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Constitutive protein content of procaspases in murine tissue

Research Article

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Abstract: Caspases are proteases most notably involved in apoptosis and inflammation. Although mRNA content is better described, the constitutive protein content of procaspases between tissue types is not well documented. Since mRNA and protein content do not necessarily correlate, we aimed to discern protein content differences between various tissues. Protein content of procaspase-1, -8, -9, and -12 was assessed in gastrocnemius, heart, liver, and kidney. Since highly expressed in skeletal muscle, content of procaspase-12 was also analyzed in muscles with different fiber type compositions to discern any fiber type differences. Furthermore, Western analysis for procaspase-12 revealed prominent bands of ~40 kDa and ~30 kDa under basal conditions, in addition to the 50 kDa band corresponding to the full-length procaspase. Therefore, the content of these caspase-12 related species in the tissue and muscle types is also described. Results show protein content of procaspase-1,-8, -9, and -12 and caspase-12 related species differs between tissue types and do not necessarily correlate with mRNA content reported in the published literature. Procaspase-12 content in skeletal muscle may be fiber-type dependent with higher expression in more oxidative fibers. Furthermore, the 40 kDa species of caspase-12 was the dominant form of the protein in most tissues analyzed.

Keywords: *Caspase • Caspase-12 • Apoptosis • Soleus • Extensor digitorum longus (EDL) • Diaphragm*

1. Introduction

Caspases are highly conserved cysteine-dependent, aspartate-specific acid proteases most notably involved in apoptosis and inflammation, although roles in other cellular processes have been identified [1,2]. There are 15 known mammalian caspases which function in specific apoptotic and/or inflammatory pathways activated by different stimuli. Caspases reside in the cell as inactive proenzymes. Upon stimulation, the procaspase is cleaved to remove the N-terminal pro-domain and again to produce a large and small subunit which are components of the stable heterotetrameric active caspase [1,3].

While mRNA content of various procaspases between tissue types is better described, the protein content is not well documented. It has been acknowledged that the tissue distribution of mRNA content does not always correlate with protein content [4]. Thus, the aim of the study was to discern constitutive protein content differences between various murine tissues.

Protein content of apical procaspase-1,-8,-9, and -12 were assessed in murine skeletal muscle (gastrocnemius), heart, liver, and kidney. In additional to procaspase-12, two other related species are described which may be cleavage products of caspase-12 or alternative isoforms. Moreover, since procaspase-12 is highly expressed in skeletal muscle, we aimed to determine muscle fiber type differences. Thus, we assessed the protein content of procaspase-12 and related species in soleus, extensor digitorum longus (EDL), and diaphragm; skeletal muscles with varying fiber type composition.

2. Experimental Procedures

Twelve-week old male BALB/c mice were purchased from Charles River (Wilmington, Ma). All procedures were approved by the institutional Research Review Board (number of approval: RRB-112010NAYLOR). Mice were sacrificed *via* cervical dislocation after

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exposure to ether in an enclosed chamber to render them unconscious. Tissues were immediately excised, rinsed in saline, snap frozen in liquid nitrogen, and stored at -80°C for future analysis.

Tissues were homogenized (Power Gen 125, Fisher Scientific, Pittsburgh, Pa) in ice-cold phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.75 mM KH_2PO_4, 5 mM EDTA) supplemented with 10 µl/ml of Halt Protease Inhibitor Cocktail and 10 µl/ml of Halt Phosphatase Inhibitor Cocktail (Pierce Biochemicals, Rockford, IL). Tissues were homogenized with a dilution of 1:25. Total tissue homogenate was centrifuged at 660x*g* for 10 minutes and the supernatant was used for biochemical analysis. Protein concentration was determined using the Bicinchoninic Acid Protein Assay Kit (Sigma, Saint Louis, Mo).

Procaspase-1, -8, -9, -12 content was determined by Western blot analysis. Proteins were separated on either a 10% or 4-12% separating polyacrylamide PAGEr Gold Precast Gels (Lonza, Rockland, Me) under denaturing conditions and transferred to nitrocellulose membranes. Nitrocellulose membranes were blocked for one hour at room temperature using a blocking solution containing 5.0% powdered milk. Membranes were incubated overnight at 4°C in primary antibody (mouse monoclonal caspase-1,-8, -9 antibodies and rat monoclonal caspase-12 antibody, Santa Cruz Biotechnology, INC, Santa Cruz, Ca). Membranes were rinsed and incubated with secondary HRP-linked antibody (anti-mouse and anti-rat IgG antibodies, Santa Cruz Biotechnology, INC, Santa Cruz, Ca) for 2 hours at room temperature. Protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biochemicals, Rockford, IL) and the Kodak IS4000R Imaging System (Carestream Health, Inc., New Haven, CT). Values are expressed as arbitrary OD units calculated by multiplying the area of each band by its average optical density. Ponceau staining (Pierce Biochemicals, Rockford, IL) of the nitrocellulose membranes were used to assure equal loading of protein.

A one-way ANOVA with a Student-Newman-Keuls post-hoc test was used for statistical analysis between groups. $P<0.050$ was considered statistically significant. All p values are listed in respective figure legends. All data is expressed mean ± SEM. n=6 for each tissue were used for all Western blots.

3. Results

3.1 Content of procaspase-1

The protein content of procaspase-1 was significantly different in each tissue with the greatest content in the heart (427650±33271) followed by kidney (299650±39061), liver (111230±18265), and skeletal muscle (20734±70), in descending order, respectively (see Figure 1).

3.2 Content of procaspase-8

The protein content of procaspase-8 was not significantly different between groups (see Figure 2).

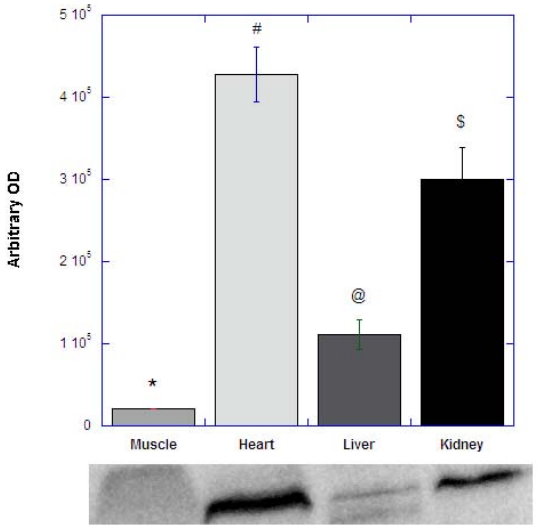

Figure 1. Procaspase-1 protein content in skeletal muscle, heart, liver, and kidney. Groups with different symbols represent statistically significant differences in mean arbitrary OD. *Muscle vs* heart $P<0.0001$, liver $P=0.0292$, kidney $P<0.0001$; *heart vs* liver $P<0.0001$, kidney $P=0.0034$; *liver vs* kidney $P<0.0001$. Each lane of representative Western blot corresponds to labeled bar graph.

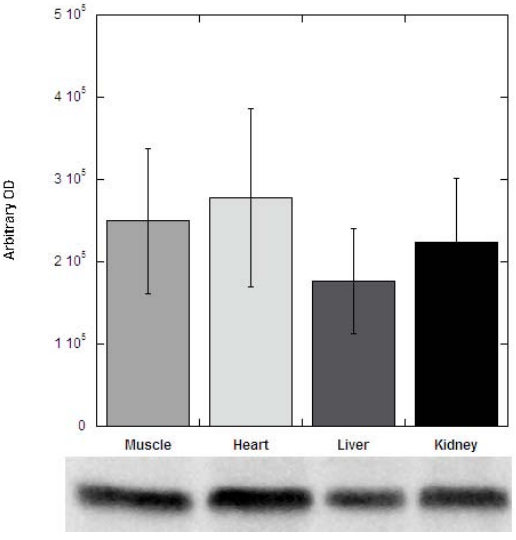

Figure 2. Procaspase-8 protein content in skeletal muscle, heart, liver, and kidney. *Muscle vs* heart $P=0.82$, liver $P=0.82$, kidney $P=0.84$; *heart vs* liver $P=0.84$, kidney $P=0.90$; *liver vs* kidney $P=0.70$. Each lane of representative Western blot corresponds to labeled bar graph.

3.3 Content of procaspase-9

The protein content of procaspase-9 was greatest in liver (405790±54892) and kidney (345460±49569), followed by heart (240720±32310) and skeletal muscle (85907±11273) (see Figure 3).

3.4 Content of procaspase-12

Procaspase-12 content was greatest in skeletal muscle (140540±23898) and heart (143300±16238) followed by liver (57136±9327) and kidney (22861±5427). Two prominent bands of ~40 kDa and ~30 kDa were also present. The content of the 40 kDa species was greatest in liver (439130±49979) compared to skeletal muscle (19755±53736), kidney (153560±28269), and heart (80621±14230). The content of the 30 kDa species was greatest in skeletal muscle (241100±52171) compared to heart (27713±5596), liver (29195±4820), and kidney. The content in the kidney was undetectable. The heart was the only tissue with the 50 kDa species as the dominant form (*i.e.*, greatest content of the 50 kDa species compared to the content of the 40 kDa or 30 kDa species). The smaller species of caspase-12 were as prominent in skeletal muscle and more prominent in liver and kidney compared to the content of the 50 kDa form (see Figure 4).

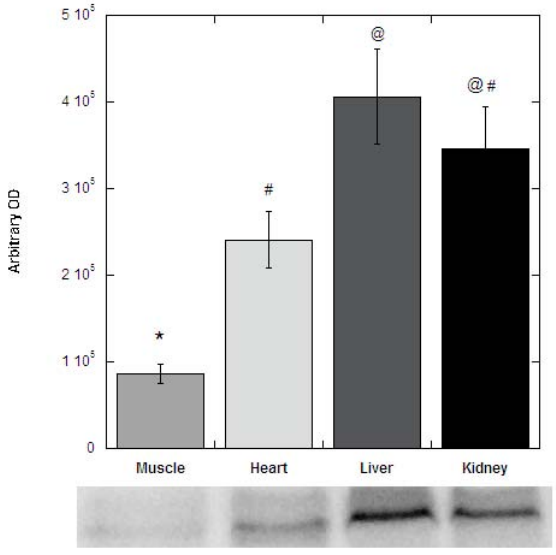

Figure 3. Procaspase-9 protein content in skeletal muscle, heart, liver, and kidney. Groups with different symbols represent statistically significant differences in mean arbitrary OD. *Muscle vs* heart P=0.0142, liver P=0.0001, kidney P=0.0006; *heart vs* liver P=0.0249, kidney P=0.0841; *liver vs* kidney P=0.3077. Each lane of representative Western blot corresponds to labeled bar graph.

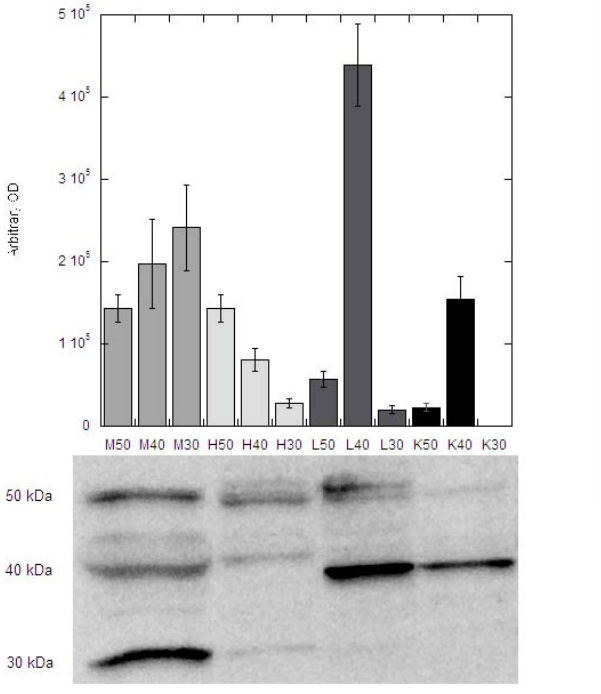

Figure 4. Protein content of procaspase-12 and related species in skeletal muscle, heart, liver, and kidney. 50 kDa: *muscle vs* heart P=0.9006, liver P=0.0011, kidney P<0.0001; *heart vs* liver P=0.0022, kidney P=0.0001; *liver vs* kidney P=0.1317. 40 kDa: muscle vs heart P=0.1219, liver P=0.0004, kidney P=0.4455; *heart vs* liver P<0.0001, kidney P=0.2115; *liver vs* kidney P=0.0002. 30 kDa: *muscle vs* heart P<0.0001, liver P<0.0001, kidney P<0.0001; *heart vs* liver P=0.8421, kidney P=0.7408; *liver vs* kidney P=0.5928. Muscle: *50 kDa vs* 40 kDa P=0.3885, 30 kDa P=0.2898; *40 kDa vs* 30 kDa P=0.5078. Heart: *50 kDa vs* 40 kDa P=0.0036, 30 kDa P<0.0001; *40 kDa vs* 30 kDa P=0.0109. Liver: *50 kDa vs* 40 kDa P<0.0001, 30 kDa P=0.3897; *40 kDa vs* 30 kDa P<0.0001. Kidney: *50 kDa vs* 40 kDa P<0.0001, 30 kDa P=0.3462; *40 kDa vs* 30 kDa P<0.0001. Each lane of representative Western blot corresponds to labeled bar graph.

The content of procaspase-12 as well as the 40 kDa and 30 kDa species was analyzed in skeletal muscles with varying fiber type compositions (see Figure 5). Procaspase-12 content was greatest in soleus (196730±9631) compared to EDL (61259±8245) and diaphragm (61810±7086). The content of the 40 kDa species was greatest in EDL (366360±13570), followed by soleus (266150±13781) and diaphragm (198560±9979) in descending order, respectively. The content of the 30 kDa species was greatest in soleus (144410±14987) and EDL (142130±18509) compared to diaphragm (54344±8158). In all three types of skeletal muscle, the form of the protein in greatest concentration was the 40 kDa species.

4. Discussion

The constitutive protein content of procaspase-1, -8, -9, and -12 was assessed in murine skeletal muscle (gastrocnemius), heart, liver, and kidney. Caspase-1 is primarily responsible for processing of pro-inflammatory cytokines, but has also been implicated in cell differentiation [5,6] and cell death [7]. In this study, procaspase-1 protein content was significantly different in each tissue with the highest content in heart followed by kidney, liver, and skeletal muscle in descending order, respectively. Kalai *et al*. reported murine tissue distribution of procaspase-1 mRNA transcript levels and protein content [4]. Procaspase-1 transcript levels were found to be higher in skeletal muscle and heart compared to liver and kidney, which had very low to undetectable levels [4]. However, Western blot analysis revealed that the protein content was significantly greater in liver compared to heart, skeletal muscle, and kidney which all had easily detectable bands [4]. These results suggest that mRNA transcript levels do not closely correlate with protein levels in murine tissues. Our results differ from those of Kalai *et al*. which may be due to differences in experimental design. Kalai *et al*. pooled organ samples from 3-6 mice and included only an n=1 for Western analysis, excluding potential for statistical analysis [4].

Caspase-8 was first identified as an apoptotic caspase activated by receptor-mediated mechanisms involving formation of the death-inducing signaling complex [1]. Thereafter, it was discovered that caspase-8 has non-apoptotic roles in cellular processes

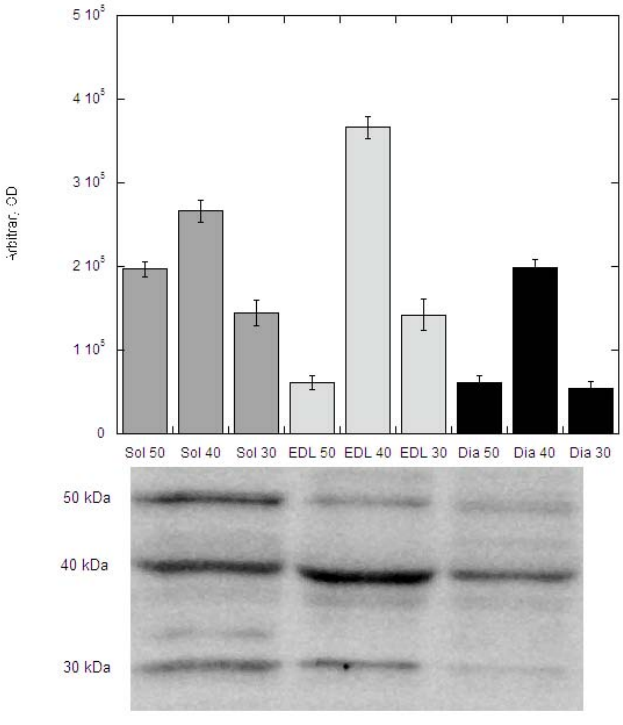

Figure 5. Protein content of procaspase-12 and related species in soleus, EDL, and diaphragm. 50 kDa: *sol vs* EDL P<0.0001, Dia P<0.0001; *EDL vs* Dia P=0.9619. 40 kDa: *sol vs* EDL P<0.0001, Dia P=0.0021; *EDL vs* Dia P<0.0001. 30 kDa: *sol vs* EDL P=0.9152, Dia P=0.002; *EDL vs* Dia P=0.0006. Sol: *50 kDa vs* 40 kDa P=0.0026, 30 kDa P=0.0148; *40 kDa vs* 30 kDa P<0.0001. EDL: *50 kDa vs* 40 kDa P<0.0001, 30 kDa P=0.0010; *40 kDa vs* 30 kDa P<0.0001. Dia: *50 kDa vs* 40 kDa P<0.0001, 30 kDa P=0.5434; *40 kDa vs* 30 kDa P<0.0001. Each lane of representative Western blot corresponds to labeled bar graph.

such as differentiation and proliferation [2]. Murine tissue distribution for procaspase-8 has only been described for transcript content [8,9]. A comparison of protein content in various tissues has not been described. Procaspase-8 protein levels were found to be similar among the tissues analyzed. In previous studies, mRNA transcript content in various murine tissues appeared to be higher in liver and kidney compared to heart and skeletal muscle [8,9].

Caspase-9 is a caspase involved with activation of the mitochondrial-mediated pathway of apoptosis [1]. Release of cytochrome c from the mitochondria, in response to various apoptotic stimuli, results in formation of the apoptosome with subsequent activation of caspase-9. Protein content of procaspase-9 between tissue types has not been previously described, however, mRNA transcript levels have been reported [10]. Procaspase-9 transcripts were present in all tissues (rat) examined, including heart, liver, and kidney; skeletal muscle was not analyzed [10]. Transcript content of procaspase-9 was lower in heart compared to liver and kidney [10]. Our results are consistent with this report in that protein content of procaspase-9 in skeletal muscle and heart was lower compared to liver and kidney, although the difference between heart and kidney did not reach statistical significance. In human tissues, mRNA transcript content was found to be higher in skeletal muscle compared to liver and kidney; heart was not analyzed [11].

Caspase-12 has been implicated in both apoptosis and inflammation. Caspase-12 has been shown to induce apoptosis in response to endoplasmic reticulum (ER) stress [12,13]. Caspase-12 has also been implicated as having a role in the inflammatory process as a dominant-negative regulator of caspase-1; indeed, caspase-12 is phylogenetically related to the inflammatory caspases [4,14]. mRNA transcript and protein content of procaspase-12 in various tissues have been previously reported and both were shown to be higher in skeletal muscle and heart compared to liver and kidney [4,15]. Our results are consistent with the published reports.

In addition to the 50 kDa band corresponding to procaspase-12, Western analysis revealed two prominent bands of ~40 kDa and ~30 kDa which may correspond to procaspase-12 cleavage products. Processing of procaspase-12 involves removal of the N-terminal pro-domain followed by a second cleavage resulting in a large and small subunit [4]. The large and small subunits form active heterotetramers. The ~40 kDa species may correspond to the fragment consisting of the large and small subunit after removal of the N-terminal pro-domain, while the ~30 kDa species corresponds to the large subunit after the second cleavage between the large and small subunit [4]. Alternatively, these bands may correspond to caspase-12 isoforms. Isoforms of caspase-12 with similar molecular weights to that of the cleavage products have been identified [15,16]. No previous studies have compared the content of these 40 kDa and 30 kDa species between tissue types under basal conditions. The content of the 40 kDa species was found to be greatest in liver compared to skeletal muscle, heart and kidney which all had similar content. The content of the 30 kDa species was found to be much greater in skeletal muscle compared to heart and liver, and undetectable in kidney. Interestingly, the prominent form of caspase-12 in skeletal muscle, liver, and kidney was not the full-length protein. In skeletal muscle, the content of the 30 kDa and 40 kDa species were similar to that of the 50 kDa full-length form. In liver and kidney, the content of the 40 kDa species greatly exceeded that of the full-length species. Heart was the only tissue in which the full-length protein was the prominent form of caspase-12 under these basal conditions. If these 40 kDa and 30 kDa species are cleavage products, it is not clear as to why the content of these products would be as great or greater than the content of the full-length procaspase under basal conditions in skeletal muscle, liver and kidney. Since the content of the 30 kDa species, which may represent complete cleavage, was greatest in skeletal muscle compared to heart, liver and kidney it may be indicative of greater ER stress under basal conditions. Indeed, a previous study reported high levels of ER stress in murine limb skeletal muscle compared to other tissues tested, including heart [17]. It is possible that the enhanced ER stress may be playing a physiologic role in the normal function and maintenance of skeletal muscle. ER stress has been shown to increase myofiber formation [18]. Further, ER stress has been shown to mediate skeletal muscle adaptation to improve muscle function and achieve metabolic benefits in response exercise [19]. Additional investigation may explain the physiologic role of heightened ER stress in skeletal muscle compared to other tissues.

Procaspase-12 and related species were also analyzed in varying types of skeletal muscles: soleus, EDL, and diaphragm. Currently, there is no published literature comparing the content of procaspase-12 or the related species between these skeletal muscle types. Murine soleus is composed of primarily oxidative fibers, both type I (slow oxidative) and type IIa fibers (fast oxidative glycolytic) [20]. EDL is composed primarily of type IIb (fast glycolytic) fibers while diaphragm is composed of a mix of fibers including type I, type IIa, type IIx (fast oxidative glycolytic, but less oxidative than IIa), and IIb [21,22]. Procaspase-12 protein content was

greatest in soleus compared to EDL and diaphragm. This may suggest that procaspase-12 is more highly expressed in type I and IIa fibers compared to less oxidative fiber types such as IIx and IIb. The content of the 40 kDa species was significantly different among all three muscle types with the greatest content in EDL followed by soleus and diaphragm, respectively. The content of the 30 kDa species was greater in soleus and EDL compared to diaphragm. Again, if these species are cleavage products, the data may suggest that the diaphragm has less ER stress compared to the soleus or EDL under basal conditions. In all three muscle types, the 40 kDa species was the dominant form of the protein. Evans *et al*. analyzed content of procaspase-12 between tibialis anterior and masseter muscles in C57BL/6 female mice. They too show prominent bands that they describe as cleavage products under basal conditions [3]. Further characterization of the 40 kDa species as a cleavage product or alternative isoform will help to elucidate the significance of our observation.

The differences in constitutive protein content of the various apical caspases between tissue types may give insight as to the propensity of that tissue to undergo apoptosis in response to the respective stimulus. Interestingly, it has been shown that in long-lived growth hormone receptor knockout mice the expression of some procaspases and other proapoptic proteins in skeletal muscle were decreased compared to wild-type mice [23]. It was suggested by the authors that this may be a protective mechanism for the loss of muscle mass that is accompanied with aging; that it decreases the propensity for apoptosis.

In summary, constitutive protein content of procaspase-1, -8, -9, and -12 varies among murine tissue types. Content of procaspase-1 was significantly different in each tissue with the highest content in the heart and the lowest in skeletal muscle. Procaspase-8 content did not vary between tissue types. Content of procaspase-9 was greatest in liver and kidney and lowest in skeletal muscle. Content of procaspase-12 was greatest in skeletal muscle and heart compared to liver and kidney. Procaspase-12 content in soleus muscle was greater than EDL and diaphragm. Thus, procaspase-12 expression in skeletal muscle may be fiber type dependent with higher expression in more oxidative fibers. Another unique observation was that the 40 kDa species of capsase-12, likely a cleavage product or isoform, was the dominant form of the protein in most tissues analyzed. Future research will elucidate the significance of this observation.

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