



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



Satoshi Kameshima, Muneyoshi Okada, Shiro Ikeda, Yuki Watanabe, Hideyuki Yamawaki*

Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034–8628, Japan

ARTICLE INFO

Article history:

Received 29 March 2016

Received in revised form

17 June 2016

Accepted 20 June 2016

Available online 28 June 2016

Keywords:

Cardiac hypertrophy

Cardiomyocyte

Eukaryotic elongation factor

Isoproterenol

Pressure overload

SHR

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.

© 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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

* Corresponding author.

E-mail address: yamawaki@vmas.kitasato-u.ac.jp (H. Yamawaki).

<http://dx.doi.org/10.1016/j.bbrep.2016.06.018>

2405-5808/© 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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 $\mu\text{g}/\text{ml}$ 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

washed in Tris buffer, the sections were incubated in biotinylated link (Dako) for 10 min and next in streptavidin-HRP (Dako) for 10 min at room temperature. The images were visualized by a liquid DAB + substrate chromogen system (Dako) and obtained using a CCD-camera equipped light microscope (BX-51). For comparing expression and phosphorylation of eEF2K and eEF2 phosphorylation between LV from ISO and Cont group, ratio of total-eEF2K-, phosphorylated eEF2K- or phosphorylated eEF2-positive area in three fields from each LV section was calculated using Image J software (NIH, Bethesda, MD, USA).

2.7. Materials

Reagent sources were as followings: propofol (Mylan N. V., Tokyo, Japan), diethyl ether (Wako Pure Chemical, Osaka, Japan), buprenorphine (Otsuka Pharmaceutical, Tokyo, Japan), isoproterenol bitartrate (Sigma-Aldrich, St. Louis, MO, USA), and pentobarbital (Sumitomo Dainippon Pharma, Tokyo, Japan).

Antibody sources were as followings: total-eEF2K (No. GTX107879) (Gene Tex, Irvine, CA, USA), phospho-eEF2K (at Ser366) (No. A0071) (Assay Biotech, Sunnyvale, CA, USA), phospho-eEF2 (at Thr56) (No. ADI-905-775-100) (Assay Designs, Ann Arbor, MI, USA), and GAPDH (No. GTX100118) (Gene Tex).

2.8. Statistics

Data are presented as means \pm SEM. Statistical evaluations were done by unpaired student's *t*-test. P values of < 0.05 were considered statistically significant.

3. Results

3.1. Changes in body weight (BW), LV weight as well as cardiac function and structure

In pressure overload-induced cardiac hypertrophy model mice, BW was not different between SHAM (21.7 ± 1.1 g, $n=8$, Table 1) and TAC group (22.8 ± 0.9 g, $n=8$, Table 1). In TAC group, LV weight (from 76.8 ± 2.6 mg to 97.5 ± 5.3 mg, $p < 0.01$, $n=8$, Table 1) and LV to BW ratio (LV/BW; from 3.52 ± 0.07 to 4.43 ± 0.28 , $p < 0.01$, $n=8$, Table 1) were significantly increased compared with SHAM group. LVID end diastole was significantly increased compared with SHAM (from 0.22 ± 0.016 cm to 0.28 ± 0.012 cm, $p < 0.05$, $n=6-8$, Table 2). LVPW end diastole (from 0.12 ± 0.007 cm to 0.15 ± 0.009 cm, $p < 0.05$, $n=6-8$, Table 2) and end systole (from 0.13 ± 0.010 cm to 0.17 ± 0.010 cm, $p < 0.05$, $n=6-8$, Table 2) in TAC mice were significantly increased compared with SHAM, suggesting the increased left ventricular wall hypertrophy. In isoproterenol-induced cardiac hypertrophy model rats, BW between control (228.0 ± 3.4 g, $n=8$, Table 1) and isoproterenol-injected group (233.4 ± 1.8 g, $n=8$, Table 1) was not different. In isoproterenol-injected group, LV weight (from 545.1 ± 10.7 mg to 717.1 ± 17.0 mg, $p < 0.01$, $n=8$, Table 1) and LV/BW (from 2.39 ± 0.04 to 3.07 ± 0.08 , $p < 0.01$, $n=8$, Table 1) were significantly increased compared with control group. We have previously shown that LV/BW was significantly increased in SHR compared with WKY at 12-week-old [8].

3.2. Phosphorylation of eEF2K and eEF2 in LV from SHR

We have recently shown that protein expression of eEF2K was significantly increased in LV from SHR compared with WKY at 12-week-old [8]. In the present study, we further examined phosphorylation states of eEF2K and its specific substrate, eEF2 in LV from SHR. eEF2K phosphorylation at Ser366 (inactive site) was

Table 1

Changes in body weight, left ventricular weight and the left ventricle to body weight ratio in pressure overload- and isoproterenol-induced cardiac hypertrophy model. TAC, transverse aortic constriction. ** $p < 0.01$ vs. SHAM, ## $p < 0.01$ vs. Control.

	Body weight (g)	Left ventricular weight (mg)	Left ventricle to body weight ratio (mg/g)
SHAM ($n=8$)	21.7 ± 1.1	76.8 ± 2.6	3.52 ± 0.07
TAC ($n=8$)	22.8 ± 0.9	$97.5 \pm 5.3^{**}$	$4.43 \pm 0.28^{**}$
Control ($n=8$)	228.0 ± 3.4	545.1 ± 10.7	2.39 ± 0.04
Isoproterenol ($n=8$)	233.4 ± 1.8	$717.1 \pm 17.0^{##}$	$3.07 \pm 0.08^{##}$

Table 2

Results of echocardiography in pressure overload-induced cardiac hypertrophy model. IVSd/s, interventricular septal end diastole/end systole; LVIDd/s, left ventricular internal diameter end diastole/end systole; LVPWd/s, left ventricular posterior wall end diastole/end systole; FS, fractional shortening; HR, heart rate. * $p < 0.05$ vs. SHAM.

	SHAM ($n=6$)	TAC ($n=8$)
IVSd (cm)	0.10 ± 0.006	0.11 ± 0.013
IVSs (cm)	0.15 ± 0.013	0.14 ± 0.011
LVIDd (cm)	0.22 ± 0.016	$0.28 \pm 0.012^*$
LVIDs (cm)	0.12 ± 0.008	0.16 ± 0.013
LVPWd (cm)	0.12 ± 0.007	$0.15 \pm 0.009^*$
LVPWs (cm)	0.13 ± 0.010	$0.17 \pm 0.010^*$
FS (%)	48.4 ± 3.5	43.5 ± 3.6
HR (bpm)	477 ± 22	457 ± 25

decreased in SHR LV compared with WKY ($p=0.0732$, $n=7$, Fig. 1). In consistent with the results, eEF2 phosphorylation at Thr56 was significantly increased in SHR LV compared with WKY ($p < 0.05$, $n=7$, Fig. 1).

3.3. Expression and phosphorylation of eEF2K and eEF2 in pressure overload-induced hypertrophied LV

We next examined protein expression and phosphorylation of eEF2K and eEF2 in TAC-induced hypertrophied LV. While eEF2K expression ($p < 0.01$, $n=8$, Fig. 2) was significantly increased in LV from TAC compared with SHAM, eEF2K phosphorylation at Ser366 was significantly decreased in LV from TAC compared with SHAM ($p < 0.05$, $n=8$, Fig. 2). In consistent with the results, eEF2 phosphorylation at Thr56 was significantly increased in LV from TAC compared with SHAM ($p < 0.01$, $n=8$, Fig. 2).

3.4. Expression and phosphorylation of eEF2K and eEF2 in isoproterenol-induced hypertrophied LV

We further examined expression and phosphorylation of eEF2K and eEF2 in LV from isoproterenol-induced cardiac hypertrophy model rats. eEF2K phosphorylation at Ser366 was significantly decreased in LV from isoproterenol-injected group (ISO) compared with control group (Cont; $p < 0.01$, $n=6$, Fig. 3). However, eEF2K expression and eEF2 phosphorylation at Thr56 were not different between the groups as determined by Western blotting ($n=6$, Fig. 3). It has been demonstrated that isoproterenol induced cardiac fibrosis in rat LV 7 days after isoproterenol injection [17]. We confirmed it by an Azan staining ($n=6$, Fig. 4A-a, e). We next examined expression and phosphorylation of eEF2K (at Ser366) and eEF2 (at Thr56) by immunohistochemistry using specific antibody. Expression of total-eEF2K and phosphorylated eEF2K as well as phosphorylated eEF2 seemed to be mainly localized to cardiomyocytes, but not fibrotic areas or cardiac fibroblasts ($n=3-4$, Fig. 4A-b-d, f-h). Of note, eEF2K-positive area was significantly

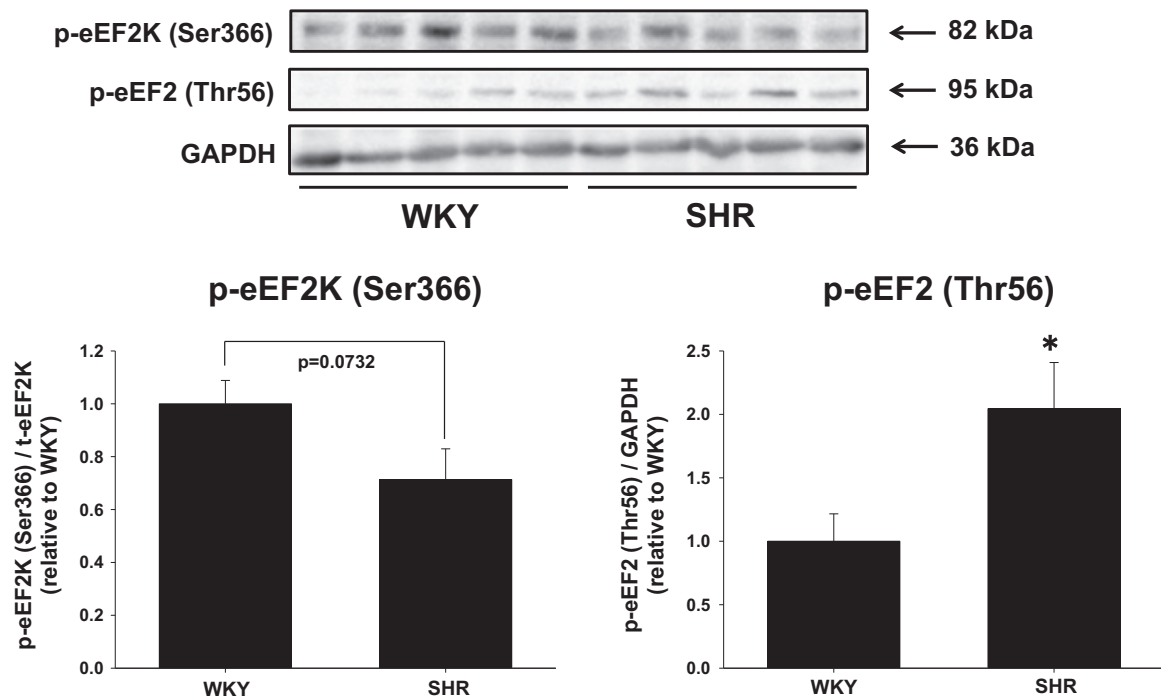


Fig. 1. Phosphorylation of eukaryotic elongation factor 2 (eEF2) kinase (eEF2K) and eEF2 in left ventricles from spontaneously hypertensive rats (SHR). After left ventricles isolated from SHR and Wistar-Kyoto rats (WKY) were immediately frozen, the protein was extracted. Phosphorylation of eEF2K (p-eEF2K at Ser366; n=7) and eEF2 (p-eEF2 at Thr56; n=7) was determined by Western blotting. The results were shown as fold increase relative to WKY. p-eEF2K or p-eEF2 was normalized to total-eEF2K or GAPDH, respectively. Our previous study has shown that protein expression of total-eEF2K was significantly increased in SHR left ventricles compared with WKY. * $p < 0.05$ vs. WKY.

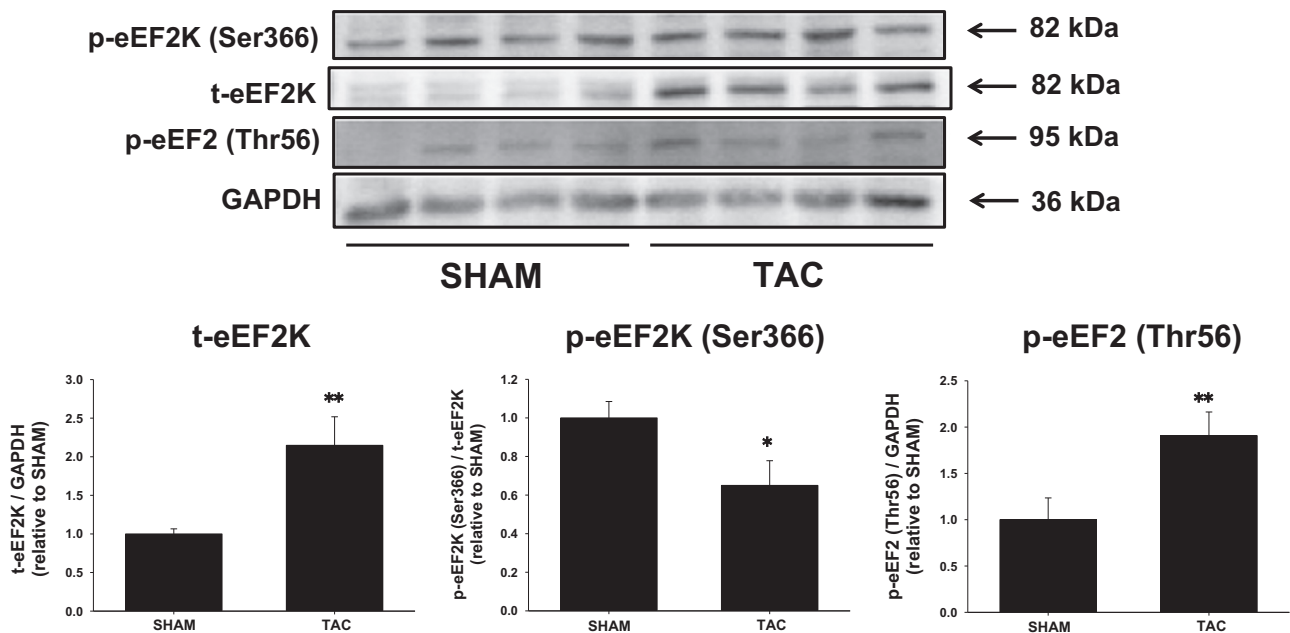


Fig. 2. Expression and phosphorylation of eEF2K and eEF2 in left ventricles from pressure overload-induced cardiac hypertrophy model mice. Transverse aortic constriction (TAC) or SHAM operation was performed in C57BL/6Njcl1 mice. After 3 days from the operation, left ventricles were isolated and immediately frozen. After extraction of protein, total-eEF2K (t-eEF2K, n=8), p-eEF2K at Ser366 (n=8) and p-eEF2 at Thr56 (n=8) were determined by Western blotting. The results were shown as fold increase relative to SHAM. p-eEF2K was normalized to t-eEF2K. t-eEF2K and p-eEF2 were normalized to GAPDH. * $p < 0.05$ vs. SHAM; ** $p < 0.01$ vs. SHAM.

increased ($p < 0.05$, n=4, Fig. 4A-b, f and B) and phosphorylated eEF2 was also increased ($p=0.0688$, n=3–4, Fig. 4A-d, h and D) in LV cardiomyocytes from ISO compared with Cont. We also confirmed that the phosphorylated eEF2K was significantly decreased in LV cardiomyocytes from ISO compared with Cont, which corresponded to the results in Western blotting ($p < 0.05$, n=3–4, Fig. 4A-c, g and C).

4. Discussion

Cardiac hypertrophy is a kind of compensatory response caused by physiological adaptation or pathological events including hypertension, cardiac myopathy and valvular disease. Cardiac hypertrophy consists of several histological changes such as an increased volume of cardiomyocyte and a fiber infiltration [3]. Protein synthesis is one of the cardinal features in cardiomyocyte

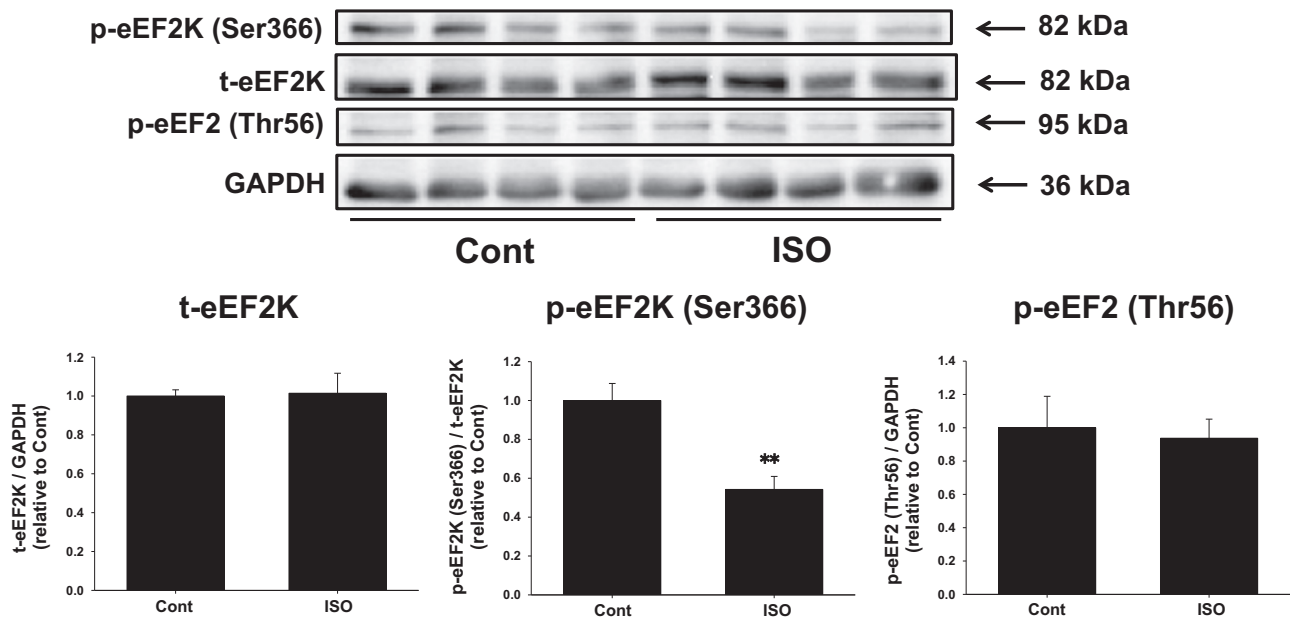


Fig. 3. Expression and phosphorylation of eEF2K and eEF2 in left ventricles from isoproterenol-induced cardiac hypertrophy model rats. Isoproterenol (5 mg/kg; ISO group) or saline (Cont group) was subcutaneously injected to rats daily. After 1 week, left ventricles were isolated and immediately frozen. After extraction of protein, t-eEF2K (n=6), p-eEF2K at Ser366 (n=6) and p-eEF2 at Thr56 (n=6) were determined by Western blotting. The results were shown as fold increases relative to Cont. p-eEF2K was normalized to t-eEF2K. t-eEF2K and p-eEF2 were normalized to GAPDH. ** $p < 0.01$ vs. Cont.

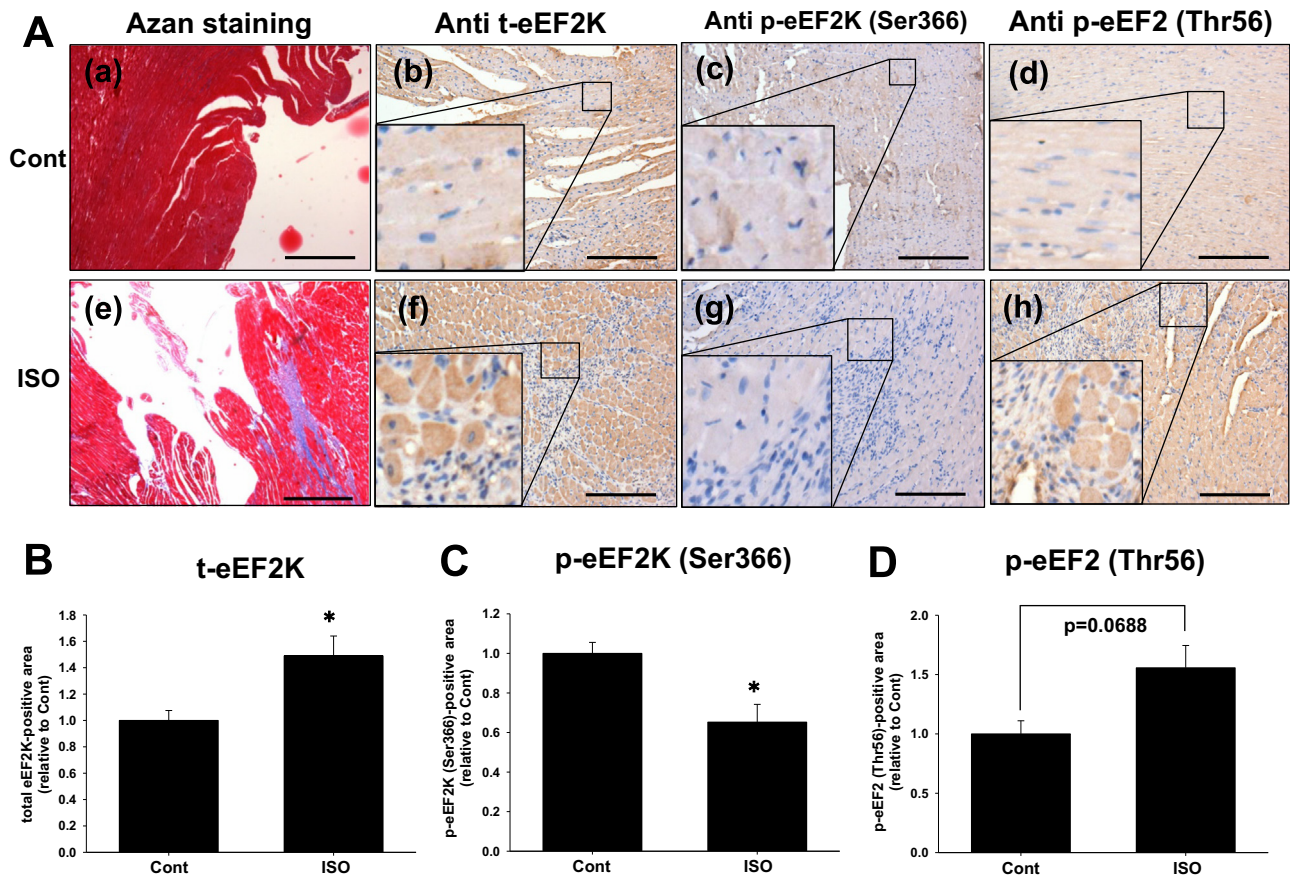


Fig. 4. Expression and phosphorylation of eEF2K (Ser366) and eEF2 (Thr56) in isoproterenol-induced hypertrophied cardiomyocytes. Isoproterenol (5 mg/kg; ISO) or saline (Cont) was subcutaneously injected to rats daily. After 1 week, left ventricles were isolated and the paraffin sections were made. (A) Representative azan-stained left ventricular sections (a: Cont, n=6; e: ISO, n=6) and immuno-stained sections using specific antibody against t-eEF2K (b: Cont, n=4; f: ISO, n=4), p-eEF2K (Ser366) (c: Cont, n=3; g: ISO, n=4) or p-eEF2 (Thr56) (d: Cont, n=3; h: ISO, n=4). Scale bar: 500 μ m (a, e), 200 μ m (b-d, f-h). (B) t-eEF2K-, (C) p-eEF2K (Ser366)- or (D) p-eEF2 (Thr56)-positive area to cross-sectional cardiomyocyte area ratio was calculated. The results were shown as fold increase relative to Cont. * $p < 0.05$ vs. Cont.

hypertrophy, and partly regulated by elongation factor, eEF2. eEF2 was phosphorylated and inactivated by eEF2K. Thus, eEF2K inactivation facilitates eEF2-dependent protein translation. Several agonists including angiotensin II, endothelin-1 and phenylephrine, which induce cardiomyocyte hypertrophy, are reported to facilitate dephosphorylation of eEF2 in rat cardiomyocytes [4,22]. These reports suggest that cardiac hypertrophy is partly regulated by increased protein synthesis via eEF2K/eEF2 signaling.

Our recent study has shown that the protein expression of eEF2K was significantly increased in hypertrophied LV from SHR compared with normal LV from WKY [8]. In the present study, we further examined phosphorylation states of eEF2K and eEF2 in LV from SHR. eEF2K phosphorylation at Ser366 was decreased in SHR LV (Fig. 1). eEF2K dephosphorylation at Ser366 makes itself an active state, and mediates phosphorylation of eEF2 [23]. In consistent with the report, eEF2 phosphorylation at Thr56 was significantly increased in LV from SHR compared with WKY (Fig. 1). In addition, we examined them in pressure overload-induced cardiac hypertrophy model mice. Increased expression and decreased phosphorylation of eEF2K at Ser366 were also found in LV from TAC mice compared with SHAM mice (Fig. 2). Consistently, eEF2 phosphorylation at Thr56 was significantly increased (Fig. 2). These results were consistent with the results obtained from SHR LV. Moreover, the eEF2K/eEF2 signaling in LV was examined in isoproterenol-induced cardiac hypertrophy model rats. While eEF2K phosphorylation at Ser366 was significantly decreased in LV from ISO rats compared with Cont rats, eEF2K expression and eEF2 phosphorylation were not different as determined by Western blotting (Fig. 3). The LV from ISO had fibrotic areas at adluminal part of free wall (Fig. 4A–a, e). In immunohistochemical analysis, we found that total- and phosphorylated eEF2K as well as phosphorylated eEF2 were localized to cardiomyocytes but not fibrotic areas (Fig. 4A–b–d, f–h). Of note, eEF2K- and phosphorylated eEF2-positive areas were increased in LV from ISO rats compared with Cont rats (Fig. 4A–b, d, f, h and B, D). Accordingly, the increased expression and the decreased phosphorylation of eEF2K and the increased phosphorylation of eEF2 in hypertrophied LV were common to all animal models in this study.

The present results suggest that protein translation might be decreased in hypertrophied LV. In general, cardiomyocytes in hypertrophied heart are exposed by hypoxic and starved condition because of microvascular rarefaction [16]. In these conditions, adenosine monophosphate (AMP) to adenosine triphosphate ratio is increased, which results in an increased activation of AMP-activated protein kinase (AMPK) in cardiomyocytes [18]. It was reported that activated AMPK facilitates eEF2 phosphorylation at Thr56 and subsequently inhibits protein synthesis in rat cardiomyocytes [1,12]. In addition, Crozier et al. revealed that phosphorylation of AMPK and eEF2 was increased by ischemia in rat isolated heart [2]. Therefore, it might be possible that AMPK/eEF2K/eEF2 pathway at least partly affects the pathogenesis of cardiac hypertrophy.

In normal cardiomyocytes, myocardial contraction, Ca²⁺ homeostasis and protein synthesis consume high proportion of cellular energy [10]. Since myocardial contractile function is impaired during the development of cardiac diseases, it is important to keep the energy consumption lower level for the maintenance of cardiac contraction [6]. Therefore, it is proposed that a decreased protein synthesis induced by eEF2 inactivation might play role for maintaining myocardial contraction during cardiac diseases. On the other hand, eEF2K/eEF2 signaling is reported to mediate apoptosis and autophagy in a certain tumor cells [24]. Thus, cardiac cell death regulated by eEF2K/eEF2 signaling might be also related to the pathogenesis of cardiac hypertrophy.

In conclusion, we for the first time show that the increased expression and the decreased phosphorylation of eEF2K and the

increased phosphorylation of eEF2 in hypertrophied LV were common to several animal models, namely SHR as well as pressure overload- and isoproterenol-induced cardiac hypertrophy. These results suggest the potential role of eEF2K/eEF2 signaling in the pathogenesis of cardiac hypertrophy development. Detailed examinations are needed to further reveal the relationships between eEF2K/eEF2 signaling pathway and cardiac hypertrophy.

Funding information

This study was supported by a Scholarship from the Tokyo Biochemical Research Foundation, Japan (to S. K.).

Transparency Document. Supporting information

Transparency data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.06.018>.

References

- [1] A.Y. Chan, C.L. Soltys, M.E. Young, C.G. Proud, J.R. Dyck, Activation of AMP-activated protein kinase inhibits protein synthesis associated with hypertrophy in the cardiac myocyte, *J. Biol. Chem.* 279 (2004) 32771–32779, <http://dx.doi.org/10.1074/jbc.M403528200>.
- [2] S.J. Crozier, T.C. Vary, S.R. Kimball, L.S. Jefferson, Cellular energy status modulates translational control mechanisms in ischemic-reperfused rat hearts, *Am. J. Physiol. Heart Circ. Physiol.* 289 (2005) H1242–H1250, <http://dx.doi.org/10.1152/ajpheart.00859.2004>.
- [3] M.H. Drazner, The progression of hypertensive heart disease, *Circulation* 123 (2011) 327–334, <http://dx.doi.org/10.1161/CIRCULATIONAHA.108.845792>.
- [4] A.D. Everett, T.D. Stoops, A.C. Nairn, D. Brautigan, Angiotensin II regulates phosphorylation of translation elongation factor-2 in cardiac myocytes, *Am. J. Physiol. Heart Circ. Physiol.* 281:H161–167. PMID 11406481 (2001).
- [5] L.L. Fu, T. Xie, S.Y. Zhang, B. Liu, Eukaryotic elongation factor-2 kinase (eEF2K): a potential therapeutic target in cancer, *Apoptosis* 19 (2014) 1527–1531, <http://dx.doi.org/10.1007/s10495-014-1019-7>.
- [6] J.S. Ingwall, Energy metabolism in heart failure and remodelling, *Cardiovasc. Res.* 81 (2009) 412–419, <http://dx.doi.org/10.1093/cvr/cvn301>.
- [7] S. Kameshima, K. Kazama, M. Okada, H. Yamawaki, Eukaryotic elongation factor 2 kinase mediates monocrotaline-induced pulmonary arterial hypertension via reactive oxygen species-dependent vascular remodeling, *Am. J. Physiol. Heart Circ. Physiol.* 308 (2015) H1298–H1305, <http://dx.doi.org/10.1152/ajpheart.00864.2014>.
- [8] S. Kameshima, M. Okada, H. Yamawaki, Expression and localization of calmodulin-related proteins in brain, heart and kidney from spontaneously hypertensive rats, *Biochem. Biophys. Res. Commun.* 469 (2016) 654–658, <http://dx.doi.org/10.1016/j.bbrc.2015.12.048>.
- [9] S. Kameshima, Y. Sakamoto, M. Okada, H. Yamawaki, Vaspin prevents elevation of blood pressure through inhibition of peripheral vascular remodeling in spontaneously hypertensive rats, *Acta Physiol.* 217 (2016) 120–129, <http://dx.doi.org/10.1111/apha.12636>.
- [10] S.C. Kolwicz Jr., S. Purohit, R. Tian, Cardiac metabolism and its interactions with contraction, growth, and survival of cardiomyocytes, *Circ. Res.* 113 (2013) 603–616, <http://dx.doi.org/10.1161/CIRCRESAHA.113.302095>.
- [11] P. Krenek, J. Kmečová, D. Kucerová, Z. Bajuszová, P. Musil, A. Gazová, P. Ochodnický, J. Klimas, J. Kyselovic, Isoproterenol-induced heart failure in the rat is associated with nitric oxide-dependent functional alterations of cardiac function, *Eur. J. Heart Fail* 11 (2009) 140–146, <http://dx.doi.org/10.1093/eurjhf/hfn026>.
- [12] L.E. McLeod, C.G. Proud, ATP depletion increases phosphorylation of elongation factor eEF2 in adult cardiomyocytes independently of inhibition of mTOR signalling, *FEBS Lett.* 531 (2002) 448–452, [http://dx.doi.org/10.1016/S0014-5793\(02\)03582-2](http://dx.doi.org/10.1016/S0014-5793(02)03582-2).
- [13] L.E. McLeod, L. Wang, C.G. Proud, beta-Adrenergic agonists increase phosphorylation of elongation factor 2 in cardiomyocytes without eliciting calcium-independent eEF2 kinase activity, *FEBS Lett.* 489 (2001) 225–228, [http://dx.doi.org/10.1016/S0014-5793\(01\)02100-7](http://dx.doi.org/10.1016/S0014-5793(01)02100-7).
- [14] C.E. Moore, S. Regufe da Mota, H. Mikolajek, C.G. Proud, A conserved loop in the catalytic domain of eukaryotic elongation factor 2 kinase plays a key role in its substrate specificity, *Mol. Cell Biol.* 34 (2014) 2294–2307, <http://dx.doi.org/10.1128/MCB.00388-14>.
- [15] A.C. Nairn, B. Bhagat, H.C. Palfrey, Identification of calmodulin-dependent protein kinase III and its major Mr 100,000 substrate in mammalian tissues, *Proc. Natl. Acad. Sci. U. S. A.* 82 (1985) 7939–7943, <http://dx.doi.org/10.1073/pnas.82.23.7939>.
- [16] T. Oka, H. Akazawa, A.T. Naito, I. Komuro, Angiogenesis and cardiac

- hypertrophy: maintenance of cardiac function and causative roles in heart failure, *Circ. Res.* 114 (2014) 565–571, <http://dx.doi.org/10.1161/CIRCRESAHA.114.300507>.
- [17] M. Okada, N. Kosaka, Y. Hoshino, H. Yamawaki, Y. Hara, Effects of captopril and telmisartan on matrix metalloproteinase-2 and -9 expressions and development of left ventricular fibrosis induced by isoprenaline in rats, *Biol. Pharm. Bull.* 33 (2010) 1517–1521, <http://dx.doi.org/10.1248/bpb.33.1517>.
- [18] D. Qi, L.H. Young, AMPK: energy sensor and survival mechanism in the ischemic heart, *Trends Endocrinol. Metab.* 26 (2015) 422–429, <http://dx.doi.org/10.1016/j.tem.2015.05.010>.
- [19] A.G. Ryazanov, Elongation factor-2 kinase and its newly discovered relatives, *FEBS Lett.* 514 (2002) 26–29, [http://dx.doi.org/10.1016/S0014-5793\(02\)02299-8](http://dx.doi.org/10.1016/S0014-5793(02)02299-8).
- [20] A.G. Ryazanov, M.D. Ward, C.E. Mendola, K.S. Pavur, M.V. Dorovkov, M. Wiedmann, H. Erdjument-Bromage, P. Tempst, T.G. Parmer, C.R. Prostko, F. J. Germino, W.N. Hait, Identification of a new class of protein kinases represented by eukaryotic elongation factor-2 kinase, *Proc. Natl. Acad. Sci. U S A* 94 (1997) 4884–4889.
- [21] J.R. Teerlink, J.M. Pfeffer, M.A. Pfeffer, Progressive ventricular remodeling in response to diffuse isoproterenol-induced myocardial necrosis in rats, *Circ. Res.* 75 (1994) 105–113, <http://dx.doi.org/10.1161/01.RES.75.1.105>.
- [22] L. Wang, C.G. Proud, Regulation of the phosphorylation of elongation factor 2 by MEK-dependent signalling in adult rat cardiomyocytes, *FEBS Lett.* 531 (2002) 285–289, [http://dx.doi.org/10.1016/S0014-5793\(02\)03536-6](http://dx.doi.org/10.1016/S0014-5793(02)03536-6).
- [23] X. Wang, W. Li, M. Williams, N. Terada, D.R. Alessi, C.G. Proud, Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase, *EMBO J.* 20 (2001) 4370–4379, <http://dx.doi.org/10.1093/emboj/20.16.4370>.
- [24] H. Zhu, X. Yang, J. Liu, L. Zhou, C. Zhang, L. Xu, Q. Qin, L. Zhan, J. Lu, H. Cheng, X. Sun, Eukaryotic elongation factor 2 kinase confers tolerance to stress conditions in cancer cells, *Cell Stress Chaperon-*. 20 (2015) 217–220, <http://dx.doi.org/10.1007/s12192-014-0545-0>.