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Biochemical and Biophysical Research Communications 368 (2008) 168–174
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Evidence for a pro-apoptotic phenotype in skeletal muscle of hypertensive rats ☆

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Received 12 January 2008
Available online 22 January 2008

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

☆ This research was supported by the Natural Sciences and Engineering Research Council of Canada (J. Quadrilatero: RGPIN 341256 and J.W.E. Rush: RGPIN 238342) and the Heart & Stroke Foundation of Ontario (J.W.E. Rush: T6009). J.W.E. Rush is the Canadian Institute of Health Research-sponsored Canada Research Chair in Integrative Vascular Biology.
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doi:10.1016/j.bbrc.2008.01.067

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

Table 1
Group characteristics

Group	Body weight (g)	Heart weight (mg)	LV weight (mg)	RV weight (mg)	Kidney weight (mg)	LV/BW (mg/g)	MAP (mm Hg)
WKY	300.8 ± 6.7	890.0 ± 21.3	686.4 ± 20.4	203.6 ± 10.0	1030.6 ± 12.8	2.284 ± 0.050	89.8 ± 4.0
SHR	333.5 ± 6.4[a]	1042.3 ± 22.2[b]	850.1 ± 19.5[b]	192.1 ± 10.0	1048.6 ± 26.4	2.549 ± 0.044[a]	177.9 ± 8.5[b]

Body weight, tissue weights, and mean arterial pressure of WKY and SHR. Values are means ± SEM for WKY ($n = 6$–8) and SHR ($n = 7$–8).
[a] $P < 0.005$ vs. WKY.
[b] $P < 0.001$ vs. WKY.

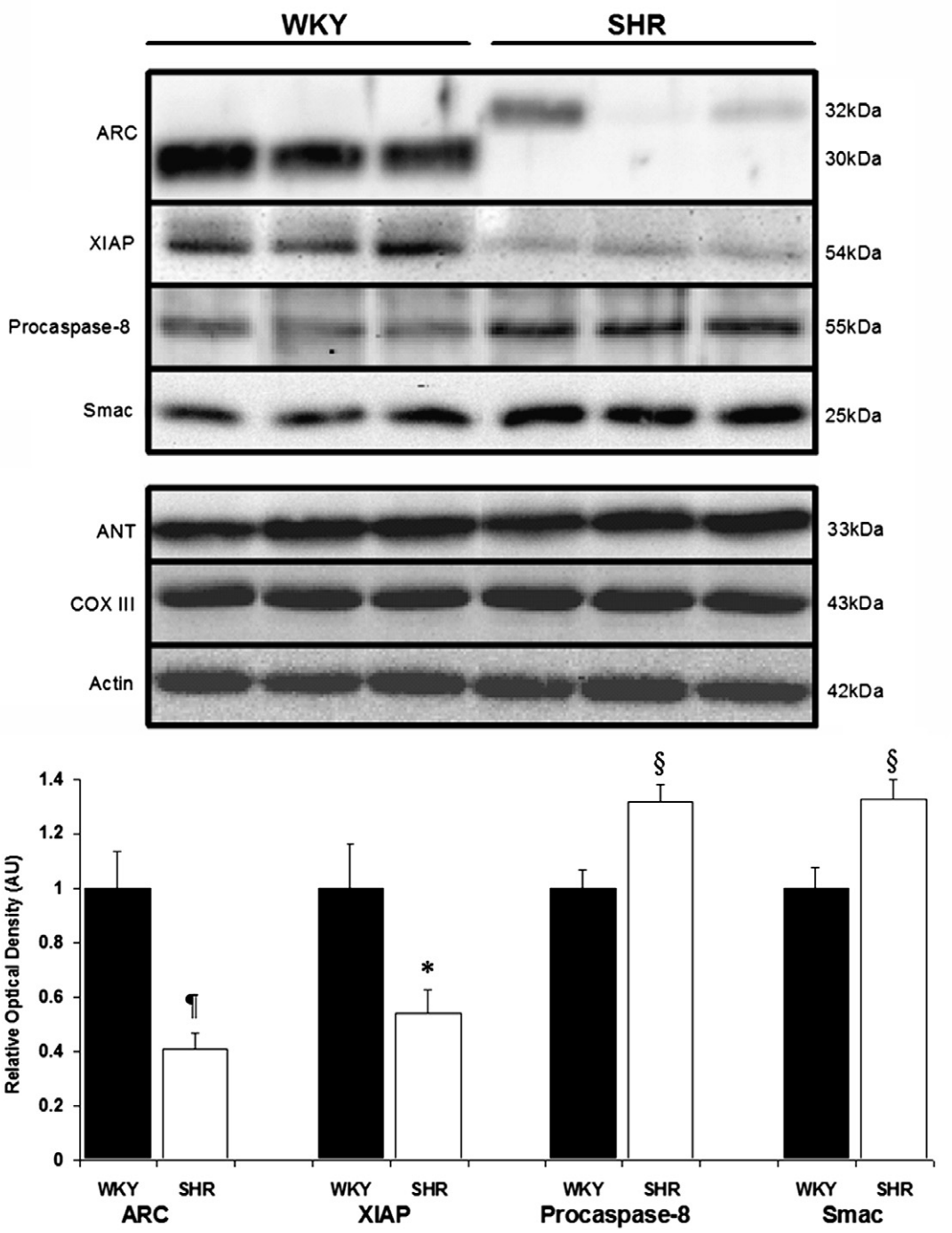

Fig. 1. Representative immunoblot data and quantitative analysis of ARC, XIAP, Smac, and procaspase-8 protein expression in WKY and SHR soleus muscle. Also shown are representative immunoblot data of ANT and COX III (mitochondrial membrane proteins) as well as actin expression in WKY and SHR soleus muscle (*quantitative analysis not shown*). Values are means ± SEM for WKY ($n = 18$) and SHR ($n = 18$). $^{*}P < 0.05$ vs. WKY, $^{\S}P < 0.005$ vs. WKY, $^{\P}P < 0.001$ vs. WKY.

SHR animals. Given the large differences in protein expression as well as the apparent alterations in molecular weight between groups, we examined these findings further using an additional commercially available primary antibody against ARC protein. Both the decreased expression and higher molecular weight of ARC protein in SHR muscle were confirmed using this alternate antibody (*data not shown*). Immunoblot analysis also revealed that XIAP protein in soleus muscle was reduced (−46%, $P < 0.05$) in SHR compared to WKY rats (Fig. 1).

There was a significant increase in both procaspase-8 (+32%, $P < 0.005$) and Smac (+33%, $P < 0.005$) protein content in SHR compared to WKY soleus muscle (Fig. 1). The activity of the proteolytic enzyme caspase-8, was also significantly elevated (+16%, $P < 0.001$) in SHR compared to WKY soleus muscle (Fig. 2). The levels of the mitochondrial membrane proteins, ANT and COX III, were not different between groups (Fig. 1).

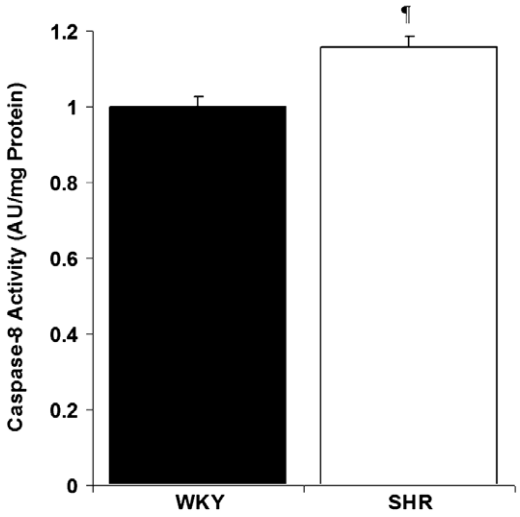

Fig. 2. Caspase-8 enzyme activity in WKY and SHR soleus muscle. Values are means ± SEM for WKY ($n = 17$) and SHR ($n = 18$). ¶$P < 0.001$ vs. WKY.

ROS generation, H_2O_2 content, and antioxidant protein levels

ROS generation in soleus muscle homogenate was significantly increased (+38%; $P < 0.005$) in SHR compared to WKY rats (Fig. 3). Similarly, there was a trend towards higher H_2O_2 content in soleus muscle of SHR compared to WKY rats (+39%; $P < 0.10$); however, this did not reach statistical significance (Fig. 3). The protein levels of the antioxidant enzymes, catalase, MnSOD, and CuZnSOD were not significantly different between groups (Fig. 4).

Discussion

The major findings of this study are that soleus muscle of hypertensive rats (SHR) had significantly lower levels of the anti-apoptotic proteins, ARC and XIAP, but higher protein levels of the pro-apoptotic proteins, procaspase-8 and Smac, compared to normotensive (WKY) control rats. Consistent with these findings, we also found that the activity of caspase-8 was significantly elevated in muscle of hypertensive animals. This extends our recent data demonstrating increased DNA fragmentation (a hallmark of apoptosis) and a pro-apoptotic protein profile in soleus muscle of SHR compared to WKY rats [7]. In addition, we provide new data showing that soleus muscle of hypertensive rats had elevated H_2O_2 content and ROS generation but similar protein levels of the antioxidant enzymes, catalase, MnSOD, and CuZnSOD. These data suggest that augmented ROS generation may play an important role in the pro-apoptotic environment and increased apoptosis observed in SHR skeletal muscle in this and our previous work [7].

Apoptosis is regulated primarily by two central pathways. The extrinsic pathway involves activation of several death-receptors (i.e., tumor necrosis factor receptor 1; TNFR1 and Fas) by their respective ligands [20]. Once activated, this stimulates the assembly of the death-inducing

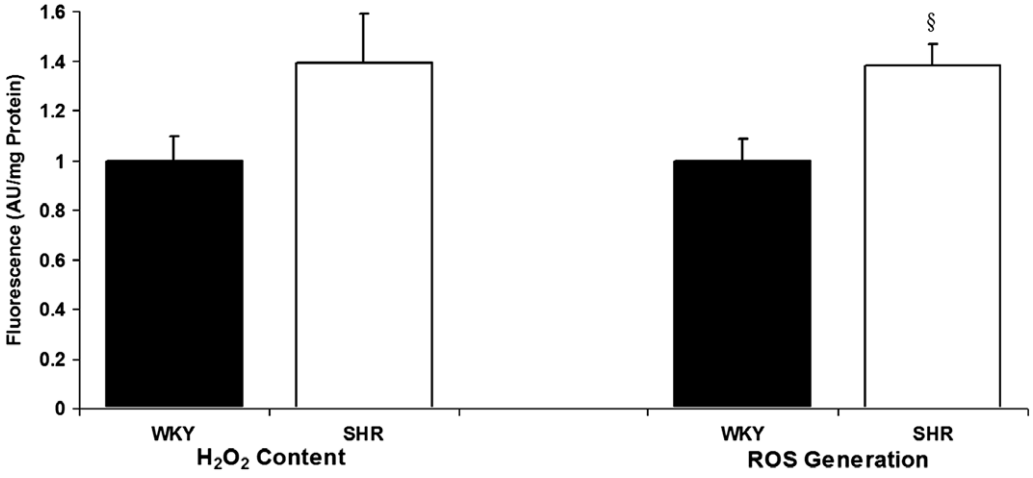

Fig. 3. H_2O_2 content and ROS generation in WKY and SHR soleus muscle. Values are means ± SEM for WKY ($n = 15$) and SHR ($n = 17$). §$P < 0.005$ vs. WKY.

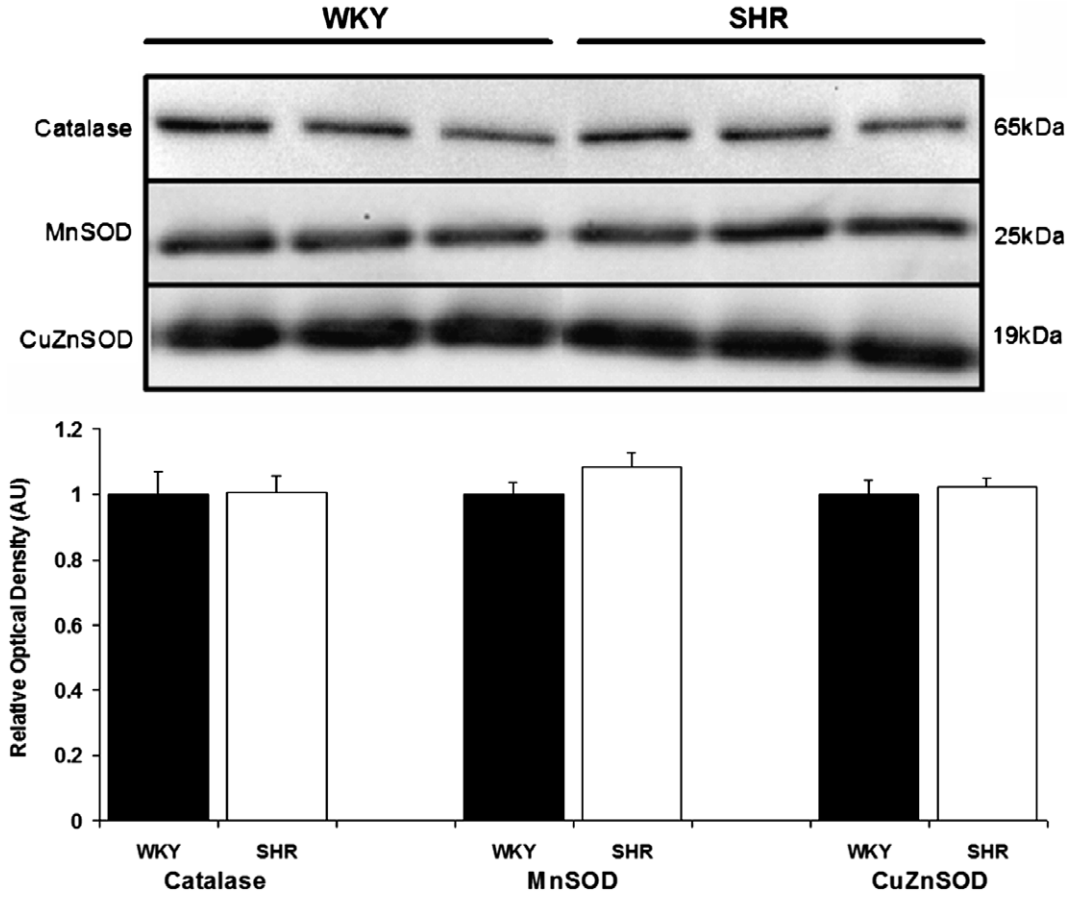

Fig. 4. Representative immunoblot data and quantitative analysis of catalase, MnSOD, and CuZnSOD protein expression in WKY and SHR soleus muscle. Values are means ± SEM for WKY ($n = 18$) and SHR ($n = 18$).

signaling complex (DISC) via the interaction of several adaptor molecules including FADD or TNFR1-associated death domain (TRADD) and procaspase-8, ultimately resulting in caspase-8 activation, caspase-3 activation, and subsequent apoptosis [9,21]. In the intrinsic pathway, pro-apoptotic proteins such as Bax and Bak can translocate to the mitochondria and cause the release of apoptogenic proteins such as cytochrome *c* and Smac [22]. In the cytosol, cytochrome *c* initiates a cascade of events which results in caspase-9 activation and eventually caspase-3 mediated apoptosis [23]. Smac can also promote caspase-mediated apoptosis by blocking the action of the caspase inhibitor, XIAP [13].

ARC is highly expressed in skeletal and cardiac muscle [8] and can inhibit apoptosis mediated by both the death-receptor and mitochondrial pathway [9]. ARC has been shown to regulate the death-receptor pathway by inhibiting apoptosis following stimulation of the TNFR1 and Fas receptors or induction by FADD and TRADD [8]. In addition, ARC can bind Fas, FADD, procaspase-8, and inhibit DISC assembly [9] as well as bind caspase-8, reduce caspase-8 enzymatic activity, and inhibit apoptosis induced by caspase-8 [8]. ARC can also influence the mitochondrial pathway by interacting with Bax [9,10]. ARC overexpression in cardiomyocytes inhibits mitochondrial Bax translocation, cytochrome *c* release, and subsequent caspase-2 and -3 activity following induction of apoptosis [24]. Others have also reported that ARC can inhibit Bax activation [9,10,25], mitochondrial Bax translocation [9], cytochrome *c* release [10], and loss of mitochondrial membrane potential [9] in response to apoptotic stimulus. Moreover, ARC knockdown causes spontaneous activation of Bax and cell death in the absence of apoptotic stimuli [9].

The present investigation demonstrates that ARC protein is significantly lower in soleus muscle of hypertensive animals. Reduced ARC levels would likely influence the death-receptor pathway. In support of this, we show increased death-receptor apoptotic signaling as evident by elevated procaspase-8 protein levels and caspase-8 enzymatic activity. Decreased ARC levels would also be expected to promote Bax activation and cell death [9]. Consistent with this report, we have previously demonstrated that increased DNA fragmentation in soleus muscle of hypertensive animals was associated with significantly increased total and nuclear Bax content as well as elevated mitochondrial Bax content (+43%, $P < 0.09$) [7]. Our previous work coupled with the present findings, suggest that apoptotic signaling via the mitochondrial and death-receptor pathway are elevated in skeletal muscle of hypertensive animals, possibly a consequence of decreased ARC protein

levels. Although we did not measure mitochondrial Smac release, the elevated Smac levels in muscle of hypertensive rats provides further support for a bias towards increased mitochondrial mediated apoptotic signaling. XIAP can undergo ubiquitination and proteasome-dependent degradation in response to apoptotic stimuli [26], which would be expected to promote caspase-3 activity and apoptosis [12]. Therefore, the decreased XIAP protein levels observed in hypertensive muscle could lead to increased caspase signaling. This present finding is consistent with and extends our previous findings which demonstrated increased procaspase-3 protein levels, caspase-3 activity, PARP cleavage, and DNA fragmentation in soleus muscle of hypertensive rats [7]. Collectively, these observations point towards a pro-apoptotic phenotype in soleus muscle of hypertensive animals.

It is possible that differences in mitochondrial content could influence some of our findings given that Smac is a mitochondrial protein [13] and ARC can be localized to the mitochondria [11]. Procaspase-8 has also been shown to be localized in mitochondria [27]; however, this has been disputed [28]. Recognizing this potential confounding factor, we determined the levels of two mitochondrial membrane proteins, COX III and ANT, as indices of mitochondrial content. We did not find differences in COX III and ANT protein expression, suggesting that alterations in mitochondrial content are not likely to be involved in the decreased ARC as well as elevated Smac and procapase-8 protein levels observed in soleus muscle of hypertensive animals.

Previous research has demonstrated that ARC not only influences oxidative stress-mediated apoptosis but that ARC protein is responsive to oxidative stress. For example, ARC can inhibit H_2O_2-induced apoptosis in a variety of cell systems [9,17], whereas reduced ARC expression increases apoptotic sensitivity to H_2O_2 [17,24], *tert*-butylhydroperoxide (*t*BOOH), and ROS mediated by mitochondrial complex I or complex III inhibition [24]. In addition, ARC is downregulated following exposure to H_2O_2 [9,15–17]; an affect mediated through ubiquitin-proteasome-dependent degradation [15,16]. In the present study, we found that soleus muscle from hypertensive rats had significantly elevated ROS generation and a higher H_2O_2 content along with reduced ARC protein levels. Interestingly, Siu and Alway [18] found decreased ARC protein levels along with increased H_2O_2 content in 7-day loaded skeletal muscle from aged animals; however, no change in ARC protein was found in experimental conditions associated with lower H_2O_2 content. Collectively, the present data support the hypothesis that increased oxidative stress may play an important role in regulating apoptosis in skeletal muscle during hypertension, possibly by influencing ARC protein levels.

Immunoblot analysis revealed that ARC protein migrated differentially between groups and was detected at approximately 32 kDa in SHR and 30 kDa in WKY rats. Wang et al. [29] reported that ARC protein in rat heart and brain as well as a number of rat cell lines was approximately 30 kDa, which is in agreement with our data from normotensive WKY rats. ARC protein from rat embryonic heart cells (H9c2) has a lower molecular weight than ARC protein from adult rat tissue [17,29]. In addition, Wang and colleagues [29] found that ARC protein in human skeletal muscle and a number of adherent human cancer cell lines migrated at approximately 34 kDa but at 38 kDa in several human lymphoma cell lines. Interestingly, the molecular weight of human ARC protein would be expected to be smaller given that human ARC contains 208 amino acids whereas rat ARC contains 221 amino acids [8]. These data suggest that ARC may undergo post-translational modifications in different species and between different cell types. At the post-translational level, ARC can be phosphorylated at threonine 149 by protein kinase CK2 [30]. It remains to be determined what accounts for this molecular weight alteration and if the altered molecular weight of ARC protein (independent of the absolute content) has a functional significance in skeletal muscle apoptotic signaling during hypertension.

In conclusion, this investigation provides further support for the hypothesis that apoptotic signaling is altered towards a pro-apoptotic phenotype in skeletal muscle of hypertensive animals. We extend our previous research and show for the first time that the anti-apoptotic proteins, ARC and XIAP, are significantly lower while the pro-apoptotic protein, Smac, is significantly elevated in soleus muscle of hypertensive animals. We also show evidence for increased death-receptor signaling in skeletal muscle during hypertension, as demonstrated by elevated procaspase-8 protein levels and caspase-8 enzyme activity. It is currently unclear what accounts for the observed pro-apoptotic phenotype; however, our data suggests that augmented skeletal muscle ROS generation may be involved. It remains to be determined if this pro-apoptotic environment plays an important role in the morphological and functional alterations that occur in skeletal muscle during hypertension.

Acknowledgment

We thank Steven G. Denniss for technical assistance with the blood pressure measures.

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