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 p44ERK1 with lower amounts p42MAPK in rat ventricle, and no apparent changes with hypertrophy. In hypertrophied hearts, ang II-induced MAP kinase activity was less markedly increased and more rapidly 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, 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 x1-adrenergic receptors [10–12] that also induce cardiac hypertrophy. Inhibition of MAP kinases prevent x1-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 neonatal 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 Lab-chow 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 ± 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 mm3 and the pieces rinsed 2 × 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% O2 and 5% CO2.

**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 200,000g 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 200,000g for 15 min and also stored frozen at −70 °C.

**MonoQ chromatography.** For MonoQ chromatography, ~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× concen-