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Coordination of changes in expression and phosphorylation of eukaryotic elongation factor 2 (eEF2) and eEF2 kinase in hypertrophied cardiomyocytes

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ABSTRACT

Eukaryotic elongation factor 2 (eEF2) kinase (eEF2K) is one of the Ca^{2+}/calmodulin-dependent protein kinases. Activated eEF2K phosphorylates its specific substrate, eEF2, which results in inhibition of protein translation. We have recently shown that protein expression of eEF2K was specifically increased in hypertrophied left ventricles (LV) from spontaneously hypertensive rats (SHR). However, phosphorylation state of eEF2K and eEF2 in hypertrophied LV is not determined. In the present study, we examined expression and phosphorylation of eEF2K and eEF2 in LV from SHR as well as the pressure overload (transverse aortic constriction: TAC)- and isoproterenol (ISO)-induced cardiac hypertrophy model. In LV from TAC mice, eEF2K expression was increased as determined by Western blotting. In LV from TAC mice and SHR, eEF2K phosphorylation at Ser366 (inactive site) was decreased. Consistently, eEF2 phosphorylation at Thr56 was increased. In LV from ISO rats, while eEF2K phosphorylation was decreased, eEF2K expression and eEF2 phosphorylation were not different as determined by Western blotting. In the results obtained from immunohistochemistry, however, total eEF2K and phosphorylated eEF2 (at Thr56) localized to cardiomyocytes were increased in LV cardiomyocytes from ISO rats. Accordingly, the increased expression and the decreased phosphorylation of eEF2K and the increased phosphorylation of eEF2 in hypertrophied LV were common to all models in this study. The present results thus suggest that cardiac hypertrophy may be regulated at least partly via eEF2K-eEF2 signaling pathway.

1. Introduction

Eukaryotic elongation factor 2 (eEF2) kinase (eEF2K) is one of the Ca^{2+}/calmodulin (CaM)-dependent protein kinases, and its amino-acid sequences are highly conserved among mammals. The homology is 97% between mice and rats, and 90% between human and rodents [20]. eEF2K belongs to a small group with α-kinase catalytic domains [19]. The α-kinase catalytic domain plays an important role for substrate specificity to eEF2K [14]. Besides that domain, a CaM-binding region and an unstructured 'linker' domain were identified in eEF2K, and these regions include several phosphorylation sites such as Ser78, Thr348 and Ser366, which regulate eEF2K activity both positively and negatively. Activated eEF2K phosphorylates its only known substrate, eEF2 at Thr56. eEF2 was also highly conserved in mammals [15]. eEF2 mediates protein translation by translocating polypeptidyl-tRNAs from the A to P site on ribosome. Of note, phosphorylation of eEF2 makes itself an inactive state and subsequently inhibits protein translation. Thus activated eEF2K inhibits eEF2 function via phosphorylation [5].

Cardiac hypertrophy is a kind of compensatory response caused by several diseases including hypertension, cardiac myopathy, valvular disease, and congenital abnormality, which eventually leads to heart failure and sudden death. It is recognized that the increased protein synthesis is one of the primary causes for cardiac hypertrophy. Angiotensin II, a peptide hormone inducing cardiomyocyte hypertrophy, was reported to facilitate eEF2 dephosphorylation at Thr56 via activating protein phosphatase 2A and mitogen-activated protein kinases signaling in rat neonatal cardiomyocytes [4]. On the other hand, a β-adrenergic agonist, isoproterenol decreased protein synthesis concomitant with an increased Ca^{2+}/CaM-dependent eEF2 phosphorylation in ventricular

Abbreviations: eEF2, eukaryotic elongation factor 2; eEF2K, eEF2 kinase; CAM, calmodulin; LV, left ventricles; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats; TAC, transverse aortic constriction; IVS, interventricular septum; LVID, left ventricular internal diameter; LVPW, left ventricular posterior wall; ISO, isoproterenol; FS, fractioning shortening; BW, body weight; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase

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cardiomyocytes from adult rats [13]. We have recently shown that eEF2K expression was specifically increased in hypertrophied left ventricles (LV) from spontaneously hypertensive rats (SHR) compared with Wistar-Kyoto rats (WKY) [8]. However, little is known about the expression and phosphorylation states of eEF2K and eEF2 in LV from in vivo cardiac hypertrophy models. The aim of this study was therefore to explore them in several animal models, namely SHR as well as pressure overload- and isoproterenol-induced cardiac hypertrophy. Accordingly, we for the first time revealed in this study that the increased expression and the decreased phosphorylation of eEF2K and the increased phosphorylation of eEF2 in hypertrophied LV were common to all models, suggesting the potential role of eEF2K/eEF2 signal in the pathogenesis of cardiac hypertrophy.

2. Material and methods

2.1. Animal study

Care and treatment of experimental animals were performed in accordance with the institutional guidelines of The Kitasato University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal experiment was approved by the ethical committee of School of Veterinary Medicine, The Kitasato University. After 12-week-old male SHR (Hoshino Laboratory Animals, Inc., Ibaraki, Japan) and age-matched WKY were euthanized by exsanguination under a deep urethane (1.5 g/kg i.p.) anesthesia, LV were isolated and immediately frozen in −80 °C. After protein extraction, the samples were used for Western blot analysis.

2.2. Pressure overload-induced cardiac hypertrophy model mice

Male C57BL/6NJcl mice weighing 15–27 g (Clea Japan, Tokyo, Japan) received an operation of transverse aortic constriction (TAC). After propofol (100 mg/kg) was pretreated intraperitoneally, mice were anesthetized by an inhalation of diethyl ether. The jugulum of mice was vertically incised and transverse aorta was displayed. A blunted 27G needle was tied with 7–0 silk suture to the aorta between brachiocephalic artery and left common carotid artery. The needle was immediately withdrawn after the ligation. Then, skin was closed with 6–0 nylon suture and buprenorphine (0.12 mg/kg) was subcutaneously injected. SHAM operated mice received an identical surgery except for aortic ligation. After 3 days from TAC operation, echocardiography was performed under diethyl ether anesthesia using SONOS 5500 (Hewlett-Packard Co., Andover, MA, USA) with a dynamically focused S12 probe (5–12 MHz. Hewlett Packard Co.). Heart rate was maintained in 420–480 bpm. Interventricular septum (IVS), left ventricular internal diameter (LVID) and left ventricular posterior wall (LVPW) in both diastolic and systolic phases as well as fractional shortening (FS) were measured by an M-mode. Subsequently LV were isolated and weighed. The isolated LV were immediately frozen in −80 °C for protein extraction and used for Western blot analysis.

2.3. Isoproterenol-induced cardiac hypertrophy model rats

Isoproterenol (5 mg/kg) was subcutaneously injected to male Wistar rats weighing 150–180 g (Clea Japan; ISO) [17]. In the control group, rats received a saline injection (Cont). We have utilized rats because rats are easy to handle and widely used to make an isoproterenol-induced cardiac hypertrophy model. After 1 week, LV were isolated under a deep pentobarbital (50 mg/kg, i.p.) anesthesia and weighed. The isolated LV were then immediately frozen in −80 °C for Western blotting and also fixed in 10% neutral buffered formalin for histological analysis. We chose a subcutaneous rather than intraperitoneal route because the effects of intraperitoneal injection are possibly stronger than subcutaneous injection [21]. In general, a high-dose isoproterenol might cause a myocardial infarction in rats. Since this is not the pathogenesis which we focused on, we did not choose the intraperitoneal route. In this study, we did not examine the cardiac function of ISO rats because we focused on cardiac hypertrophy rather than dysfunction. Since Krenek et al. [11] previously reported that isoproterenol (5 mg/kg) injection to rats for 7 days induced cardiac dysfunction (decreased systolic left ventricular pressure, dp/dt_{max}, dp/dt_{min} and heart rate), there might be a similar cardiac dysfunction in our ISO rats.

2.4. Western blotting

Western blotting was done as described previously [7,8]. Protein lysates were obtained by homogenizing tissue samples with lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA-2Na, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin; Cell Signaling Technology, Danvers, MA, USA) containing 1% proteinase inhibitor mix (Nacalai Tesque, Kyoto, Japan). Protein concentration was measured using a bicinchoninic acid method (Pierce, Rockford, IL, USA). Equal amount of proteins (8–10 μg) was separated by SDS-PAGE (10%) and transferred to nitrocellulose membranes (Pall, Ann Arbor, MI, USA). After being blocked with 3% bovine serum albumin for phosphorylation-specific antibodies or 0.5% skim milk for others for 1 h, membranes were incubated with the following primary antibodies (1:500 dilution): total-eEF2K, phospho-eEF2K (at Ser366), phospho-eEF2 (at Thr56) at 4 °C overnight. Then, the membrane was visualized using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, 45 min at room temperature) and the EZ-ECL system (Biological Industries, Kibbutz Beit Haemek, Israel). Anti-GAPDH antibody (1:1000 dilution) was used for normalizing the expression of total-eEF2K and phospho-eEF2. The resulting bands were analyzed using CS Analyzer 3.0 software (ATTO, Tokyo, Japan).

2.5. Azan staining

Azan staining was done as described previously [17]. LV tissues were fixed in 10% neutral buffered formalin. The tissues were dehydrated and embedded in paraffin and thin tissue sections (4 μm) were made. Deparaffinized sections were soaked in 5% potassium dichromate solution for 1 h and stained with azocarmine G (Waldeck, Division Chroma, Munster, Germany) at room temperature overnight. Sections were soaked in 12-tungsto-(VI)-phosphoric acid n-hydrate solution for 1 h and stained with aniline blue-orange G (Waldeck, Division Chroma) for 15 min. Images were obtained using a CCD-camera equipped light microscope (BX-51, Olympus, Tokyo, Japan).

2.6. Immunohistochemistry

Immunohistochemistry was done as described previously [9]. LV tissues were fixed in 10% neutral buffered formalin. The tissues were dehydrated and embedded in paraffin and thin tissue sections (4 μm) were made. After the deparaffinized sections were heated using a microwave for antigen retrieval, endogenous peroxidase activity was blocked by incubating in Dako REAL peroxidase-blocking solution (Dako, Glostrup, Denmark) for 15 min. Then, the sections were blocked with 5% normal goat serum for 60 min and subsequently incubated with specific primary antibody against total-eEF2K (1:250 dilution), phospho-eEF2K (1:250 dilution) or phospho-eEF2 (1:200 dilution) at 4 °C overnight. After