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Original article

Transient activation of P38 MAP kinase and up-regulation of Pim-1 kinase in cardiac hypertrophy despite no activation of AMPK

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ABSTRACT

AMP-activated protein kinase (AMPK), is an important regulator of cardiac metabolism, but its role is not clearly understood in pressure overload induced hypertrophy. In addition, the relationship between AMPK and other important protein kinases such as p38 MAP kinase, Akt and Pim-1 is unclear. Thus we studied the time course of AMPK activity and phosphorylation of Thr-172 of its α-subunit during the development of cardiac hypertrophy. In parallel, we examined the expression and activation of key kinases known to be involved in cardiac hypertrophy that could interact with AMPK (i.e. p38 MAP kinase, Akt and Pim-1). Male C57BL/6J mice underwent sham or transverse aortic constriction (TAC) surgery and the hearts were harvested 2, 4, 6 and 8 weeks later. Despite significant left ventricular (LV) hypertrophy, LV dilation and impaired LV contractile function at all time points in TAC compared to sham mice, the activity and phosphorylation of AMPK were similar to sham. In contrast, p38 and Pim-1 protein expression was transiently increased in TAC mice at 2 and 4 weeks and at 2, 4 and 6 weeks, respectively. In addition, p38 activation by phosphorylation was also transiently increased at 2 to 6 weeks. There were no differences between sham and TAC mice in p38, Akt or Pim-1 at 8 weeks. In conclusion, TAC resulted in a transient up-regulation in the expression of p38 and Pim-1 despite no activation of AMPK or Akt.

1. Introduction

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that is activated in the heart in response to acute stress by phosphorylation at Thr-172 of its α-subunit either by upstream kinases, or allosterically by increased intracellular AMP level. Acute metabolic stress, such as ischemia and exercise, can activate AMPK and stimulate glucose uptake and glycolysis [1]. Pharmacological activation of AMPK is also linked to inhibition of protein synthesis in isolated neonatal rat cardiomyocytes [2], although it is unclear if a decrease in AMPK activity plays a role in cardiac hypertrophy in vivo [3]. Kantor et al. reported a 30% decrease in AMPK activity in freeze-clamped left ventricular myocardium from juvenile pigs with volume overload induced cardiac hypertrophy [4]. More recently, we observed no change in the activation state of AMPK, as assessed by Western blot using antibodies to AMPK phosphorylated at Thr-172, in rats and mice with cardiac hypertrophy and heart failure due to 8–16 weeks of chronic pressure overload induced by aortic constriction [5–8]. In contrast, Tian et al. found that AMPK activity was increased approximately threefold in tissue taken from isolated buffer-perfused hearts from rats subjected to 17–19 weeks of aortic banding [9]. The differences between the results of these studies could be due to the experimental conditions prior to tissue sampling. In addition, it is possible that in our studies there was a transient activation of AMPK during the initial period of pressure overload that dissipated by the 8-week sampling time. The time course of AMPK activity over the development of LV hypertrophy and remodeling has not been reported.

AMPK has recently been shown to interact with other key signally kinases in the heart. p38 belongs to a family of mitogen-activated protein kinases (MAPK) that are activated by various stresses. Although early in vitro studies with constitutively active p38 suggest that p38 exerts pro-hypertrophic effects [10], recent studies using transgenic mice (dominant-negative and knockout) and pressure overload induced LV hypertrophy suggesting p38 is actually an inhibitory factor against cardiac hypertrophy [11,12]. While AMPK may activate P38 during acute ischemia [13,14], a dissociation between the two has also been reported recently [15]. The relationship between AMPK and p38 during cardiac hypertrophy is not known.

The activity of AMPK can also be regulated by Akt, a key serine/threonine protein kinase involved in cardiac growth, metabolism and survival [16,17]. Akt inactivation has been observed in LV hypertrophy after prolonged pressure overload [18]. Recent studies show that Akt activity is partially regulated by the downstream effector Pim-1 kinase [19]. Pim-1 is up-regulated at the transcript level in the early phase of

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pressure overload in mice and appears to be protective during the early response to aortic pressure overload [19]. The relationship between the expression of Pim-1 protein and expression and activation of Akt in response to chronic pressure overload has not been reported.

The goal of the present investigation was to assess expression and activation of AMPK, p38, Akt and Pim-1 during the development of cardiac hypertrophy, left ventricular dilation and contractile dysfunction in response to chronic aortic pressure overload. Studies were performed in a well established model of transverse aortic constriction (TAC) in mice. We hypothesized that with the onset of pressure overload there would be a transient activation of AMPK and an associated increase in p38 expression and activation that would return to sham values as LV hypertrophy and chamber remodeling stabilized. Further, it was anticipated that Pim-1 would be transiently up-regulated at the protein level, which would correspond to increased activation of Akt.

2. Methods

2.1. Animal model

Male (6–8 weeks) C57BL/6J mice were purchased from Charles River and maintained on a 12:12-h light: dark cycle. Anesthesia was induced by nose-cone inhalation of 5% isoflurane in 60% oxygen. After endotracheal intubation with a 20-gauge catheter (Angiocath™, BD), ventilation was supported (Harvard Apparatus Inspira) with 2% isoflurane in 60% oxygen. Transverse aortic constriction (TAC) was made with 27-gauge needles, as described previously [5]. Sham surgery was performed without aortic constriction. Mice were given analgesic (Buprenex 0.1 mg/kg i.p.) for 3 days following surgery. In the acute phase (within 72 h after surgery), the mortality was 0 % in sham operated mice and 15% in mice subjected to TAC. Echocardiographic examinations and tissue harvests were performed at 2, 4, 6, and 8 weeks after surgery in both sham and TAC mice. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland at Baltimore and conformed to the guiding principles for the care and use of laboratory animals published by the National Institutes of Health.

2.2. Echocardiography

Two-dimensional M-mode and pulsed-wave Doppler studies were performed with a Vevo 770™ high-resolution imaging system (VisualSonics, Toronto, Ontario) featuring a 30-MHz central transducer. Briefly, mice were anesthetized with 5% isoflurane and placed supine on a heated platform with legs taped to ECG electrodes for heart rate monitoring. Hair was removed from the chest using a topical depilatory agent. After cleaning with alcohol, the chest was covered with a pre-warmed, centrifuged ultrasound gel (Aquasonic 100; Parker Laboratories, Orange NJ). Imaging began following a 1–2-min stabilization period. Anesthesia was maintained with isoflurane, 1.5–2.0% by mask. Parasternal short-axis M-mode images at the mid-papillary level were collected for measurement of LV end diastolic diameter (LVEDD) and LV end systolic diameter (LVESD). Ejection fraction was calculated as [(end diastolic volume−end systolic volume)/end diastolic volume]]×100%. End systolic and diastolic volumes were calculated from the LVEDD and LVESD as previously described [20].

2.3. Terminal surgery

Mice were anesthetized and ventilated as described above and the heart was exposed by a midline sternotomy. A piece of LV tissue (~30 mg) was cut off the apex of the beating heart and immediately freeze-clamped; this sample was used for immunoblot analysis and measurement of AMPK activity. The remainder of the heart was dissected into total atria (left and right), RV (free wall) and the remaining LV. The total LV mass was calculated as the combination of the freeze-clamped LV tissue plus the remaining LV. Tibia length was measured.

2.4. Immunoblot analysis

All homogenization steps were performed at 4 °C. Homogenization buffer (Tris–HCl 50 mM, NaCl 150 mM, EDTA 5 mM, Triton-100 0.5%, SDS 0.1%, protease inhibitor cocktail (Sigma P8340) 1%, phosphatase inhibitor cocktail (Sigma P2850) 4%) was added to the tissue sample in a 200 μl:10 mg buffer:tissue ratio immediately prior to homogenization by high-frequency shaking (Invitrogen tissue-lyzer, 30 Hz for 2 min) with pre-cooled stainless steel beads (Qiagen). The crude homogenate was centrifuged at 20 000 ×g for 5 min, and the supernatant collected. Protein concentration was measured with a modified Bradford method (Bio-Rad DC protein assay kit). Seventy-five micrograms of protein of each sample was loaded on polyacrylamide gel (Bio-Rad precast 10% Tris–HCl gel) for electrophoresis. Gels were subsequently transferred to nitrocellulose membrane. After blocking with 5% non-fat milk, membranes were incubated with specific antibodies (phospho-AMPK α Thr-172, AMPK α, ACC, phospho-ACC Ser79, phospho-Akt Ser473, Akt, phospho-p38 Thr180/Tyr182, p38, from Cell Signaling; Pim-1 from Invitrogen) according to the manufacturers' instruction. Infra-red dye labeled secondary antibody (LICOR Biotechnology) incubation was performed in darkness for 1 h at room temperature. After sufficient washing, membranes were scanned and analyzed on the Odyssey™ scanner (LICOR Biotechnology). After probing for phosphorylated proteins, the membranes were stripped (Restore Plus™ Western blot strip buffer, Pierce) and re-probed with antibodies against their respective total protein. Equally loading was confirmed with blotting for calsequestrin (Affinity Bioreagents, MA).

2.5. AMPK activity assay

Approximately 20 mg of frozen LV tissue was homogenized for 30 s using a Polytron Homogenizer in 80 μl of homogenization buffer containing 50 mM Tris–HCl (pH 8 at 4 °C), 1 mM EDTA, 10% (wt/vol) glycerol, 1 mM dithiothreitol (DTT), 0.02% (vol/vol) Brij-35, protease inhibitor cocktail (Sigma P8340) and phosphatase inhibitor cocktail (Sigma P2850, P5726). The homogenate was then centrifuged at 10 000 ×g for 20 min at 4 °C. The supernatant was collected and 5 μg of protein from the supernatant was used to assay AMPK activity as previously described [21,22] with the synthetic AMARA peptide (AMARAASAAALARRR) as the substrate.

2.6. Statistical analysis

Data are presented as mean±standard error of the mean (SEM). Statistical analysis was performed with commercially available software (Sigma Stat 3.0). The effects of time and TAC were assessed using a 2-way ANOVA and the Bonferroni post hoc test. A p value of <0.05 was considered significant.

3. Results

3.1. Acute and chronic mortality after surgery

LV function and biochemical parameters were assessed at 2, 4, 6 and 8 weeks post-surgery. The overall mortality was 0% in all sham mice, and 29% in TAC mice. The final surviving number was 5–6 for sham mice and 8–10 for TAC mice at each time point.

3.2. Cardiac LV and echocardiographic measurements

TAC resulted in a significant increase in LV mass at 2 weeks after surgery (30% increase compared to the sham group), which