mammalian cells. Genes for the HSPs are induced by the denatured proteins that are produced during elevation of body temperature (heat shock), ischemia, intense exercise, training exercise, and other stresses. All known HSPs [ubiquitin, HSP10, HSP27, HSP32, HSP47, HSP60, HSP70 (HSC73 and HSP72), HSP90, and HSP10] are chaperones, molecules which help to restore the function and structure of damaged/denatured proteins (24). HSPs also appear to protect a vast number of cell types in vivo against a diverse number of insults. Some examples are the protection of the retina against light-induced injury, protection of the brain against ischemia, protection of the heart against ischemia/reperfusion, and protection of the hippocampal neurons against focal ischemia (24). The constitutively expressed HSC73 member of the HSP70 family of HSPs is one of the major chaperones found in normal cells. The other member of this family, HSP72, is the major inducible HSP (24). All organisms contain proteins of the HSP70 family, the most extensively studied family of HSPs. However, little is known about the relationship between muscle function and the HSP70 family.

Many laboratories have reported that intense physical exercise causes stress-related alterations of muscle cytosolic homeostatic systems. Brooks et al. (2) reported that exercise increased the temperature of active muscles to 45°C, a condition that is equivalent to heat shock and that, in turn, can cause uncoupling of muscle mitochondria. Exercise can also increase the production of reactive oxygen species (6, 15, 22), which in turn, can increase the synthesis of HSPs (5, 27). Salo et al. (23) found that extensor digitorum longus (EDL) and soleus (SOL) muscles dissected from rats exercised to exhaustion had an increase in mRNA for HSP72, with the increase in EDL being the larger and most persistent.

However, there is no known relationship between the level of HSP72 within skeletal muscles and its functions, in particular on its response to and recovery from fatiguing stimulation. Thomas and Noble (28), using whole body hyperthermia (heat shock), have recently investigated whether a preconditioning heat
shock heat could provide subsequent protection to skeletal muscle contractility against low-frequency fatigue. In their study, male rats were heat shocked at 41.5°C for 15 min either 24 h or 4 days prior to fatiguing stimulation to compare the contractile responses of the plantaris muscle with those of a non-heat-shocked group. Either 24 h or 4 days following heat shock, HSP72 was elevated above control levels. The authors found no differences between the heat-shocked muscles and the control muscles in measurements of contractility prior to fatiguing stimulation or in resistance to fatigue. However, whole body heat shocking is a non-specific stress that affects many different cellular proteins and increases receptor sensitivity to catecholamines and steroids as well (4, 21, 25).

The present study utilized transgenic mice in which HSP72 was genetically upregulated to specifically determine how increased concentration of this particular heat shock protein affects the contractility and fatigue characteristics of muscles containing fast (EDL), slow (SOL), and mixed (diaphragm, DPH) fibers. Previous studies on hearts from these same transgenic animals have shown that HSP70 provides protection from the damaging effects of ischemia/reperfusion (20).

When stress proteins come upon a denatured (misfolded) protein, they bind to the protein via the peptide domain to prevent protein-protein aggregation. As documented for heat-damaged RNA polymerase, HSP72 disaggregates and reactivates heat-damaged proteins (26). This repair is important because only properly folded proteins have their normal physiological function. Black and Subject (1) suggest the HSC73 functions during nonstressed conditions, whereas HSP72 is synthesized to meet the extra demands imposed on the cell by stress.

Because both ischemia and fatigue are considered to be oxidative forms of insults that damage proteins, we hypothesize that increased synthesis of heat shock proteins will prevent oxidant damage or assist in repair of damaged proteins during repeated muscle contractions (9, 17, 23). Preliminary reports of this data have previously appeared in abstract form (14).

METHODS

Experimental animals. Transgenic B6 × SJL mice were produced using a chimeric transgene consisting of a rat inducible HSP72 inserted into the vector pCAGGS (20); these animals, developed by Dillmann and colleagues (20), were used in all experiments. Transgene-negative littermate mice were used as controls. All animals (males and females) were allowed to recover from the stress of shipment and to acclimate to their new environment for at least 7 days before any procedures were begun. Animals were given free access to water and food and were housed in a facility with a controlled 12:12-h light/dark cycle. All mice were weighed immediately before they were killed. Care was taken to ensure that all procedures relating to the living animals were in accordance with the “Guiding Principles in the Care and Use of Animals” approved by the American Physiological Society.

Intact muscle preparation. The mice were killed by cervical dislocation, and the intact EDL, DPH, and SOL muscles were removed and placed in a dissecting dish containing a modified Krebs-Henseleit solution with the following composition (in mM): 119.5 NaCl, 5.9 KCl, 1.8 CaCl₂, 0.4 NaH₂PO₄, 0.5 MgCl₂, 11.9 NaHCO₃, and 10 glucose. Fetal calf serum (0.2%) was added to the solution to increase viability of the dissected muscle (12). The final solution was continuously aerated with a 95% O₂-5% CO₂ gas mixture to maintain a pH of 7.35. EDL and SOL muscles were used as removed from the animal. Experiments on the DPH were conducted on small strips dissected from the costal region between the central tendon and the rib cage. Sutures were attached to each end of the muscles, to tendons when possible. All experiments were carried out at room temperature (~22°C).

Intact whole muscles or muscle strips were mounted vertically on a glass stimulating apparatus (Radnoti, Monrovia, CA) with platinum electrodes and were immersed in a 20-mL bathing chamber containing the incubation medium. The muscle sutures were attached to an isometric force transducer and to the stationary post of the stimulating apparatus. The contractile status of the muscle was monitored on a strip-chart recorder. The output of the force transducer was digitized and stored in a computer and was analyzed with DADiSP Software for contractile characteristics. The resting tension and the stimulatory voltage (provided by a Grass digital stimulator) were adjusted to produce a maximal isometric tetanic force (Tmax). Upon completion of an experimental protocol, the muscles were removed from the bathing medium, rapidly frozen in liquid nitrogen, and stored for Western blot analysis.

Intact force normalization. Because of the differences in muscle shapes used in this study and the uncertainties that can occur from measurements of muscle length and wet weight, we decided to normalize force (Tmax) using the total amount of protein as the normalization index. The muscles from control and transgenic mice were homogenized and analyzed total protein. Total protein was determined by the method of Lowry et al. (19), and normalized force was expressed as grams of force per milligram of total protein.

Force vs. frequency relationships. The muscles were allowed to equilibrate for 20 min, receiving a maximal tetanic frequency of stimulation (100–140 Hz) once per minute. After equilibration, the muscles were subjected to several frequencies of stimulation between 1 and 140 Hz to produce the force vs. frequency relationship.

Caffeine stimulation. Following the 20-min equilibration period, the muscles were subjected to alternated train-pairs of tetanic stimulation (frequencies that produced 50% Tmax for each muscle) at 1-min intervals. The muscles were then exposed to increasing concentrations of caffeine (0.1–10 mM). The muscles were exposed to each caffeine concentration for at least 5 min before the force generated was recorded.

Intermittent fatiguing stimulation protocol. In this work, we used an intermittent stimulation protocol that was initially established by Edwards et al. (8). In this protocol, muscles are alternately stimulated with a high frequency (HF: 140 Hz for EDL, and 100 Hz for both the DPH and SOL) that produced Tmax and a low frequency (LF) of tetanic stimulation (21–44 Hz, which produced 50% Tmax in an attempt to study the relative contributions from both the contractile proteins and the sarcoplasmic reticulum (SR) to the response of fatigue stimulation. The duration of the stimulation trains was 350 ms (for EDL and DPH) or 700 ms (for SOL). After Tmax was initially determined, the intact muscles were allowed to equilibrate for 20 min, during which time they were stimulated with pairs of high- and low-frequency pulse trains administered with a periodicity of 1 min. Following equilibration, the muscles were subjected to a
5-min intermittent stimulation fatiguing protocol consisting only of the high-frequency (HF) stimulatory tetanic trains (100 or 140 Hz) administered at a 1-s periodicity. Thereafter, the periodicity of the training was returned to 1-min intervals of high- and low-frequency stimulation, and the muscles were allowed to recover for 30 min. All force data were normalized to the single tetanic event (Tmax) measured just prior to the fatiguing protocol.

Measurements of heat shock proteins. The muscles used in the fatiguing experiments were homogenized and analyzed for HSP72 and HSC73. Samples of the muscle homogenate were denatured in 4% SDS buffer with dithiothreitol at 100°C and were subjected to SDS-PAGE. Care was taken that equal amounts of total protein were loaded onto the gel for each sample. For quantification purposes, a known amount of a purified protein standard was also loaded onto the gel. Electrophoresis was performed under reducing conditions on an 8% Tris-glycine gel. The band densities were evaluated by densitometric scanning (Bio-Rad model GS-700 Imaging Densitometer). Samples were normalized against the amount of total protein loaded. Total protein was determined by the method of Lowry et al. (19). After electrophoresis, the protein samples were transferred to a nylon membrane by electroblotting (Bio-Rad Immun-Lite Assay Kit) and were blocked for 2 h with a 5% nonfat milk solution. The membrane were washed again with TTBS and probed for 2 h at room temperature with a 1:3,000 dilution of goat-anti mouse secondary antibody conjugated to alkaline phosphatase (Bio-Rad). The membrane was finally washed with TTBS, and the antibody visualized with enhanced chemiluminescence.

RESULTS

Heat shock protein content. Figure 1 shows the HSP72 and HSC73 content (normalized to total protein) of the EDL, SOL, and DPH muscles isolated from both control and transgenic B6 × SJL mice. HSP72 was detectable (>0.034 ng/μg protein) immediately upon arrival of the control animals at our animal facility. However, it became undetectable after the first week of acclimation (data not shown). Therefore, all animals were allowed to acclimate to their new environment and recover from the stress of shipment before they were used in any experiments. As expected, HSP72 was significantly elevated in muscles from transgenic-positive mice compared with those of the nontransgenic (control) littermates (<0.034 ng/μg protein). The concentration of HSP72 in transgenic mice was significantly greater than that of HSC73 in the control mice. Upregulation of HSP72 in all muscles from transgenic mice was accompanied by a significant downregulation in all muscle types of HSC73, a protein whose synthesis is controlled by a separate gene.

Size of intact muscles. To compare the size of the muscles, we analyzed the total protein content (an indicator of muscle size) of the EDL and SOL isolated from control and transgenic animals. (DPH muscle strips could not be included in this comparison, because they were cut from the isolated diaphragms). EDL and SOL muscles were of approximately equal size. The total protein content of the muscles from transgenic animals was no different from the respective control animals; i.e., there was neither wasting nor hypertrophy of muscles because of the upregulation of the inducible form of the 72-kDa heat shock protein, HSP72. This occurred even though the total body weight of the transgenic animals was on average 17% larger (34.5 ± 1.3 g) than the control animals (29.5 ± 1.3 g).

Contractile properties of intact muscles. The maximum isometric force-generating capabilities (Tmax, measured at the stimulation frequency that produced maximal tetanic force), normalized to total muscle protein, of the various intact muscles are shown in Fig. 2. The EDL produced the greatest Tmax. The SOL and DPH muscles were of approximately equal strength. There was no significant difference between Tmax in control and transgenic muscles for any of the muscles studied.

Figure 3, A–C, illustrates the force-frequency relationships for the three muscle types studied. The force displayed is normalized to Tmax for each muscle. The 50% Tmax level was achieved at 21.8 ± 3.5 Hz in control DPH muscles and at progressively higher frequencies in SOL (26.5 ± 2.5 Hz) and EDL (44.0 ± 1.9 Hz) muscles. We found no differences in these 50% Tmax
frequencies when comparing muscles isolated from control animals to those isolated from transgenic animals.

In a separate series of experiments, the dose-dependent influence of caffeine on the force generated by the isolated muscles was measured and is displayed in Fig. 4, A–C. As noted in the Methods, each muscle was exposed to increasing concentrations of caffeine (a 3-min exposure to each concentration between 0.1–10 mM). Each muscle was subjected, alternately, to HF (frequency chosen to give maximal force) and LF (chosen to produce 50% of maximal force, see Fig. 3, A–C) stimulation. The HF stimulation allowed monitoring of the caffeine effect on $T_{\text{max}}$, whereas LF stimulation allowed indirect monitoring of the caffeine effect on SR calcium release. There was no significant effect of caffeine on the HF force generated by EDL and DPH muscles isolated from either control or transgenic animals. However, caffeine caused a significant increase in the HF force generated by SOL muscles from both control and transgenic animals. Because caffeine does not affect the maximum calcium-activated force of skinned fibers (10), these results suggest that HF stimulation of EDL and DPH muscles is capable of releasing sufficient calcium from the SR to maximally activate the contractile apparatus of these intact muscles. Therefore, the $T_{\text{max}}$ values reported in Fig. 2 accurately represent the maximal force that can be generated by these intact muscles. However, because there was a caffeine-related increase in the HF force generated by SOL muscles, the $T_{\text{max}}$ reported in Fig. 2 is ~15% less than the true maximal force that the SOL is capable of generating. For all three muscle types studied, the caffeine effect on HF stimulation was the same in muscles from control and transgenic animals.

The series of experiments described in Fig. 4, A–C, also shows the influence of caffeine on the contractile response of the isolated muscles to LF stimulation. As expected, we found that caffeine produced a dose-dependent increase in the force generated by all muscle types in response to LF stimulation by increasing calcium release from the SR. In muscles from control animals, the percentage increase at 10 mM caffeine was greatest in DPH (increase of 101 ± 7%) and progressively less in SOL (90 ± 8%) and EDL (71 ± 3%) muscles. Since caffeine increases the calcium sensitivity of calcium-induced calcium release (CICR), these results indicate that the degree of CICR contributing to the total calcium release from the SR sensitivity follows the intensity of the effect of caffeine: DPH > SOL > EDL. The effect of caffeine on the response to LF stimulation was not different between control and transgenic DPH and SOL muscles. In contrast, EDL muscles from transgenic animals exhibited a statistically greater sensitivity to caffeine (see Fig. 4C). These results suggest that upregulation of the HSP72 gene in some way selectively influences the sensitivity of the SR to caffeine in fast-twitch skeletal muscles.

Response of intact muscles to intermittent fatiguing stimulation. In the next series of experiments, we determined how EDL, SOL, and DPH muscles from control and transgenic animals respond to, and recover from, a 5-min intermittent fatigue protocol. Figure 5, A–C, illustrates the quantitative differences among the three muscle types evaluated. The greatest depression at the end of the 5-min fatigue period in the response to the HF stimulation was found in EDL muscles; only 11 ± 1% of the prefatiguing HF response level remained, compared with 20 ± 4% for the DPH and 46 ± 3% for the SOL. Maximum recovery was to 74 ± 3% after 22 ± 2 min for EDL, to 88 ± 3% after 8 ± 1 min for DPH, and to 101 ± 2% after 7 ± 1 min for SOL; slow muscles recovery was more complete than fast muscles recovery.

The decrease in the response to LF stimulation was also greatest in EDL muscles after the 5-min period of intermittent fatiguing stimulation; starting from 50% of the prefatiguing HF response level, only 14 ± 1% remained in the EDL compared with 21 ± 1% for the DPH and 29 ± 2% for the SOL. Compared with the other two muscles studied, recovery of the response to LF stimulation was least in EDL muscles; a return to only 26 ± 1% of the prefatigue HF level was achieved at an average time of 21 ± 2 min, compared with a return to 32 ± 2% at 6 ± 1 min in the DPH and to 45 ± 3% (essentially full recovery) after 7 ± 1 min in the SOL. Figures 5, A–C, also shows that the responses to HF and LF stimulation were statistically the same in muscles from control and transgenic animals.

**DISCUSSION**

To the best of our knowledge, this is the first report of the effects of an increased concentration by means of genetic overexpression of HSP72 on the contractility and fatigability properties of EDL, SOL, and DPH.
muscles. Using these same animals, Marber et al. (20) have previously demonstrated that hearts overexpressing HSP72 were protected against the deleterious effects of ischemia. Because both fatigue and ischemia are believed to produce oxidative damage, we postulated that the skeletal muscles from these animals would also be protected against the short-term acute detrimental effects of fatiguing stimulation, in that increased synthesis of HSP70 would prevent oxidant damage or assist in the rapid repair of damaged proteins due to repeated muscle contractions (9, 18, 23).

Muscle fiber type and HSP72 and HSC73. Locke et al. (18) found constitutive expression of HSP72 (typically the induced protein of the HSP family) in unstressed rat muscle comprised primarily of type I muscle fibers (SOL) but not in muscles comprised primarily of type IIB fibers (white gastrocnemius). In muscles of mixed type, HSP72 content was roughly proportional to the percentage of type I fibers. They suggested that increased levels of HSP72 in type I, slow oxidative, muscles is possibly related to the fact that this fiber type is continuously subjected to a more stressful environment.

Fig. 3. Force vs. frequency relationship for DPH (A), SOL (B), and EDL (C) muscles from control (open circles) and transgenic animals (solid circles). The force at each frequency was normalized to T_max for each muscle.
vironment than other fiber types and requires the function served by HSP72. We only found detectable (≥0.034 ng/μg) amounts of HSP72 in the muscles we studied upon arrival of the shipped animals, and within 1 wk of acclimation the levels of HSP72 became undetectable. We believe that shipment produced a stress condition, and this may explain the difference between our findings and those of Locke and colleagues (18). In addition, this group (18) also reported that muscles containing more type I myosin heavy chain contain more HSC73. We did not find this to be the case in our study, all control muscles contained essentially the same level of HSC73.

**Response to intermittent fatiguing stimulation.** Edwards et al. (8) were the first to report that after a brief period of recovery from fatiguing exercise, there remains a significant deficit in the force produced by human muscle in response to a subsequent single, LF,
A submaximal, tetanic stimulus, which releases a submaximal amount of calcium from the SR. At the same time, there was no decrease in force produced in response to a single, HF, maximal, tetanic stimulus, because the HF train releases sufficient calcium to saturate the contractile proteins. The contractile response of skeletal muscle to electrical stimulation follows a well-defined “force-frequency” relationship, with “frequency” being the independent variable. As the frequency of stimulation increases, the contractile response also increases, as more calcium is released from the SR. The contractile response of the muscle to lower frequencies of stimulation reflects the processes involved with the calcium-release mechanisms. The contractile response of the muscle to higher frequencies plateaus as the calcium released from the SR saturates the contractile proteins.

The deficit in force in response to an intermittent fatiguing stimulation protocol required ~24–48 h to return to normal in the studies of Edwards et al. (8).
We found that the SOL muscle recovered rapidly to both HF and LF stimulation, and the EDL recovered only slightly to LF stimulation and recovered incompletely to HF, whereas the DPH displayed an intermediate level of recovery when compared with the SOL and the EDL muscles. This long-lasting effect after intermittent fatigue has been observed in a number of isolated muscles (13) and appears to result from a reduction in the release of Ca\(^{2+}\) from the SR (11, 29). In agreement with these findings, Brotero and Nosek (3) demonstrated that exposure of EDL saponin-skinned muscle fibers to H\(_2\)O\(_2\) disrupts the calcium release process. Brotero and Nosek (3) have postulated that under fatiguing stimulation, the intracellular concentration of H\(_2\)O\(_2\) increases (as a result of increased production of superoxide and the normally low intracellular concentration of catalase in skeletal muscles), which leads to oxidation/modification of essential proteins of the excitation-contraction coupling (ECC) process. The time of recovery from intermittent fatiguing stimulation in fast-twitch (EDL) and mixed muscle (DPH) is consistent with the time required to resynthesize potentially damaged or denatured SR calcium release proteins, whereas the full recovery displayed by the SOL indicates that slow-twitch muscles are better equipped against oxidative damage.

**Caffeine and HSP72.** We found that caffeine sensitivity was enhanced only in EDL muscles from the transgenic mice. Although we have not investigated the cellular and molecular mechanisms that could be associated with these findings, we speculate that HSP72 might either affect the Ca\(^{2+}\) loading capacity of the SR (via an increased activity of the SR/Ca\(^{2+}\)-ATPase pump) or the calcium sensitivity to CICR (either by a direct effect on the ryanodine receptor (RyR) or via modulation of accessory SR proteins, such as FKBP12, calmodulin, or triadin) in fast-twitch muscles. It is also conceivable that the potential influence of the upregulated HSP72 was offset by the diminished influence of the downregulated HSC73 and that the observed effects were indirect effects that resulted from modulation of additional genes/proteins induced by the manipulation of the HSP70 family.

In conclusion, our present study unequivocally demonstrates that the overexpression of HSP70 does not influence the basic isometric contractile properties of skeletal muscle or protect them under short-term, acute effects of 5 min of fatiguing stimulation. Because other laboratories have found a protective role of HSPs from ischemia in transgenic mice overexpressing HSP70 and both ischemia and fatigue are considered to be types of oxidative stress, we expected to find a protective role in skeletal muscles. Therefore, it is possible that HSPs may play a different role in skeletal muscles when compared with cardiac muscle or that this type of protective role is specific for the ischemic insult.

Yet another possibility is that in skeletal muscles HSP72 and HSC73 are not equally potent and the combination of the observed upregulation of HSP72 and the downregulation of HSC73 somehow contributed to the lack of protection reported here. To the best of our knowledge, nobody has addressed this important question. What we can infer from the literature is that the stress protein-related responses are very complex. Recently, Lee and Vierling (16) have demonstrated that a small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. Demidov et al. (7) concluded that in cardiosurgery patients, HSP72 was implicated in cardioprotection, whereas HSC73 was not implicated. Therefore, it is possible that under certain conditions these proteins will have additive/synergistic effects, whereas under other conditions they will not.

Finally, this study shows that in EDL muscle, HSP72 may be directly or indirectly involved with the modulation of the ECC process (probably the calcium release process from the SR), since an enhanced sensitivity to caffeine was observed in the EDL muscle in the transgenic muscles overexpressing this protein. Additional studies will be required to further investigate this possibility and whether HSP72 can assist skeletal muscles to recover in a faster fashion from repetitive stimulation under chronic or long-term conditions and whether other families of HSPs might be involved with the protection of skeletal muscles against the damaging effects of fatiguing stimulation.

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