HEAT SHOCK PROTEINS (HSPs) are highly conserved proteins that are induced in cells when exposed to stress, such as elevated temperatures, and are considered to be involved in a general mechanism that helps cells to survive many lethal stresses (11, 18). Many HSPs also are present in unstressed cells and are thought to play a critical role in normal cellular function. For example, it was found in vitro that HSP72 functions as a molecular chaperone that protects the aggregation of nascent polypeptide, accelerates the maturation of proteins, and refolds denatured proteins (4, 10).

Western blot analyses have shown that, in adult rats, the soleus muscle, which is comprised predominantly of slow fibers, has a relatively higher amount of HSP72 than the plantaris and white gastrocnemius muscles, which are comprised predominantly of fast fibers (21). Furthermore, immunohistochemical techniques have been used to localize HSP72 in the slow fibers of skeletal muscles of sedentary adult rats (26, 29). The levels of HSP72 are increased in both fast and slow muscles after heat stress (30), glucose depletion (9), or exercise (8, 12, 19, 22). Thus, in adult mammalian skeletal muscles, HSP72 is associated with a slow-fiber phenotype and is induced by exogenous stressors such as heat stress and exercise.

It is not known, however, whether HSP72 is expressed in rat skeletal muscles when there are no exogenous stresses. To address this issue, we determined the HSP72 levels in the slow soleus and fast plantaris muscles of embryonic rats, i.e., at a developmental stage when there are minimal exogenous influences on the muscles and when the metabolic and contractile properties of fast and slow muscles are similar (3, 15, 23–25). In addition, we used immunohistochemical procedures to determine whether there was fiber-type-specific expression of HSP72 in embryonic muscles. Because the mechanical and phenotypic properties of the soleus, but not the plantaris, muscle shift toward a “slower” profile during postnatal development (5, 15, 27), we also determined the HSP72 expression pattern in the soleus and plantaris from embryonic day 22 (E22) to postnatal day 56 (56d). Our working hypothesis was that HSP72 levels would increase in parallel with the increase in the percent composition of slow myosin heavy chain (MHC). In general, the data support our hypothesis.

METHODS

Experimental animals and design. The soleus and plantaris muscles from Wistar rats were sampled from E22 (n = 67) and at postnatal days 3 (3d; n = 44), 7 (n = 13), 10 (10d; n = 12), 14 (14d; n = 8), 28 (28d; n = 8), 42 (n = 9), and 56d

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All experimental and animal care procedures were conducted in accordance with the Committee on Animal Care and Use at Kumamoto University and followed the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences established by Physiological Society of Japan and the American Physiological Society. The temperature and humidity of the animal room were controlled at 22 ± 1°C and 60%, respectively, throughout the study.

All rats were weighed just before the removal of the muscle tissues or hindlimbs. E22 (n = 13) and 3d (n = 14) rats were anesthetized with pentobarbital sodium (40 mg/kg ip), and the hindlimbs were removed bilaterally at the level of the knee. The hindlimbs were quickly frozen in isopentane cooled by liquid nitrogen and stored at −85°C until the analysis for immunohistochemistry. All other rats were anesthetized with pentobarbital sodium (40 mg/kg ip), and the soleus and plantaris muscles were removed bilaterally. The muscles were cleaned of excess fat and connective tissue, wet weighed, quickly frozen with liquid nitrogen, and stored at −85°C until the analysis for HSP and MHC.

Muscle samples were homogenized with 10 volumes of buffer containing (in mM) 10 Tris-HCl, 10 NaCl, 0.1 EDTA, and 15 mercaptoethanol (pH 7.6), according to the procedures of Kilgore et al. (16), and then centrifuged at 12,000 g for 20 min. The supernatants were boiled in sample buffer (17) for 2 min at a final protein concentration of 1 µg/µl, according to the method of Bradford (2), and then subjected to HSP analysis. The remaining pellet was boiled in sample buffer for 2 min and then subjected to MHC analysis.

Immunohistochemical procedures. The expression patterns of slow type I MHC and HSP72 in individual fibers in selected muscles were analyzed in serial cross sections by using monoclonal antibodies specific for slow type I MHC (M8421, Sigma Chemical, St. Louis, MO) and HSP72 (SPA-810, StressGen), respectively. Serial sections (10 µm thick) of the frozen hindlimbs of E22 and 3d rats, and the plantaris muscles of 56d rats, were cut at a midbelly region in a cryostat at −20°C and mounted on glass slides coated with gelatin. The sections were dried at room temperature for 30 min and then incubated in 0.3% Triton X-100 and 1 mM phosphate-buffered saline (PBS) for 60 min. After three rinses in PBS, the sections were incubated with 0.3% H2O2 and PBS for 30 min and then incubated three times in PBS. The sections were then blocked in 1% BSA and PBS for 10 min and were reacted with the primary antibodies diluted 1:100 in PBS overnight at 4°C. The sections were washed three times in PBS, reacted for 30 min with the second biotinylated anti-mouse IgG antibody (Vectastain ABC kit, PK-6102, VECTOR Laboratory) diluted 1:100 in PBS, and then washed three times in PBS. Finally, the sections were incubated with avidin-biotin-horseradish peroxidase complex (Vectastain ABC kit, PK-6102) for 60 min, washed three times in PBS, and stained for ~5 min with H2O2 solution [diluted 1:1,000 with Tris-buffered saline (TBS); 100 mM Tris-HCl, pH 7.5, 0.9% NaCl] by using 3,3-diaminobenzidine (Sigma Chemical) as a substrate. Quantification of the HSPs was performed by using the National Institutes of Health Imaging System. HSP content in the soleus and plantaris muscles at each time point was expressed relative to that observed in the soleus muscle of 56d rats. To ensure uniformity, only bands within the same blot were used to compare the relative amount of each protein among groups.

Statistical analyses. All data are presented as means ± SE. Significant differences between time points for each HSP and each muscle were determined by using one-way ANOVA followed by Scheffe’s post hoc test. All differences were determined to be significant at the P < 0.05 level.

RESULTS

Expression of HSP72 in embryonic muscles. Figure 1 shows the immunohistochemical staining patterns for the anti-HSP72 and anti-type I MHC antibodies in the muscles of the hindlimbs of a representative E22 and 3d rat. The soleus and plantaris muscles were identified based on the study of Condon et al. (7). In the E22 rats, fibers reacting positively to the anti-HSP72 antibody were observed throughout the soleus muscle but only in the deep (close to the fibula) region of the plantaris muscle (Fig. 1, top left). Similar staining patterns of the fibers to the anti-type I MHC antibody were observed in the E22 soleus and plantaris muscles (Fig. 1, top middle), i.e., type I MHC positive fibers were observed throughout the entire cross section of the soleus, but the density of positive fibers was higher in the deep than in the superficial region of the plantaris. In the 3d rats, the staining patterns of fibers in the soleus and plantaris muscles to anti-HSP72 and anti-type I MHC antibodies were similar to those observed in the E22 rats (Fig. 1, bottom, right and left). Higher magnification representations of the staining patterns for the two antibodies for fibers in the deep region of the plantaris in the E22 and 56d rats are shown in Fig. 2. It is clear that, in the plantaris muscle
Fig. 1. Immunohistochemical staining of hindlimb muscles in embryonic day 22 (E22) and postnatal day 3 (3d) rats with anti-heat shock protein (HSP)72 and anti-type I myosin heavy chain (MHC) antibodies. Note that fibers throughout the entire soleus muscle cross section react to both antibodies, whereas only fibers in the deep (close to the fibula) region of the plantaris and gastrocnemius muscles react to both antibodies. The majority of fibers in the superficial region of the plantaris and gastrocnemius muscles did not react with either antibody. Negative control (Neg Cont) section of E22 limb that has not reacted with primary antibody shows the inhibition of endogenous peroxidase activity. S, soleus; PD, deep region of the plantaris; PS, superficial region of the plantaris; GD, deep region of the gastrocnemius; GS, superficial region of the gastrocnemius; F, fibula. Scale bars, 400 μm.

Fig. 2. Fiber type-specific expression of HSP72 in the rat plantaris muscle. Immunohistochemical staining was performed on postnatal day 56 (56d) and E22 plantaris muscles by anti-HSP72 and anti-type I MHC antibodies. Note that only the fibers containing type I MHC express HSP72 at both ages. Arrows indicate the same fibers in the serial sections at each age. Scale bar in right of each age, 50 μm.
at both ages, only the fibers positive for the anti-type I MHC antibody reacted positively to the anti-HSP72 antibody. This type I fiber-specific expression of HSP72 also was observed in the soleus muscle. As shown in Table 1, almost all type I fibers counted in the immunohistochemical sections expressed HSP72.

**Body and muscle weight changes during development.** The mean body weight of the rats progressively increased throughout the developmental period and reached 281 ± 4 g at 56d (Fig. 3). The muscle weight for the soleus and plantaris also increased throughout the developmental period and reached 138 ± 3 and 306 ± 7 mg, respectively, at 56d.

**Developmental changes in MHC isoform composition.** The soleus muscles from E22 rats contained two MHC isoforms, i.e., type I (24 ± 2%) and embryonic/neonatal (76 ± 2%) MHCs (Fig. 4A). The percentage of type I MHC progressively increased to reach ~65% at 10 days and then fluctuated between ~60 and 70% thereafter. The percentage of embryonic/neonatal MHC progressively decreased and was undetectable at 56d. Type IIa MHC was initially detected at postnatal day 7, with the percentage increasing gradually up to 28d, and then fluctuating between 30 and 40% thereafter. At 56d, the soleus was composed of ~70 and 30% type I and IIa MHC, respectively.

The plantaris muscles from the rat embryos also contained two MHC isoforms, i.e., type I (4 ± 1%) and embryonic/neonatal (96 ± 1%) MHCs (Fig. 4B). The percentage of embryonic/neonatal MHC progressively decreased with age, with the steepest decline observed between 14d and 28d. There was no detectable embryonic/neonatal MHC I in the plantaris muscle at 28d and thereafter. The percentage of slow type I MHC reached a peak of ~10% at 10d and 14d and then decreased to ~1% at 56d. There was a progressive increase in all adult fast MHCs during the experimental period, such that the plantaris was composed of ~10, 34, and 56% of type IIa, IIx, and IIb MHC isoform, respectively, at 56d.

**Developmental changes in HSP72 and HSC73 content.** HSP72 was not detectable in the soleus muscle of embryonic rats but gradually increased from 3d to 56d.

### Table 1. Quantification of fibers that positively reacted to anti-MHC I and HSP72 antibodies in the immunohistochemical sections

<table>
<thead>
<tr>
<th></th>
<th>No. of MHC I Positive Fibers (Type I Fiber)</th>
<th>No. of HSP72 Positive Fibers in Type I Fibers</th>
<th>HSP72 Positive Fibers in Type I Fibers, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>E22 soleus</td>
<td>501</td>
<td>492</td>
<td>98.2</td>
</tr>
<tr>
<td>E22 plantaris</td>
<td>283</td>
<td>281</td>
<td>99.3</td>
</tr>
<tr>
<td>3d Soleus</td>
<td>472</td>
<td>461</td>
<td>97.7</td>
</tr>
<tr>
<td>3d Plantaris</td>
<td>403</td>
<td>398</td>
<td>98.8</td>
</tr>
</tbody>
</table>

MHC, myosin heavy chain; HSP, heat shock protein; E22, embryonic day 22; 3d, postnatal day 3.

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**Fig. 3. Changes in body weight and absolute soleus and plantaris weights during the developmental period.** Values are means ± SE. The number of samples are 54 for E22, 30 for 3d, 13 for postnatal day 7 (7d), 12 for postnatal day 10 (10d), 8 for postnatal day 14 (14d), 8 for postnatal day 28 (28d), 9 for postnatal day 42 (42d), and 8 for postnatal day 56 (56d).

**Fig. 4. Changes in MHC composition (gel electrophoresis) in the soleus (A) and plantaris (B) muscles during the developmental period.** Values are means ± SE. Emb/Neo, embryonic and neonatal MHC; I, I MHC I; IIa, MHC IIa; IIx, MHC IIx; IIb, MHC IIb.
(Fig. 5). In the plantaris muscle, HSP72 could not be detected until 10d and remained at that level thereafter (Fig. 6). Relative to the soleus muscle, the HSP72 levels in the plantaris muscle were very low, i.e., between 8 and 15% of the levels observed in the soleus of 56d (Fig. 6).

In contrast to HSP72, HSC73 was detectable in both the soleus (Fig. 7) and plantaris (Fig. 8) muscles of embryonic rats. The levels of HSC73 in the soleus and plantaris muscles at E22 were ~60 and 80%, respect-
tively, of the 56d soleus muscle and were significantly lower than those at any other time point. There were no significant changes in the HSC73 levels in either the soleus or plantaris muscles during the postnatal developmental period (Figs. 7 and 8).

DISCUSSION

A novel aspect of this investigation was the determination of the expression patterns for HSP72 in embryonic rat skeletal muscles, a developmental stage when the rats are considered to have minimal exogenous stress. Our immunohistochemical data clearly show the presence of HSP72 in the soleus and plantaris muscles of E22 rat embryos. This observation indicates that the expression of HSP72 in muscles can be influenced by factors other than exogenous stress. We also showed that the expression of HSP72 was specific to fibers expressing type I MHC in the plantaris muscles of embryonic as well as 56d rats, a finding consistent with that observed in adult muscles (26, 29). Although we provide a comparison between the earliest (embryonic and neonatal) and adult stages, a limitation in the immunohistochemical analyses is the lack of data for the midrange time points.

During the embryonic and early neonatal periods, the muscle fibers contain multiple MHC isoforms (including embryonic/neonatal) and are polyinnervated; however, most muscles have similar metabolic and contractile properties. For example, Nemeth et al. (25) reported that the metabolic profiles of the rat soleus and extensor digitorum longus muscles were similar at birth. In addition, Navarrete and Vrbova (23, 24) reported that motor units in both the soleus and extensor digitorum longus muscles of rats at early developmental stages fire at relatively low frequencies and for short periods of time, suggesting that their contractile properties are similar at this time. Consistent with this view is the observation that, during the first 2 wk after birth, both muscles increase their speed of contraction, whereas the extensor digitorum longus continues to become faster and the soleus gradually becomes slower during the following postnatal weeks (6). All of these observations indicate that HSP72 expression at these early stages of development may be derived from endogenous factors specific to fibers containing type I MHC.

We could not detect HSP72 by using the Western blotting technique in either the embryonic soleus or plantaris muscles, suggesting that the sensitivity of the Western blots was lower than that of the staining reaction to the monoclonal antibodies used for the immunohistochemical analyses. In addition, the embryonic soleus and plantaris muscles contained a very small amount of type I MHC (Fig. 4) and thus most likely very small amounts of HSP72. The changes in the MHC isoform profile of the rat soleus muscle observed in the present study are similar to those reported previously (20). In addition, Butler-Browne and Whalen (5) found that the amount of neonatal MHC isoform in the rat soleus muscle was drastically decreased during postnatal days 21–28, similar to that observed in the present study.

Using Western blots, we determined the levels of HSP72 in the soleus and plantaris muscles during the first 2 postnatal months. In the soleus muscle, HSP72 was detected as early as 3d (Fig. 5), and this may reflect a stress response to the environmental changes that occur at birth. The HSP72 levels were similar during the first 2 wk after birth and then gradually increased from 14d to 56d. This expression pattern appears to be related to at least two interrelated factors: 1) the development of postural and locomotor capabilities and 2) the associated increase in the percentage of slow fibers. Walking is adopted as a major form of locomotion in rat pups at postnatal days 12–13, and then vertical movements are started, and the quantity of locomotion is rapidly increased at 14d, at which time the eyes begin to open (1, 36). Thus the increase in locomotion and physical activity parallels the large increase in HSP72 observed between 10d and 14d. After 14d, the body weight begins to rapidly increase (Fig. 3), and the mechanical stresses on the antigavity soleus muscle accompanying locomotor and postural tasks also progressively increase. Again, there is a parallel increase in HSP72 content during this period. There also is a progressive increase in the percentage of type I MHC composition of the soleus muscle throughout the developmental period (Fig. 4A), which most likely reflects the requirement for additional slow fibers to perform locomotor and postural tasks in these growing animals.

In adult rats, Locke et al. (20) reported a significant increase in the content of both HSP72 and type I MHC protein in hypertrophied plantaris muscle and a decrease in HSP72 content and the number of slow fibers in the soleus muscle of hyperthyroid rats. These findings support a reciprocal relationship between HSP72 and type I MHC in skeletal muscles. The increases in type I MHC and HSP72, however, were not completely in parallel in the present study; the steepest increase in type I MHC occurs between E22 and 10d, whereas the steepest increase in HSP72 occurs between 14d and 56d. Because HSP72 immunoreactivity was localized specifically to fibers containing type I MHC, these findings suggest that the amount of HSP72 in fibers expressing type I MHC may be very low at the early compared with the later time points studied. Although this issue cannot be resolved in the present study, the low amount of type I MHC observed may be related to the size of the fibers. The soleus fibers are undifferentiated at postnatal day 5, and thereafter the fibers differentiate to slow or fast between postnatal day 7 and postnatal week 10, i.e., there is an increasing percentage of slow fibers and a simultaneous increase in fiber size (15). Thus the progressive increase in HSP72 content in the rat soleus muscle during development may be related to an increase in both the number and size of slow fibers. An additional possibility is that the HSP72 content per microgram of protein was increased in the slow fibers of the soleus muscles...
because of the increased stress associated with increased locomotion and/or weight bearing (see above). The developmental changes in the amount of HSP72 in the plantaris muscle were very different from those observed in the soleus muscle. We could not detect the presence of HSP72 in this fast extensor muscle until 10d. The amount of HSP72 was consistently very low in the plantaris muscle compared with the soleus at all time points, i.e., between 8 and 15%, relative to the levels in the 56d soleus muscle. Some possible explanations for this muscle-specific response include that 1) the plantaris muscle is most likely recruited to a much lesser degree than the soleus for the postural and slow locomotor tasks performed during the developmental period and thus may have less of a stress response than the soleus muscle at each time point; and 2) the percent composition of type I MHC of the plantaris muscle was consistently very low, i.e., <10% of the total MHC throughout the developmental period. Because HSP72 was localized in the slow fibers of the plantaris, the small amount of type I MHC combined with the relatively low stressor levels are consistent with the observed low levels of HSP72. It also appears that this differential expression level between fast and slow muscles is maintained in adulthood, i.e., the HSP72 content in predominantly fast muscles such as the extensor digitorum longus and tibialis anterior is between 5 and 16% of that in the predominantly slow soleus muscle of adult rats (14). Combined, these observations indicate that both endogenous (presence of type I MHC) and exogenous (increasing weight-bearing activity) factors are important determinants of the HSP72 content in skeletal muscles of rats during the developmental period.

HSC73 is constitutively expressed in all cells and tissues that have been analyzed in unstressed adult rats and in a variety of tissues in several species at a young postnatal age (13, 33). In addition, there were no differences in HSC73 content among a variety of skeletal muscles or between skeletal and cardiac muscles in adult rats (14). In the present study, we observed relatively high levels (relative to that determined in the soleus muscle at 56d) of HSC73 in both muscles at all time points, including E22 (Figs. 7 and 8). In fact, by 3d, the HSC73 levels in both muscles were similar to that of the 56d soleus muscle. The present and previous results showing the expression of HSC73 at early developmental stages reflect its universal function, i.e., HSC73 functions as a molecular chaperone to maintain cell and tissue homeostasis (10), even under resting or sedentary conditions (35).

In summary, we show for the first time that HSP72 is expressed specifically in muscle fibers that contain some type I MHC at an embryonic stage of development in rats. This relationship was observed in both a slow (soleus) and fast (plantaris) ankle extensor muscle. Because these muscles are impacted minimally by exogenous stressors at this stage of development, the presence of HSP72 must be influenced by endogenous factor(s). Furthermore, it is highly likely that these endogenous factors are closely associated with type I MHC. Recent evidence indicates that calcineurin, a calcium-calmodulin-dependent protein phosphatase, plays an important role in the expression of the slow phenotype, including type I MHC, in skeletal muscles in vitro and in vivo (32, 34). Thus one possibility is that calcineurin may affect the expression of HSP72 as well as the type I MHC isoform. The present data are also the first to show muscle-specific changes in HSP72 during the early postnatal period in rats. Although these changes appear to be closely related to exogenous stresses associated with weight-bearing postural and locomotor tasks, the possibility that endogenous factor(s) may be involved cannot be ruled out at this time.

REFERENCES


J Appl Physiol • VOL 95 • SEPTEMBER 2003 • www.jap.org


