Angiotensin II—Induced Protein Phosphorylation in the Hypertrophic Heart of the Dahl Rat

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Angiotensin II—induced phosphorylation of proteins was examined in isolated myocytes from hearts of Dahl rats. A high salt diet induced cardiac hypertrophy in Dahl salt-sensitive rats. Angiotensin II–induced phosphorylation of a 42-kd protein (pp42) was detected by two-dimensional electrophoresis in hypertrophic but not normal ventricular myocytes. Angiotensin II stimulation was time-dependent, with a peak effect at 30 minutes. The half-maximal and maximal concentrations of angiotensin II that stimulated pp42 phosphorylation were 1 and 10 nM, respectively. Phosphorylation of pp42 was a function of cardiac hypertrophy. Phorbol 12-myristate 13-acetate–induced phosphorylation of pp42 indicates the possibility of an association between protein kinase C and the signal transduction pathway of angiotensin II–induced pp42 phosphorylation. Ionomycin and A23187 (both at 1 μM) did not stimulate phosphorylation of pp42. Angiotensin II produced a small increase in the synthesis of myocyte proteins in both normal and hypertrophic cells as shown by [3H]methionine incorporation. However, this increase could not account for the increase in the phosphate content of pp42. This protein was not an isoform of actin nor was it of platelet origin. These results raise the possibility that angiotensin II may play a role in the activation of factors in hypertrophic myocytes; however, further study is required to define a link between phosphorylation of pp42 and the hypertrophic process. (Hypertension 1992;20:633–642)

KEY WORDS • angiotensin II • phosphorylation • hypertrophy • rats, Dahl salt-sensitive

The effects of the octapeptide angiotensin II (Ang II) on the cardiovascular system have been investigated mainly from the perspective of hemodynamic actions on the heart and peripheral vasculature. The direct effects of Ang II on the heart include an increase in the force of myocardial contraction in several species including humans.1–4 In rats, the contractile response to Ang II is dependent on the stage of development. In cultured neonatal rat cardiac myocytes Ang II has a negative inotropic effect,5 whereas in adult rats Ang II produces a small increase in contractile force in right ventricular strips6 and an increase in contraction and a decrease in relaxation rate in isolated ventricular myocytes.7 The cellular mechanism of Ang II's action in neonatal cardiac myocytes includes increased calcium current, increased accumulations of inositol monophosphate and inositol bisphosphate,8 and stimulation of the Na+–H+ pump.9 In adult ventricular myocytes Ang II stimulates intracellular calcium release9 and induces transcription of c-fos through a pathway involving protein kinase C.10 In the vasculature, Ang II increases systemic vascular resistance by eliciting contraction of vascular smooth muscle.11 Cellular responses in the vasculature include stimulation of phospholipase C,12 mobilization of Ca2+,13 activation of Na+–H+ exchange,14 induction of expression of nuclear oncogenes such as c-fos,15,16 and phosphorylation of nuclear and cytoskeletal intermediate filaments.17,18 Although some of these responses are common to those elicited by mitogens such as platelet-derived growth factor (PDGF),19,20 Ang II causes vascular smooth muscle cell hypertrophy rather than cell proliferation.21 Thus, while the intracellular mechanism of action of Ang II on cardiac myocytes has not been entirely elucidated, important similarities between vascular smooth muscle cells and cardiac muscle cells are apparent.

We postulated that Ang II has direct effects on hypertrophic ventricular myocytes. Since in vascular smooth muscle cells protein phosphorylation occurs in response to Ang II treatment,17,18 we analyzed phosphoproteins in Ang II–treated isolated ventricular myocytes. We chose the Dahl salt-sensitive (DS) rat as a model of cardiac hypertrophy for two reasons: first, this rat is characterized by an enhanced pressor response to Ang II22 due to low plasma renin levels independent of high blood pressure23 partly because of a restriction length polymorphism in the renin gene,24 and second, the development of cardiac hypertrophy in the DS rat can be regulated by the administration of various concentrations of salt in the diet.25 Thus, it is possible to study various stages of development of cardiac myocyte hypertrophy and use appropriate intrastrain controls. We report on Ang II–induced protein phosphorylation in the heart, focusing especially on an Mw=42,000 phosphoprotein found predominantly in hypertrophic ventricular myocytes.

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Methods

Dahl Rats

Inbred SR/Jr and SS/Jr and outbred DR and DS rats from Harlan Sprague Dawley, Inc. (Indianapolis, Ind.) were maintained from weaning on a diet of powdered Purina Labchow (Mississauga, Canada) or the same diet supplemented with 6% NaCl. The animals were permitted to drink tap water ad libitum.

Isolation of Myocytes

Ventricular myocytes from age- and diet-matched normal and hypertrophied hearts were isolated simultaneously and the same media was used. Viable adult cardiac myocytes were isolated from rats weighing 150–300 g, as previously described.26 The rats were anesthetized with halothane and killed by cervical dislocation. The hearts were rapidly excised; rinsed in oxygenated Joklik modified minimum essential medium (GIBCO Canada, Burlington, Canada) at 35°C to which had been added 1.2 mM MgSO₄, 23.8 mM NaHCO₃, and 0.5 mM L-carnitine (solution A); and perfused through the aortas with oxygenated solution A at 35°C and 7 ml/min for 5 minutes. The pumped solution was changed to solution B (solution A plus 238 mg/ml type II collagenase, 1 mg/ml fatty acid-free bovine serum albumin, and 25 μM CaCl₂) and perfusion continued for 20 minutes, during which time the perfusate was collected, warmed, oxygenated, and recycled. After being trimmed of major vessels and atria, the ventricles were placed in solution C (solution A plus 1% bovine serum albumin and 1.5 mM CaCl₂) and quickly blotted and weighed. The ventricles were minced and shaken for 10 minutes, and the supernatant was discarded. Subsequently, the perfusate of solution B was added for a further 15 minutes of shaking. Isolated myocytes were harvested, diluted 1:10 in solution C, and centrifuged at 45g for 1 minute. The isolated cells were washed twice in solution C before being resuspended in Dulbecco's modified Eagle medium (DMEM) and stored under 95% O₂ and 5% CO₂. Isolated myocytes were washed with inorganic phosphate-free DMEM twice, then incubated in this medium for 2 hours. Agonists were added to 0.5-ml samples containing 0.5×10⁶ cells for various times during the final portion of the labeling period so that samples were harvested together. The reactions were stopped by centrifuging at up to 13,500g for 5 seconds, aspirating the labeling medium, and adding either sodium dodecyl sulfate (SDS) gel electrophoresis buffer or isoelectric focusing gel buffer to the cell pellet.

Immunoprecipitation

Cell pellets were lysed in a buffer containing 10 mM Tris, 1% Triton X-100, 5 mM ethylenediaminetetraacetic acid, 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml aprotinin (pH 7.6 at 4°C). The lysate was rocked overnight with antiactin monoclonal antibody-linked protein G agarose beads (Genex, Gaithersburg, Md.) at 4°C. The slurry was spun at 10,000g for 10 minutes and washed twice with 1.0 ml of 0.01 M sodium phosphate (pH 7.4), 0.15 M NaCl, and 0.05% sodium azide. SDS gel electrophoresis buffer was added, and the mixture was heated to 90°C for 10 minutes. The immunoprecipitates were then analyzed on 10.5% SDS polyacrylamide gels.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SDS gels were run according to the method of Laemmli.28 Samples were dissolved in SDS gel electrophoresis buffer, boiled for 5 minutes, and loaded onto vertical 10.5% polyacrylamide slabs. Following electrophoresis, the gels were stained with Coomassie brilliant blue R-250, destained, and dried.

Two-Dimensional Gel Electrophoresis

Two-dimensional electrophoresis was performed according to the method of O'Farrell.29 Samples for isoelectric focusing gels were dissolved in 9.5 M urea, 2% Triton X-100, 5% mercaptoethanol, 1.6% pH 5–7 ampholytes, and 0.4% pH 3–10 ampholytes and then frozen at –80°C overnight. Samples were assayed for protein content by a modification of the Bradford method,28 and 30 μg protein was loaded onto tube gels. Isolelectric focusing tube gels were run at a constant 750 V for 4.5 hours, after which time the current stabilized at 0.02 mA/gel. Tubes were frozen at –80°C overnight. Second-dimension 10.5% SDS polyacrylamide slab gels were run as described.

Platelet Preparation

Blood from the rats was diluted to 25 mM sodium citrate before being centrifuged at 150g for 10 minutes; the platelet-containing plasma was removed. Platelets were examined by phase-contrast microscopy and counted using a hemocytometer before centrifugation for 10 minutes at 1,500g. Platelets were resuspended in phosphate-free DMEM containing 250 μCi/ml carrier-free [³²P]orthophosphate for 60 minutes. Samples were then centrifuged at 12,000g, the labeling medium was aspirated, and the pellet was dissolved in 200 μl isoelectric focusing gel buffer (9.5 M urea, 2% Triton X-100, 5% mercaptoethanol, and 2 ml ampholytes). Protein concentration was determined by a modification of the method of Bradford,27 and two-dimensional gel electrophoresis was performed as described.

[³²P]Orthophosphate Incorporation

Isolated myocytes were washed with inorganic phosphate (Pᵢ)-free DMEM twice and incubated in Fᵢ-free DMEM for 30 minutes to reduce the intracellular phosphate content. Cells were labeled with 250 μCi/ml carrier-free [³²P]orthophosphate in DMEM for 2 hours. Agonists were added to 0.5-ml samples containing 0.5×10⁶ wells for 5 seconds, aspirating the labeling medium, and adding either sodium dodecyl sulfate (SDS) gel electrophoresis buffer or isoelectric focusing gel buffer to the cell pellet.
Quantification of Radiolabeled Proteins

$[^3]P$Phosphate-labeled protein gels were mounted, dried, and autoradiographed on Kodak X-AR5 x-ray film (Rochester, N.Y.) using intensifier screens at $-80^\circ$C for 7 days. $[^35]S$Methionine-labeled protein gels were soaked in Amplify (Amersham, Oakville, Canada) for 10 minutes before being dried and exposed to film for 5 days. Densitometric analysis was performed on a Bio-Rad video densitometer (Bio-Rad Canada, Mississauga, Canada) to quantify two-dimensional and SDS gel autoradiographs. The analysis was based on computation of the densitometric area of interest divided by the densitometric area represented by the total phosphoprotein profile. Internal comparison was also made with an $M_r=42,000$ protein (pp42). As a means of validating this method of quantification, spots from selected gels were excised and dissolved in ACS (Amersham Canada) and scintillants were counted. Similar results were obtained in experiments in which both methods were used. The densitometric method was preferred because excising selected spots from the gel presented more difficulties with consistency for the $M_r=42,000$ phosphoprotein (pp42) of interest that was present in only small amounts. Seeing the phosphoprotein on autoradiographs was easier than deciding its precise location on gels.

Materials

Carrier-free $[^32]P$orthophosphate and $[^35]S$methionine were from Amersham Canada. Ang II, saralasin, and ionomycin were from Peninsula Laboratories, Belmont, Calif. Phorbol 12-myristate 13-acetate (PMA) and A23187 were from Sigma Chemical Co., St. Louis, Mo. Anti–muscle actin (clone C4) and antiactin (clone B4) monoclonal antibodies were obtained from ICN Biomedicals, Toronto, Canada.

Data Analysis

Analysis of variance was used to compare groups. Linear regression analysis was also used, and analysis of variance assessed the underlying relation. The 5% level was considered significant.

Results

Ventricular Hypertrophy

Inbred Dahl salt-sensitive (SS/Jr) rats and outbred DS rats fed a high salt diet developed ventricular hypertrophy as determined by an increased ventricular weight relative to total body weight (ventricle to body weight index) (Figure 1); DS rats displayed greater variability in the degree of cardiac hypertrophy than SS/Jr rats. Age- and sex-matched inbred Dahl salt-resistant (SR/Jr) rats or outbred Dahl salt-resistant (DR) rats fed an identical diet maintained a constant ventricle to body weight index, with no signs of left ventricular hypertrophy. In SS/Jr rats there was a significant ($p<0.05$) relation ($r=0.811$) between duration of the high salt diet and degree of ventricular hypertrophy.

Viability of Myocytes

Viability of ventricular myocytes was usually 75–85% as determined by trypan blue exclusion. The difference in viability between normal and hypertrophied myocytes isolated in the same experiment was <5%. Phosphoprotein differences between hypertrophied and normal myocytes were not attributable to viability differences. Isolated cells were characteristically rod-shaped and quiescent. The yield of viable cells was 15–20 million/heart.

Electrophoretic Analysis of Angiotensin II–Stimulated Myocytes

Ang II increased the phosphorylation of a number of proteins in hypertrophic myocytes from SS/Jr rats but not myocytes from SR/Jr rats (Figures 2H and 2D, respectively). Especially prominent was pp42. This protein resolved as a series of at least seven discrete points within a pH range of 5.0–5.5 and displayed an acidic isoelectric shift with Ang II stimulation. No differences were evident in phosphoproteins from unstimulated myocytes from SR/Jr and SS/Jr rats (Figures 2B and 2F, respectively). Myocardial proteins from SR/Jr and hypertrophic SS/Jr rat hearts were similar when analyzed by O’Farrell gels and stained with Coomassie brilliant blue (Figures 2A and 2E, respectively). No apparent alteration in the protein profile occurred after 30 minutes of Ang II stimulation (Figures 2C and 2G, respectively).

Time Course and Concentration-Dependence of Angiotensin II–Stimulated Phosphorylation

The phosphorylation of pp42 from hypertrophic SS/Jr rat myocytes was detected by an increase in the two-dimensional autoradiographic density and a shift in the isoelectric point of the predominantly phosphorylated isoforms. Phosphorylation of pp42 was evident after 5 minutes of exposure to Ang II (Figure 3). Peak phosphorylation of 1.7 times the basal level was reached after 30 minutes of Ang II stimulation, after which phosphorylation declined gradually in parallel with a
retreat of the isoelectric point of the phosphorylated isoforms by 120 minutes. Myocytes from SR/Jr rats showed only slight stimulation of pp42 phosphorylation, to 1.27 times the basal level, by 5 minutes followed by a rapid decline to below the basal level by 30 minutes (Figure 3). Over the observation period, Ang II produced a significant (p<0.05) increase in phosphorylation of pp42 in myocytes from SR/Jr rats. The prominent pp32 was quantified for densitometrically compared with control hearts from SS/Jr rats; panels C, D, G, and H: Ang II-stimulated rats. Arrow indicates M_r=42,000 phosphoprotein.

The phosphorylation of pp42 in myocytes from SS/Jr rats was a function of Ang II concentration and was evident at 0.1 nM Ang II; maximal concentration was 10 nM and half-maximal concentration (EC_{50}) was 1 nM (Figure 4). Prior treatment of myocytes from SS/Jr rats with the Ang II receptor antagonist saralasin (10 μM for 5 minutes) markedly attenuated the induction of phosphorylation with Ang II (Figure 4). At 1 μM saralasin alone had no effect on either the isoelectric point or the [$^{32}$P]phosphate content of pp42.

**pp42 Is Neither Actin nor of Platelet Origin**

It was conjectured that pp42 is a minor phosphorylated component of soluble G-actin or nascent actin chains. To test this hypothesis, cells were prelabeled with [$^{32}$P]orthophosphate and extracted with 1% Triton X-100, and cell extracts were immunoprecipitated using monoclonal antibodies specific for actin and muscle actin. Although G-actin fractions were isolated as assayed by SDS polyacrylamide gel electrophoresis, no phosphoprotein was detected on autoradiographs (Figure 5A) or by scintillation counting of gel slices. Pellets of extracts, which contained 1% Triton X-100-insoluble F-actin, were also analyzed by SDS polyacrylamide gel electrophoresis and autoradiography and were found not to contain phosphoprotein at the 42-kd band (Figure 5B).

Since pp42 bears isoelectric and M_r similarities to the prominent platelet phosphoprotein pleckstrin, we tested the hypothesis that pp42 originates from adherence of platelets to myocytes during the isolation procedure. Rat platelets were therefore isolated and allowed to take up [$^{32}$P]orthophosphate, and total proteins were subjected to two-dimensional gel electrophoresis and autoradiography. Samples were run separately and combined with myocyte samples. The platelet phosphoprotein profile was distinct from that of the myocytes; pp42 was not apparent among the platelet phosphoproteins (Figure 6).

**Relation Between Angiotensin II–Induced Phosphorylation and Cardiac Hypertrophy**

Ang II–induced phosphorylation of pp42 was a function of ventricular hypertrophy as measured by the ventricle to body weight index (Figure 7). There was little response to Ang II in salt-sensitive rats on the normal salt diet that did not have left ventricular hypertrophy and displayed ventricle to body weight indexes similar to those of salt-resistant rats.

**Effect of Phorbol 12-Myristate 13-Acetate and Calcium Ionophores on Phosphorylation**

The phorbol ester PMA induced phosphorylation of pp42 in both SR/Jr and SS/Jr rats (Figure 8); pp32 was unaffected by PMA stimulation, but a number of other proteins, including an acidic M_r=85,000 protein, were phosphorylated in a time-dependent manner by PMA (Figure 8). The time course of PMA-induced phosphorylation of pp42 in myocytes from SS/Jr rats showed a rapid increase, to 1.62-fold over the basal level, for up to 30 minutes followed by a slight decline; by 120 minutes there was still a 1.32-fold increase in incorporated [$^{32}$P]phosphate. In contrast, myocytes from SS/Jr rats displayed a modest rise in phosphorylation of pp42 that reached 1.3-fold over the basal level by 30 minutes and then decreased to below the basal level (Figure 9). PMA produced a significant (p<0.05) increase in phosphor-
Sung and Rabkin Phosphoproteins in Cardiac Hypertrophy

**FIGURE 3.** Time course of angiotensin II (Ang II) stimulation of phosphorylation. Left panel: Radiographs. Isolated ventricular myocytes from inbred Dahl salt-resistant (SR/Jr) and salt-sensitive (SS/Jr) rats with ventricle to body weight indexes of 6.3 and 9.1 g/kg, respectively, were prelabeled with 250 μCi/ml [32P]orthophosphate for 2 hours followed by stimulation with 100 nM Ang II for times indicated. All samples were harvested at same time. Amount of protein loaded onto each isoelectric focusing gel was 30 μg. Arrow marked 1 indicates Mr=42,000 protein (pp42); arrow marked 2 indicates Mr=32,000 protein (pp32). R, SR/Jr; S, SS/Jr. Data are from representative experiment. Right panel: Line graph shows densitometric analysis of time course of phosphorylation. Phosphorylation levels of pp42 and pp32 were quantified by video densitometry. Data are expressed as fold change from basal level. Bars indicate range of data from two separate experiments.

**[35S]Methionine Incorporation**

[35S]Methionine incorporation, two-dimensional gel analysis, and autoradiography revealed relative de novo synthesis of cardiac proteins over 2 hours. Ang II-stimulated myocytes from SR/Jr and SS/Jr rats (Figure 10) had visibly increased [35S]methionine incorporation in many proteins; pp42 was poorly visible, indicating that it is of relatively low abundance and that its synthesis was not increased relative to that of other cell proteins by Ang II stimulation. Slightly more [35S]methionine was incorporated into myocytes from SR/Jr rats than from hypertrophic SS/Jr rats; however, the relative incorporation into pp42 was the same.

**Discussion**

We have demonstrated that Ang II induces protein phosphorylation in ventricular myocytes from hypertrophic Dahl rats. Of special interest was an Mr=42,000 protein that was evident in radiographs from two-dimensional electrophoresis. This action of Ang II in myocytes from Dahl rats is likely mediated through Ang II receptors since first the response of pp42 phosphorylation was dependent on the Ang II concentration and displayed an EC50 of 10^{-7} M, which is in accord with studies of the contractile response of isolated adult myocytes to Ang II. Second, the competitive inhibitor saralasin blocked Ang II-induced phosphorylation of pp42.

The inbred Dahl rat has been of special interest as a model of hypertension because of alterations in the renin-angiotensin system. Low plasma renin activity may predispose the cardiovascular system to Ang II sensitivity in the DS rat. For this reason this model is...
Figure 4. Line graph shows concentration-dependence of angiotensin II (Ang II)-induced phosphorylation of Mr=42,000 protein (pp42) and inhibition in presence of [Sar\(^{1}\), Ala\(^{8}\)] angiotensin. Isolated ventricular myocytes from inbred Dahl salt-sensitive (SS/Jr) rat with ventricle to body weight index of 10.3 g/kg were prelabeled with 250 μCi/ml \([^{32}P]\)orthophosphate for 2 hours followed by 10 minutes of stimulation with various concentrations of Ang II alone (o) or in presence of 10 μM [Sar\(^{1}\), Ala\(^{8}\)]angiotensin (©). Phosphorylation of pp42 was quantified by video densitometric scanning of radiographs. Data are expressed as fold change from basal level. Data shown are from two separate experiments, and bars indicate range. ATII, angiotensin II.

ideal to study in vitro the magnified actions and signaling pathway of Ang II. Cardiac hypertrophy gradually develops in the DS rat concomitant with the development of hypertension. Furthermore, Pfeffer et al\(^{25}\) found that cardiac hypertrophy in this strain is in proportion to the increase in blood pressure, although the exact nature of the link or links between blood pressure and hypertrophy is unresolved.

We found that the Ang II-sensitive pp42 was not actin nor was it of platelet origin. The protein, although present in low abundance in myocytes, is a prominent feature of the Ang II-stimulated phosphoprotein profile and is characterized by a series of at least seven discrete points that display a charge shift on autoradiographs of two-dimensional gels. A number of diverse mitogens (such as epidermal growth factor, PDGF, and PMA) phosphorylate an Mr=42,000 protein in a variety of cell types.\(^{34-41}\) An important difference may be due to a difference or differences in the cell types used in those studies and in ours; whereas the previous studies were done in established fibroblast cell lines, our study was done in primary cultures of isolated cardiomyocytes. An Mr=42,000 protein in chick and mouse fibroblasts is phosphorylated in response to PMA but has an isoelectric point of pH 6.8,\(^{41}\) which distinguishes it from the pp42 described here, one that has an isoelectric point of pH 5.0–5.5. Another difference is in the number of isoforms. We observed seven isoforms, which is distinct from the Mr=42,000 protein previously observed in 3T3 cells that apparently has two or three isoforms.\(^{37}\) An Mr=42,000 cardiac sarcolemmal protein has also been

Figure 5. Gels demonstrating absence of angiotensin II (Ang II) stimulation of phosphate incorporation into immunoprecipitated soluble G-actin and Triton X-100-insoluble F-actin. Isolated ventricular myocytes from inbred Dahl salt-resistant (SR/Jr) and salt-sensitive (SS/Jr) rats were prelabeled with \([^{32}P]\)orthophosphate for 2 hours and then exposed to 1 μM Ang II for 30 minutes. Cell were lysed with 1% Triton X-100, and samples of supernatant were immunoprecipitated with anti-muscle actin antibody. R, SR/Jr; S, SS/Jr. Top row (a): Upper left shows 10.5% sodium dodecyl sulfate (SDS) gel of immunoprecipitated protein; 42-kDa position is indicated. Upper right shows 5-day autoradiograph of gel in upper left. Bottom row (b): 1% Triton X-100-insoluble material was centrifuged at 13,500g for 5 minutes, and pellet was dissolved in SDS gel electrophoresis buffer and analyzed on 10.5% SDS gels. Lower left shows Coomassie brilliant blue staining of pellet proteins. Lower right is same gel autoradiographed for 5 days. The 42-kDa position is indicated. Experiment was repeated five times with antibodies to skeletal and cardiac alpha actin as well as gamma actin.
Sunga and Rabkin  Phosphoproteins in Cardiac Hypertrophy  639

FIGURE 6. Radiographs compare platelet and myocyte phosphoproteins. Platelets and ventricular myocytes from inbred Dahl salt-sensitive (SS/Jr) rat were isolated and prelabeled with \[^{32}P\]orthophosphate for 2 hours. Platelet and myocyte protein samples were analyzed separately (20 μg/gel) (left) or mixed together (10 μg from each cell type) (right) for two-dimensional gel analysis and autoradiography. Arrow indicates M,=42,000 protein. Experiment was repeated three times with similar results.

reported as a major substrate for protein kinase C in chick hearts,39 and an M,=46,000 protein is among those phosphorylated by protein kinase C in canine cardiac sarcolemma.40

The function of pp42 in the heart is unknown. However, it is possible that pp42 is a component of the signaling pathway mediating the action of Ang II in cardiac hypertrophy.41,42 The issue of a causal relation between Ang II-induced pp42 phosphorylation and cardiac hypertrophy has not been resolved, and it is possible that pp42 phosphorylation occurs concomitant with but is not causally related to the development of left ventricular hypertrophy. However, some data suggest an involvement of Ang II signal transduction in the hypertrophic heart of the DS rat; increased sensitivity of hypertrophic myocytes to Ang II–induced phosphorylation of pp42 was evident, in contrast to limited phosphorylation of the same protein in normal myocytes from age-, sex-, and diet-matched DR rats or in myocytes from DS rats fed a low salt diet with no hypertrophy. Furthermore, the degree of phosphorylation of pp42 was proportional to the degree of cardiac hypertrophy. Third, other factors such as age or salt intake alone are likely to play direct roles in the Ang II–induced phosphorylation of pp42 since age-matched DR rats fed a high salt diet display no increase in pp42 phosphorylation. It is unlikely that pp42 is involved in the positive inotropic action of Ang II,43,44 which is believed to be mediated through stimulation of slow Ca\(^{2+}\) channels.43 Neither calcium ionophore A23187 nor ionomycin, which increase intracellular calcium contents, altered the phosphorylation of pp42.

A link between Ang II and cardiac hypertrophy has been widely hypothesized since it is known that cardiac hypertrophy can regress with angiotensin converting enzyme inhibitor treatment, independent of changes in blood pressure.44,45 Also, peptide analogues of Ang II can stimulate cardiac hypertrophy in rats at subpressor doses,46 and more recently Ang II has been demonstrated to be a potent growth factor in fibroblasts,47 aortic smooth muscle cells,48 and cultured chick cardiac myocytes.49 This signaling pathway may involve inositol
phosphate metabolism and protein kinase C activation in rats. Moreover, the stimulation of cell growth under a number of conditions is known to involve a complex cascade of protein kinase activations as part of these signal transduction pathways.

Cardiac hypertrophy was indicated by an increase in the ventricle to body weight index. The assumption that this index is indicative of ventricular myocyte size has been validated in studies in which various techniques have shown that the remodeled, enlarged heart consists of myocyte hypertrophy. Furthermore, it is known that ventricular cardiomyocytes cease to divide during the first few weeks after birth and fulfill the increased demands on the heart by increasing their volume.

The phosphorylation of pp42 is partially mimicked by the phorbol ester PMA, suggesting that Ang II-induced phosphorylation involves activation of protein kinase C. However, this is not the only mechanism involved because PMA did not mimic phosphorylation of pp42 to the same extent or with the same time course as Ang II. Furthermore, there was a little phosphorylation of the widely distributed acidic $M_r=83,000-87,000$ protein substrate of protein kinase C in Ang II-stimulated myocytes from SS/Jr rats while noticeably more phosphorylation of the same protein occurred in myocytes from both SR/Jr and SS/Jr rats stimulated with PMA.

The phosphorylation of pp42 by PMA was rapid and attained a higher peak in hearts from SR/Jr rats than in those from hypertrophic SS/Jr rats. This is consistent with studies demonstrating that DR rats have significantly more phorbol ester receptor sites per cell than DS rats. This may also indicate that a component conferring altered Ang II sensitivity in DS myocytes is upstream from protein kinase C in the Ang II transduction pathway. To our knowledge, ventricular Ang II receptors have not been compared in DR and DS rats; however, previous studies have indicated that the vascular response of DS rats to a bolus injection of Ang II is greater than that of DR rats. We speculate that this
may be due to differences in Ang II receptors in vascular smooth muscle or to better coupling between Ang II receptors and intracellular signaling pathways in DS compared with DR rats; this vascular response difference may also be manifest as a parallel difference in the myocardial response to Ang II.

Although protein synthesis was increased in Ang II–treated myocytes compared with untreated myocytes from both SR/Jr and hypertrophic SS/Jr hearts, this was not sufficient to account for the increased phosphate content of pp42 due to Ang II stimulation. The appearance of increased quantities of pp42 was not due to Ang II stimulation of de novo synthesis of the protein since the small, nonspecific increase in cardiac proteins did not parallel changes in pp42 quantity or time course. Two-dimensional gel analysis of [35S]methionine-labeled proteins also demonstrated the low abundance of pp42 relative to other actively synthesized cardiac myocyte proteins and furthermore demonstrated that similar levels are found in ventricular myocytes from SR/Jr and hypertrophic SS/Jr rats.

In conclusion, Ang II induces phosphorylation of an M₉ = 42,000 protein in myocytes from hypertrophic hearts of DS rats. Pending its further identification and delineation of its functional role (or roles) in the heart, the data suggest that pp42 may be a component of the transduction of a trophic signal in the heart since the amount of phosphorylation of the protein was related to the degree of cardiac hypertrophy and the demonstrated potential for Ang II to regulate growth in the heart, aorta, and other vascular smooth muscle.1

References
15. Kawahara Y, Sunsko M, Tsuda T, Fukazaki H, Fukamoto Y, Takai Y: Angiotensin II induces expression of the c-fos gene through...


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