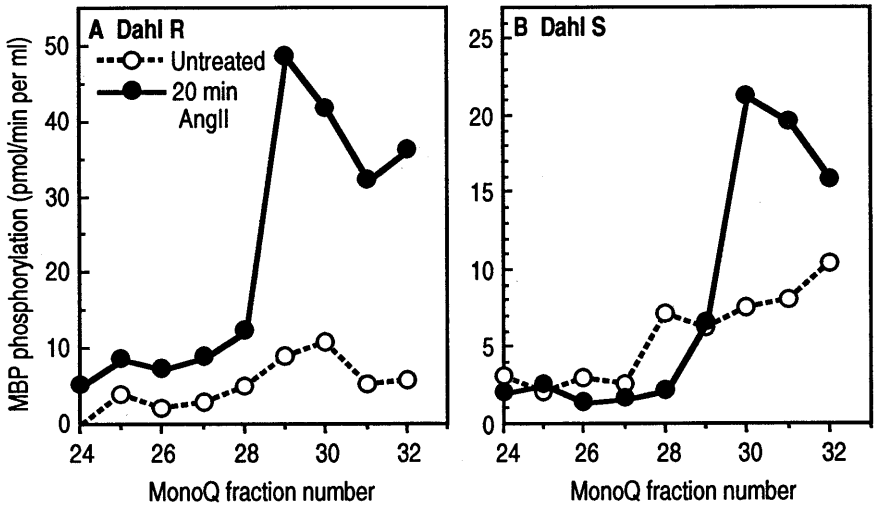

Figure 5. The MAP kinase response in ventricular muscle to ang II is shown in a pair of low-salt-fed Dahl R (*A*) and Dahl S (*B*). Cytosolic protein (5 mg) from control explants (0) and those treated with 1 μM ang II for 20 min were subjected to MonoQ chromatography.

$p42^{MAPK}$ than $p44^{ERK1}$. Ang II-induced activation of MAP kinase in the ventricular myocardium of the Dahl R was primarily directed toward $p44^{ERK1}$. A protein kinase C-dependent pathway may be responsible for $p44^{erk1}$ activation in response to ang II, since PMA, which is a potent activator of protein kinase C (PKC), produced a degree of MAP kinase activation that was comparable to that produced in response to ang II. PKC-dependent pathways are triggered by ang II-induced activation of a specific phospholipase C-catalysed hydrolysis of phosphatidylinositor-4,5-bisphosphate [30, 31]. Furthermore, ang II-induced PKC activation was demonstrated in these hearts.

In the hypertrophic ventricle, the degree and duration of ang II activation of $p44^{ERK1}$ was reduced as compared with the nonhypertrophic rat ventricle. Speculation on a mechanism would include downregulation of the MAP kinase pathway in cardiac hypertrophy. In yeast, the MAP kinase homologue, FUS3, downregulates its own phosphorylation pathway [32]. Thus MAP kinase activation produced by acute cardiac stretch or growth factors [8, 9] may have a negative feedback role through MAP kinase-mediated phosphorylation of upstream kinases. A more generalized effect on upstream factors is also suggested by the similar effect produced by phorbol ester and ang II in the Dahl S rat on a high-salt diet.

Prolonged incubation with ang II appeared to be associated with a faster disappearance of the MAP kinase activity in hypertrophied compared with nonhypertrophied hearts. Since MAP kinase proteins were unaffected by hypertrophy, ang II-induced induction and later reduction of MAP kinase activity in the Dahl S heart did not reflect proteolysis of $p44^{ERK1}$ or $p42^{MAPK}$. Rather, a post-ang II receptor-binding step in the MAP kinase activation pathway may be compromised in the myocardium of Dahl S rats by a negative regulatory mechanism that is less apparent in Dahl R rats. Alternatively, ang II-induced phosphatases [33] may be augmented in heart of Dahl S rats on high-salt diets. A MAP kinase phosphatase induced by growth factors and phorbol ester reaches a maximum approximately 30 min after stimulation [34], a time frame consistent with increased inactivation of MAP kinase in Dahl S rats on high-salt diets. The altered ang II signal transduction that led to MAP kinase inhibition appeared to be specifically linked to ventricular hypertrophy. It is not present in the skeletal muscles or hearts of Dahl S rats fed low-salt diets. The limited ability of PMA to stimulate MAP kinase activation in the Dahl S rat heart implies that PKC may be modified in the hypertrophic ventricle such that altered PKC regulation in the hypertrophic ventricular explants could account for the loss of ang II-induced MAP kinase activation. It has been proposed that activation of PKC, when it occurs simultaneously with activation of an src-like tyrosine kinase pathway, can antagonize activation of MAP kinase kinase [35].

Although there are a number of different PKC isoforms, we assessed the effect of ang II on calcium-sensitive PKC isoforms. Of the various kinds of PKC present in the heart, only the alpha- and beta-PKC isoforms are known to be calcium-sensitive [36]. We have found that the alpha-PKC isoform is the dominant one in rat cardiomyocytes (unpublished data).

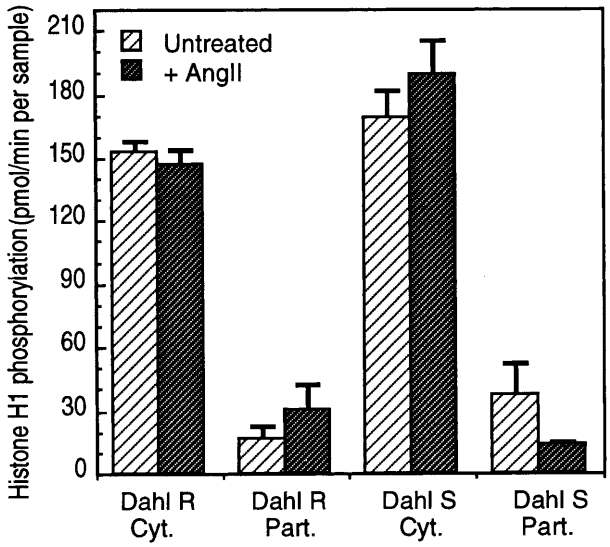

Figure 6. Effect of ang II-induced translocation of protein kinase C in ventricular explants treated with 1 μM ang II for 5 or 30 min and control explants (0). Samples containing 5 mg of protein from cytosol and microsomal fractions were loaded on MonoQ columns and each separately subjected to MonoQ chromatography. The Ca^{2+}/diolein/phosphatidylserine-stimulated histone H1 phosphotransferase activities of PKC in the monoQ fractions were determined. The data represent separate column fractionations and set of kinase activity from a pair of Dahl S and R rats. Measurements were also made both in the absence and presence of calcium, phospholipid and diacylglycerol.

The Dahl rat was selected for these studies because it is a model of genetic hypertension that develops cardiac hypertrophy with an appropriate control. The Dahl S rat has low plasma renin and ang II, which leads to enhanced response to ang II [37] and should, in principle, lead to enhanced tissue response to in vitro ang II stimulation.

The blunted ang II-induced activation of MAP kinase may be part of a generalized alteration of the signal transduction pathways that initially produce cardiac cell growth. This hypothesis is supported by the findings that the MAP kinase response to both ang II and PKC was similarly affected in cardiac hypertrophy. Cardiac hypertrophy also displays dramatically altered regulatory mechanisms involving cAMP-dependent signalling pathways [23]. Upstream abnormalities would include different distribution of ang II receptor subtypes in cardiac hypertrophy or postreceptor abnormalities in G protein composition with the development of cardiac hypertrophy. Changes in ang II-R may occur in cardiac hypertrophy [38] that may affect the interaction with various signal transduction pathways [39]. There are no data on density and binding affinity of ang II receptors or their subtypes in the Dahl rat, so conclusions about these receptors must be made with caution. However, reduction in ang II number or affinity are not consistent with the observation that ang II increases phosphorylation of some cardiac proteins in cardiac hypertrophy in Dahl S rats compared with Dahl R rats [40]. A more attractive possibility is cardiac hypertrophy-induced G protein alterations. Bohm et al. [41] reported that Dahl S rats on a high-salt diet had an increased immunodetectable $G_{i\alpha}$, whereas the activity of $G_{s\alpha}$-deficient S49 cyc-mouse lymphoma cell membranes was unchanged.

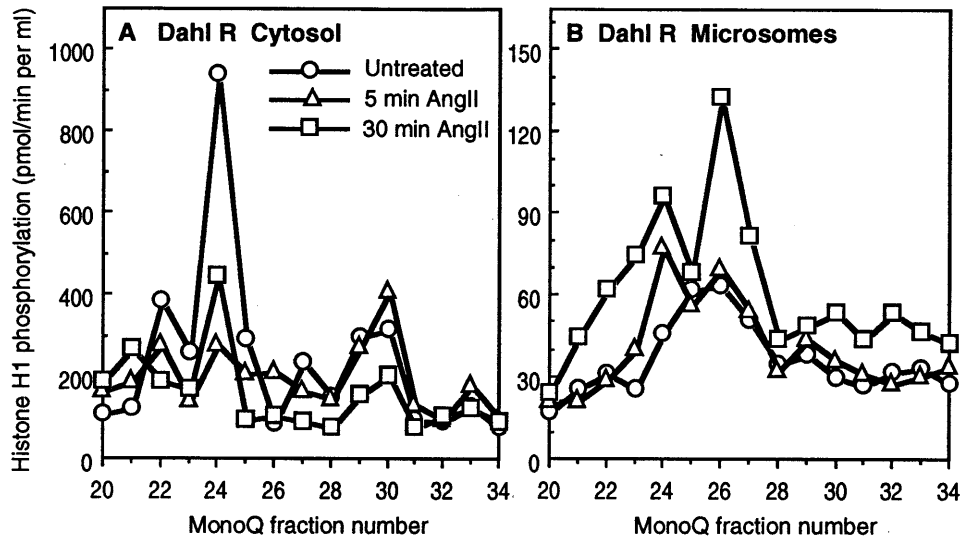

Figure 7. The effect of ang II-induced translocation of protein kinase C in ventricular explants treated with 1 μM ang II for 20 min and control explants (0) is shown for Dahl R (left) and Dahl S (right) rats on a high-salt diet. The data are the mean ± 1 SEM.

Recent data have shown a link between cAMP and MAP kinase pathways [42]. We may speculate that an interaction between cAMP and mitogen-induced activation of MAP kinases, in the heart, warrants investigation, since it might identify the underlying basis for ang II-altered regulation of MAP kinase that we found in the present study and the similar alteration in ang II effects on cAMP generation that we previously described in this model of cardiac hypertrophy [23].

In summary, this study investigated MAP kinase activation by ang II in heart that has already suffered hypertrophy. Under these conditions, ang II produced less MAP kinase activation compared with the nonhypertrophic heart. Taken together with the marked alteration in the pattern of the MAP kinase response to PKC and the PKC response to ang II, this study points to profound disturbances in intracellular regulation of signalling pathways involving MAP kinase in cardiac hypertrophy.

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