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Evidence for a pro-apoptotic phenotype in skeletal muscle of hypertensive rats ☆

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Abstract

In this report, we demonstrate that soleus muscle of spontaneously hypertensive rats (SHR) had significantly lower protein levels of apoptosis repressor with caspase recruitment domain (ARC) and X-linked inhibitor of apoptosis protein (XIAP) as well as significantly higher protein levels of second mitochondria-derived activator of caspase (Smac) and procaspase-8 compared to normotensive Wistar-Kyoto (WKY) rats. In addition, soleus muscle from hypertensive rats had significantly increased caspase-8 proteolytic enzyme activity as well as significantly elevated reactive oxygen species (ROS) generation and higher hydrogen peroxide (H_2O_2) content. There was no change in the protein levels of the antioxidant enzymes, catalase, copper-zinc superoxide dismutase (CuZnSOD), and manganese superoxide dismutase (MnSOD). Interestingly, ARC protein migrated at approximately 32 kDa in SHR but at 30 kDa in WKY rat muscle; possibly indicating a post-translational modification. These results demonstrate that soleus muscle of hypertensive rats display a pro-apoptotic phenotype and augmented ROS generation.

Keywords: Apoptosis; ARC; XIAP; Caspase-8; Smac; Oxidative stress; Skeletal muscle; Hypertension

Skeletal muscle apoptosis has been shown to be significantly elevated during a number of conditions associated with skeletal muscle wasting and dysfunction including aging [1], muscular dystrophy [2], and chronic heart failure [3]. Hypertension is often viewed only as a cardiovascular disease; however, hypertension is also associated with a number of skeletal muscle functional and morphological alterations. For example, hypertension in humans is associated with a lower percentage of slow-twitch fibers [4]. Spontaneously hypertensive rats (SHR), a model of essential hypertension, also show skeletal muscle alterations including decreased fatigue resistance [5], development of less contractile force [5], and fiber-type redistribution [6]. Recently, we have shown that DNA fragmentation (a hallmark of apoptosis) is increased in skeletal muscle of SHR compared to normotensive Wistar-Kyoto (WKY) rats [7].

Apoptosis repressor with caspase recruitment domain (ARC) is an anti-apoptotic protein that is highly expressed in cardiac and skeletal muscle [8]. ARC can interact with procaspase-8, caspase-8, Fas, and Fas-associated death domain (FADD) as well as inhibit apoptosis mediated via the extrinsic pathway [8,9]. ARC can also prevent Bax activation and translocation to the mitochondria, thus inhibiting apoptosis mediated through the intrinsic pathway [9,10]. Given that ARC is highly expressed in skeletal muscle, a tissue that does not normally undergo high rates of apoptosis [8,11], ARC may play an essential role in the regulation of skeletal muscle apoptosis. X-linked inhibitor of apoptosis protein (XIAP) can also influence apoptosis by inhibiting caspase-3 processing and activity [12]. However, release of second mitochondria-derived activator of

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caspase (Smac) from the mitochondria during apoptotic signaling can block the action of XIAP, thereby promoting caspase activation and apoptosis [13].

Currently, data regarding the effect of hypertension on skeletal muscle apoptosis is limited. We have previously shown elevated apoptosis, increased caspase-3 signaling, a decreased Bcl-2:Bax ratio, and accumulation of nuclear apoptosis inducing factor (AIF) in soleus muscle of hypertensive rats [7]. However, the expression of several important apoptotic factors as well as the involvement of death-receptor signaling during skeletal muscle apoptosis in hypertension are currently unknown. In particular, given that ARC is highly expressed in skeletal muscle and very responsive to apoptotic stimuli; it is of interest to examine this important anti-apoptotic protein in this model. The purpose of this study was to examine several pro- and anti-apoptotic proteins (ARC, XIAP, Smac, caspase-8) in soleus muscle of hypertensive animals. Furthermore, given that oxidative stress plays an important role in apoptosis [14] and in ARC degradation [15–17], we also examined whether several antioxidant enzymes including catalase, manganese superoxide dismutase (MnSOD), and copper-zinc superoxide dismutase (CuZnSOD) as well as reactive oxygen species (ROS) generation and hydrogen peroxide (H_2O_2) content were altered in skeletal muscle during hypertension. We hypothesized that anti-apoptotic factors would be suppressed while pro-apoptotic factors and oxidative stress would be elevated in soleus muscle of hypertensive rats.

Materials and methods

Experimental animals. Male normotensive WKY rats ($n = 18$) and SHR ($n = 18$) were obtained from Harlan (Indianapolis) and group housed on a 12:12 h reverse light/dark cycle in a temperature and humidity controlled environment. Standard rodent lab chow and tap water were provided ad libitum. All procedures involving animals were performed in accordance with the guidelines of the University of Waterloo Animal Care Committee.

Blood pressure measurement. At approximately 16–18 weeks of age rats were weighed and anesthetized with a pentobarbital sodium injection (0.65 mg/kg ip; MTC Pharmaceuticals). Soleus muscles were removed, immediately frozen in liquid nitrogen, and stored at −80 °C until further analysis. Blood pressure measurements were made in a subset of age- and weight-matched rats. Mean arterial blood pressure (MAP) was measured for a period of 10 min using a transducer (Harvard) attached to a PE-50 cannula inserted into the left common carotid artery. Following the blood pressure measurements, rats were sacrificed by removing the heart and right ventricle (RV), left ventricle (LV), and kidney mass were determined.

Preparation of whole muscle lysate. Whole soleus muscle lysates were prepared as previously described [7]. Briefly, soleus tissue (~20 mg wet wt) was homogenized in ice-cold lysis buffer (20 mM HEPES, 10 mM NaCl, 1.5 mM MgCl, 1 mM DTT, 20% glycerol and 0.1% Triton X-100; pH 7.4) containing protease inhibitors (Complete Cocktail; Roche Diagnostics) using a glass homogenizer. The whole tissue lysates were centrifuged at 1000*g* for 10 min at 4 °C, the supernatant collected, and protein concentration determined by the BCA protein assay.

Immunoblot analysis. Immunoblot analysis was performed as previously described [7] using primary antibodies against ARC, Smac (Assay Designs), ARC, procaspase-8 (Santa Cruz Biotechnology), XIAP, CuZnSOD, and MnSOD (Stressgen Bioreagents) in conjunction with the appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Equal loading of protein and quality of transfer were confirmed by Ponceau S staining and actin (Sigma–Aldrich) protein expression. Adenine nucleotide translocator (ANT) and cytochrome *c* oxidase subunit III (COX III) (Santa Cruz Biotechnology) were used as control markers for mitochondria protein content. Molecular weight of the immunoblotted protein was determined using a biotinylated protein ladder and anti-biotin secondary antibody (Cell Signaling Technology). Immunoblot data was expressed as relative arbitrary units (AU).

Caspase-8 activity. Caspase-8 activity was determined in soleus homogenate using a Caspase-8 Fluorimetric Assay Kit (Sigma–Aldrich) according to the manufacturer's instructions. In this assay, the peptide substrate, Ac-IETD-AMC, is weakly fluorescent but yields a highly fluorescent product following proteolytic hydrolysis by caspase-8. Briefly, soleus homogenate was incubated with Ac-IETD-AMC substrate at room temperature for 2 h. Fluorescence was measured using a SPECTRAmax Gemini XS microplate spectrofluorometer (Molecular Devices) with excitation and emission wavelengths of 360 nm and 440 nm, respectively. In control experiments, incubation of substrate with recombinant caspase-8 resulted in a large increase in fluorescence emission. In addition, incubation of recombinant caspase-8 with the caspase-8 inhibitor, Ac-IETD-CHO, completely inhibited the fluorescent signal (*data not shown*). Caspase-8 activity was expressed as AU per mg protein.

Hydrogen peroxide content and reactive oxygen species generation. Hydrogen peroxide (H_2O_2) content in soleus muscle homogenate was determined essentially as previously described [18] using the Amplex Red Hydrogen Peroxide Assay Kit (Invitrogen). This assay utilizes, 10-acetyl-3,7-dihydroxyphenoxazine, a non-fluorescent substrate that forms highly fluorescent resorufin following oxidization by H_2O_2 in the presence of horseradish peroxidase. Reactive oxygen species (ROS) generation was determined as previously described [19] with modifications using 2′,7′-dichlorofluorescin-diacteate (DCFH-DA); a non-fluorescent dye that is oxidized by a variety of ROS to form highly fluorescent dichlorofluorescin (DCF). Briefly, soleus muscle was homogenized in phosphate buffered saline (PBS) using a glass homogenizer. Muscle homogenate was incubated in the dark with either an Amplex Red cocktail at room temperature or 5 μM DCFH-DA (Invitrogen) at 37 °C. Fluorescence was measured every 15 min for 2 h using a SPECTRAmax GEMINI-XS microplate spectrofluorometer with excitation and emission wavelengths of 530 and 590 nm, respectively (Amplex Red), or 490 and 525 nm, respectively (DCFH-DA). Maximal fluorescence was expressed as AU per mg of protein.

Statistical analysis. Data were analyzed by independent sample *t*-test using SPSS analysis software (SPSS). In all cases, $P < 0.05$ was considered statistically significant. All results are expressed as means ± SEM.

Results

Anatomical data and blood pressure

As shown in Table 1, body weight ($P < 0.005$), heart weight ($P < 0.001$), LV weight ($P < 0.001$), left ventricle-to-body weight ratio (LV/BW) ($P < 0.005$), and mean arterial pressure ($P < 0.001$) were significantly higher in SHR compared to WKY rats. Kidney weight and RV weight were not significantly different between groups.

Apoptotic protein expression and caspase-8 activity

Immunoblot analysis of soleus muscle revealed that ARC protein was depressed (−59%; $P < 0.001$) in SHR compared to WKY rats. Moreover, ARC protein migrated at a molecular weight of approximately 32 kDa in SHR and at 30 kDa in WKY rats (Fig. 1). This 2 kDa shift in the molecular weight of ARC protein was observed in all