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Differential expression of cardiac uncoupling proteins 2 and 3 in response to myocardial ischemia-reperfusion in rats

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ABSTRACT

Aims: We aimed to evaluate the transcription and translation of genes for uncoupling protein 2 (UCP2) & uncoupling protein 3 (UCP3) in rat heart mitochondria of both ventricles after myocardial ischemia followed by various periods of reperfusion.
Main methods: Seven groups of 8 male Wistar rats were evaluated for the effects of ischemia and also reperfusion, using Western blot of isolated mitochondrial proteins in addition to RNA extraction followed by real-time RT-PCR analysis.
Key findings: In rats with 30 min of reperfusion (R30) UCP2 protein was increased 213 ± 33%, which is meaningfully more than the control group ($P < 0.001$). Western blot showed increase in UCP2 protein level in groups receiving reperfusion for 60 min (R60), 120 min (R120) and 180 min (R180) as much as 152 ± 28% ($P < 0.001$ vs. control), 123 ± 19% ($P < 0.01$ vs. control) and 131 ± 30% ($P < 0.01$ vs. control), respectively. There was no statistically important difference in UCP2 mRNA between either right or left ventricles of ischemic and ischemia-reperfusion (IR) groups vs. control group. In the groups R180 and R240, UCP3 protein levels showed 131 ± 27% and 102 ± 18% increase, respectively (both $P < 0.001$ vs. control group). However, the change in UCP3 level in other groups was not significantly different from the control group.
Significance: UCP2 and UCP3 protein levels are considerably increased in the ischemic area early after acute myocardial IR. The right ventricular UCP2 protein expression does not change, that is, effect of IR on UCP2 protein is a local process. However, UCP3 protein level increased both in ischemic area of the left ventricle and in non-ischemic area of the right ventricle.

Introduction

Myocardial ischemia is associated with activation of many complex physiologic and pathologic processes. Numerous studies including our recently published data have shown that the levels of many proteins including anticoagulant factors, structural proteins, mediators of inflammation, proteins related to transcription and translation, and also proteins involved in regulation of metabolism are significantly increased after acute myocardial ischemia-reperfusion (IR) (Fert-Bober et al., 2008; De Celle et al., 2005; Safari et al., 2012, 2013). Obviously, accurate identification of these changes is quite necessary for development of more effective drugs for injuries induced by acute myocardial IR.

Uncoupling protein-2 (UCP2) and 3 (UCP3) are in the mitochondrial anion carrier family of proteins that catalyze translocation of protons across the inner mitochondrial membrane. It has been shown that decrease in proton motive force will reduce generation of mitochondrial reactive oxygen species (ROS), and will diminish production of adenosine triphosphate (ATP) by uncoupling of phosphorylation and oxidation pathways (Cioffi et al., 2009). It is also thought that UCPs are involved in free fatty acid (FFA) metabolism since they catalyze export of fatty acid peroxides to outside of the inner mitochondrial membrane (Himms-Hagen and Harper, 2001; Murray et al., 2005). Some studies have recently suggested the fundamental role of UCP2 and UCP3 in mitochondrial Ca^{2+} uniport, too (Trenker et al., 2007). So, these proteins can be regarded as multi-task cellular factors.

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There is some evidences that ROS (Brand et al., 2004; Azzu and Brand, 2010) and FFA (Murray et al., 2005; Cole et al. 2011) can induce expression of UCP2 and UCP3. The presence of UCP2 and UCP3 proteins in the rat heart has been shown in several studies including ours, but the cardiac regulation of their expression in ischemic myocardium remains unclear. Increased mitochondrial ROS production, FFA accumulation and calcium overload, and concomitantly decreased ATP have been implicated in myocardial damage induced by IR (Dorweiler et al., 2007; Asano et al., 2003). Acknowledging the crucial role of mitochondria in cardiac cells, assessment of changes in mitochondrial genes & proteins which affect physiological parameters such as free radical production, ATP generation, Ca^{2++} handling and FFA production in cardiomyocytes would be of great importance. Therefore, the aim of the current study was to evaluate the transcription of UCP2 & UCP3 in rat heart mitochondria after myocardial ischemia followed by various periods of reperfusion, and also assessment of the level of expression of their relevant proteins.

Materials and methods

Experimental groups

Seven groups ($n = 8$ in each group) of male Wistar rats were maintained on a reverse 12-h: 12-h light/dark cycle in a temperature-controlled room. All experimental procedures were done in accordance with the EU Directive 2010/63/EU for animal experiments and approved instructions of the Ethics Committee on Animal Experiments of Shahid Sadoughi University, Yazd, Iran.

Myocardial ischemia reperfusion was induced by ligation of the left anterior descending (LAD) branch of coronary artery. Briefly, the rats weighing 250–300 g were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal), intubated and ventilated artificially with room air at a frequency of 80 inflations/min and a tidal volume of 1 mL/100 g. Lead II of electrocardiogram (ECG) was recorded using cutaneous needle electrodes. Mean arterial blood pressure in the left carotid artery was monitored by a blood pressure transducer. A left thoracotomy was performed in the 4th intercostal space, the pericardium incised, and the heart exposed. In ischemic group (I) the LAD branch was occluded for 30 min by lifting a 5/0 silk thread which was passed below the LAD through a piece of polyethylene tube. The criteria for confirmation of successful coronary occlusion were development of a pale color in the distal myocardium, ST elevation on ECG, and reduction of the mean arterial pressure. In five ischemia-reperfusion groups (IR groups) after 30 min of ischemia, we allowed reperfusion of ischemic myocardium for 30, 60, 120, 180 and 240 min by loosening the silk thread. In order to recognize the ischemic area at the end of the reperfusion, the LAD was occluded again and the pale ischemic area from the left ventricle was cut immediately. At the end of the reperfusion the heart was immediately removed and washed in cold isotonic saline. Since UCP measurement in right ventricle was one of the main goals of this study, Evans blue staining was not used for determining ischemic area. In the I and IR groups, the ischemic area from the left ventricle was biopsied, but in the sham-operated and the control animals corresponding normal left ventricular region was cut out. In all groups a right ventricle sample was also collected. Liquid nitrogen was used for rapid freezing of the tissue samples at −80 °C for further studies. The same surgical procedures were performed in sham-operated rats, except that the suture around the coronary artery was not tied. The seventh group included intact animals which served as control.

Cardiac mitochondrial isolation

According to the modified method of Butz et al., rat heart mitochondria were isolated using differential centrifugation (Butz et al., 2004). In this way, tissue was placed in ice-cold cardiac homogenization and isolation buffer A (2 mM EGTA, 40 mM NaCl, 210 mM sucrose, and 30 mM HEPES, pH 7.4) supplemented with protease inhibitors. Tissue was disrupted by the use of a Teflon-glass homogenizer. To eliminate nuclei and cell debris, the homogenate was centrifuged at 600 ×*g* for 10 min at 4 °C. One mL of supernatant was centrifuged at 10,000 g at 4 °C for 20 min. The pellet from this step was washed in 1 mL of isolation medium B which contained 10 mM Tris and 1 mM EDTA, pH 7.4. The final pellet containing mitochondria was resuspended in 66 μL of 16% SDS and 200 μL of buffer B, and centrifuged at 1100 *g* for 20 min at room temperature to remove insoluble materials. The supernatants which contained mitochondria were stored at −80 °C.

Western blot analysis

By the use of the Bradford method, protein concentrations of homogenates were determined (Bradford, 1976). The sample buffer contained 10% (v/v) glycerol, 5% (w/v) SDS, 0.25% (w/v) bromophenol blue, 5% (v/v) 2-mercaptoethanol, and 0.0625 M Tris–HCl, pH 6.8, and the protein extract was incubated for 7 min at 100 °C in it before being loaded onto the wells. The homogenate proteins were loaded in equal amounts on a 12.5% SDS-polyacrylamide gel electrophoresis mini-gel in each lane (Bio-Rad laboratories, Hercules CA, USA). Electrophoresis was performed for about 1.5 h at 110 V in running buffer (0.025 M Tris–HCl, 0.2 M glycine, 1 mM EDTA, and 3.5 mM SDS). Using a blot apparatus (Bio-Rad, USA), proteins were transferred from the gels to polyvinylidene fluoride membrane (Amersham Bioscience Co., UK). The membranes were blocked with 2% ECL Advance Blocking Milk (Amersham Bioscience Co., UK) and 1% bovine serum albumin in TBST (0.01 M Tris–HCl, pH 7.6, 1.5 mM NaCl, 0.1% (v/v) Tween-20) for 1 h. Western blotting was performed with polyclonal goat anti-UCP2 and anti-UCP3 primary antibodies (Santa Cruz Biotechnology, Inc., USA) and with donkey anti-goat horseradish peroxidase-conjugated secondary antibody (from the same manufacturer) at a dilution of 1:500 and 1:10,000, respectively. After this step, chemiluminescent substrate (ECL Advance reagents, Amersham Bioscience Co., UK) was added, and blots were exposed on film. Bands were scanned, and their densities determined using ImageJ software (version 1.43). Data were normalized to control bands.

RNA extraction and real-time RT-PCR analysis

Expression levels of the genes were determined by real-time RT-PCR. The RNeasy fibrous tissue mini kit from QIAGEN, USA was used for total RNA extraction according to the manufacturer's instructions. Concentrations of RNA were determined by measuring the absorbance at 260 nm, and its purity was assessed by 260/280 nm absorbance ratio (Eppendorf, Hamburg, Germany). For synthesis of first strand cDNA, we used 1 μg of total RNAs using random hexamers, dNTP and Moloney murine leukemia virus reverse transcriptase (Fermentas), in a total volume of 20 μL. The RT-PCR was performed using the Rotor Gene system (Corbett Research 2004, Australia) and SYBR Green I. Relative quantity of gene expression was analyzed according to the Pfaffl method. The nucleotide sequences of the PCR primers were as follows: UCP2: forward 5′-GCCC GGGCTGGTGGTGGTC-3′ and reverse 5′-CCCCGAAGGCAGAAGTGAAG TGG-3′; UCP3: forward 5′-CGTCTCGGTACATCCTGACTA-3′ and reverse 5′-TTCTTCCCTGGCGTGGTTCTGTA-3′; β-actin: forward 5′-GAACCCTAAG GCCAACCGTGAAAAGAT-3′ and reverse 5′-ACCGCTCGTTGCCAATAGTG ATG-3′.

Statistical analysis

For comparison of the differences between control, sham and IR groups, one-way analysis of variance (ANOVA) was used. Post hoc analysis was performed using the Tukey multiple comparison test. To compare hemodynamic parameters in each group, we used paired *t*-test.