

## Response of Heat Shock Protein 72 to Repeated Bouts of Hyperthermia in Rat Skeletal Muscle

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### Summary

We investigated the effects of repeated hyperthermic bouts on the heat shock response of heat shock protein (HSP) 72 in skeletal muscle. Rats were assigned to control and hyperthermia groups which were exposed to heated water at 42 °C. The hyperthermia group was further divided into sub-groups: a single bout (H30) or four bouts of hyperthermia for 30 min (H30x4). There was an increase in HSP72 protein content of the H30 groups in both *extensor digitorum longus* (EDL) and *soleus* muscles. Moreover, HSP72 protein expression in H30x4 group was significantly higher than in H30 group in both EDL and *soleus* muscles. The HSP72 mRNA was markedly increased from control levels in the H30 and H30x4 group in both types of muscles. However, HSP72 mRNA of the H30x4 group was lower than that of the H30 group in *soleus* muscles. Heat shock response of HSP72 is activated even after repeated bouts of hyperthermia, with a differential regulation between muscle types.

### Key words

Hyperthermia • Heat shock response • Heat shock protein • Skeletal muscle

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Heat shock protein (HSP) 72 is the most thermally sensitive isoform in skeletal muscle. HSP72 plays essential roles in cellular homeostasis by acting as molecular chaperones and facilitating the folding, transportation, and conformational maturation of newly

synthesized proteins. Recently, it has been suggested that the induction of HSP72 in skeletal muscle has broad therapeutic benefits in the treatment of various types of disease such as type 2 diabetes (Chung *et al.* 2008) and muscular dystrophy (Gehrig *et al.* 2012).

The duration of the hyperthermic bout was shown to be important in modulating HSP72 induction in several tissues, with significantly greater levels of HSP72 protein following longer heating periods (Ruell *et al.* 2004). In contrast, although clinical application of hyperthermia generally involves intermittent repeated applications (Chung *et al.* 2008, Gehrig *et al.* 2012), little is known as to whether the transcriptional response to hyperthermia is preserved and HSP72 levels increase cumulatively after repeated bouts of hyperthermia. Thus, in the present study, we examined the effects of a single bout or multiple bouts of hyperthermia on the HSP72 expression in skeletal muscles.

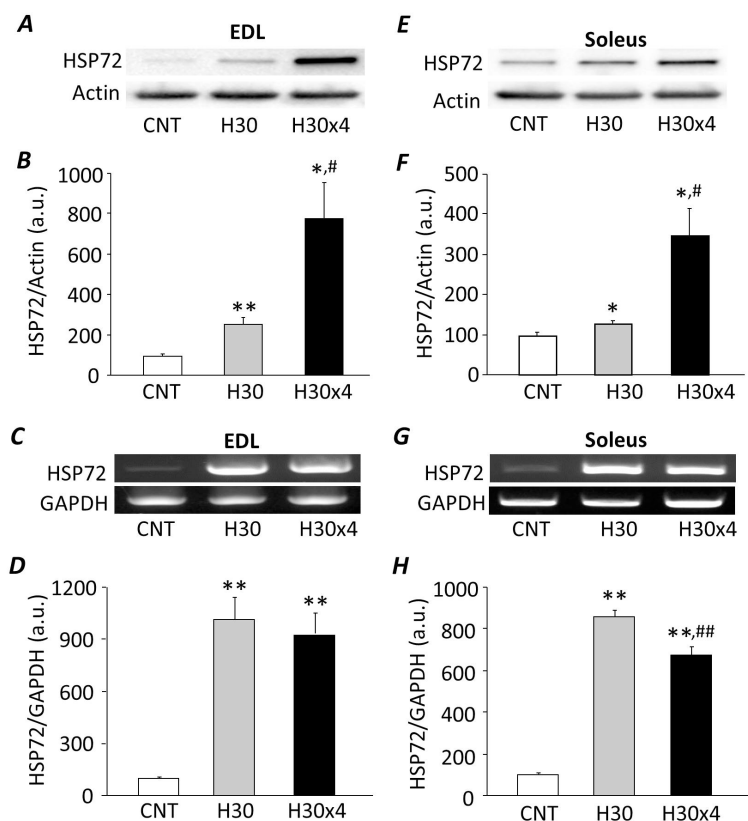
All experimental procedures were approved by the *Committee on Animal Experiments of Sapporo Medical University*. Animal care was in accordance with institutional guidelines. Male Lewis rats (9-week-old, n=42) were randomly assigned to control (CNT) and hyperthermia group which was subdivided into a single bout (H30) or 4 bouts of hyperthermia for 30 min every other day (H30x4). Hyperthermia was given by placing their hindlimbs into a heated water bath at 42 °C under isoflurane anesthesia. The time intervals of repeated hyperthermia were chosen according to the results of Selsby and Dodd (2005) who found that HSP72 expression peaks between 24 and 48 h following hyperthermia.

Rats were killed by cervical dislocation under isoflurane anesthesia and both EDL and *soleus* muscles were excised 24 h after hyperthermia and were used for protein analysis. Western blotting was performed using primary antibodies (anti-HSP72, Stressgen; anti-actin, Sigma) as previously described (Yamada *et al.* 2012). In another set of animals, HSP mRNA level was examined 1 h after hyperthermia because it peaks 1-2 h following hyperthermia (Welc *et al.* 2012). RT-PCR was performed as described previously in detail (Konturek *et al.* 2005).

Data are presented as mean  $\pm$  SEM. Differences between groups were determined by using an unpaired

Student's *t*-test. A *P* value less than 0.05 was regarded as statistically significant.

The time course changes in temperature for the deep gastrocnemius and rectum were measured in one anesthetized rat using a thermistor probe (Microcomputer Thermometer 700 1H, Physitem) during 30 min of hyperthermia at 42 °C and recovery at room temperature. The muscle temperature and core temperature was gradually increased from 35.9 °C and 36.0 °C to 41.3 °C and 40.9 °C after 30 min of hyperthermia, respectively. They decreased back below the initial temperature 30 min after recovery (muscle: 34.6 °C, core: 34.8 °C).



**Fig. 1.** Effects of the number of hyperthermia bouts on HSP72 expression of EDL and *soleus* muscles. The levels of HSP72 protein expression in EDL (**A, B**) and *soleus* (**E, F**) muscles were quantified as arbitrary units (a.u.) normalized to actin content. The levels of HSP72 mRNA expression in EDL (**C, D**) and *soleus* (**G, H**) muscles were quantified as arbitrary units (a.u.) normalized to GAPDH mRNA content. Bars show the mean and SEM results from 5-8 muscles per group. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. CNT, #  $p < 0.05$ , ##  $p < 0.01$  vs. H30.

Figs 1A and 1E show representative immunoblots of HSP72 after a single bout or after four bouts of hyperthermia for 30 min in EDL and *soleus* muscles. There was an increase in HSP72 protein content of the H30 groups in both EDL (2.7-fold) and *soleus* (1.3-fold) muscles (Figs 1B and 1F). Moreover, compared to the H30 group, HSP72 expression was significantly higher in EDL (3-fold) and *soleus* (2.7-fold) muscles from the H30x4 group.

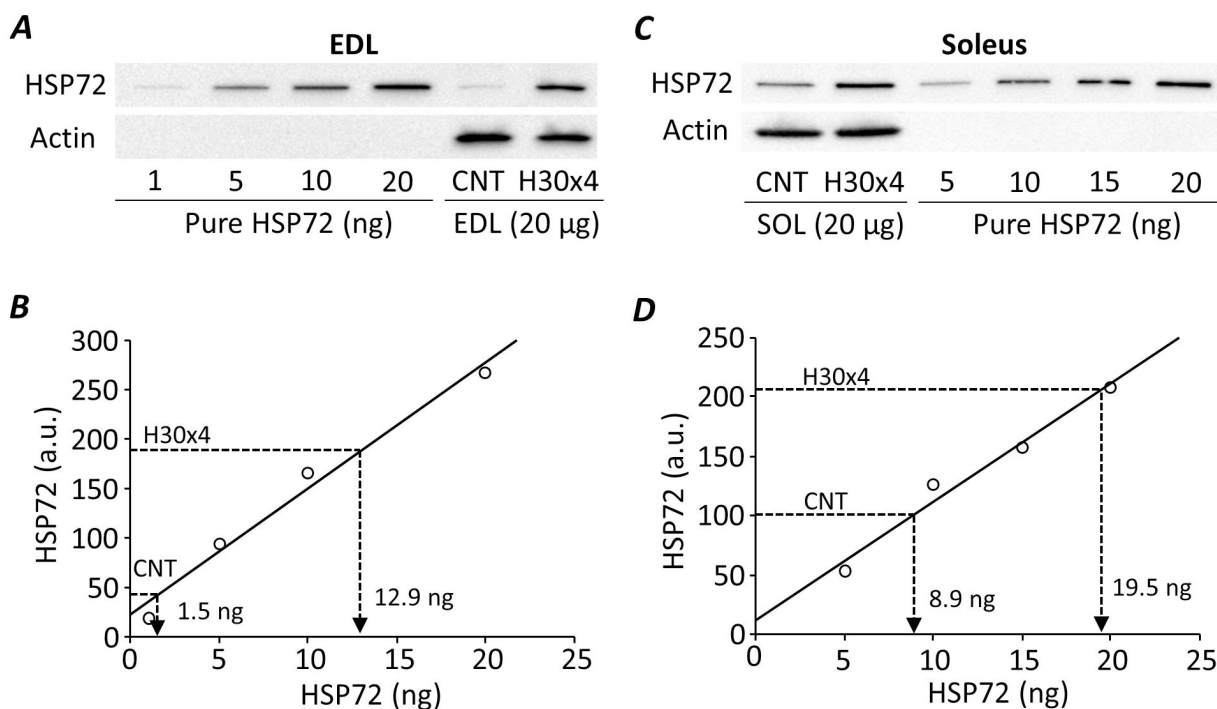
To further assess whether the transcriptional reactivity of HSP72 is affected by repeated bouts of hyperthermia, we compared the HSP72 mRNA levels

after a single bout and after four bouts of hyperthermia for 30 min. The HSP72 mRNA was markedly increased in the H30 and H30x4 groups, whereas there was a reduction in HSP72 mRNA of the H30x4 group compared to the H30 group in *soleus* muscles (Figs 1C, 1D, 1G, and 1H). Moreover, compared to the CNT group, there was no change in the expression level of HSP72 mRNA after repeated hyperthermic bouts without the last acute hyperthermia (i.e. 49 h following three boats of hyperthermia) (EDL:  $100.0 \pm 12.5$  vs.  $100.8 \pm 19.5$  arbitrary units (a.u.) [ $n=4$ ];  $P > 0.05$ ), *soleus*:  $100.0 \pm 25.7$  vs.  $112.4 \pm 13.7$  a.u. [ $n=6$ ];  $P > 0.05$ ), suggesting that baseline

level of HSP72 transcription is not affected by repeated hyperthermia.

It has been shown that slow-twitch muscle has a higher HSP72 content compared with fast-twitch muscle (Locke *et al.* 1994). To compare the absolute amount of HSP72 in EDL and *soleus* muscles following repeated hyperthermia, the entire muscle homogenate (20  $\mu$ g) was run with known amounts of purified HSP72 (1-20 ng, Enzo Life Sciences), which allowing a calibration curve to be generated (Figs 2A-2D). The relative amount loaded was verified by reprobing the

membrane for actin. HSP72 content of EDL and *soleus* muscles was  $75 \pm 11$  and  $381 \pm 89$   $\mu$ g/g protein in CNT groups and  $530 \pm 138$  and  $793 \pm 57$   $\mu$ g/g protein in H30x4 groups, respectively ( $n=4-6$ ). It was shown, using an ELISA assay, that HSP72 content in rat fast-twitch *flexor digitorum* muscles is  $\sim 63$   $\mu$ g/g protein (Barbe *et al.* 2013), which is very similar to that of EDL muscles in our study, verifying the reliability of the quantification method. Thus, the absolute amount of HSP72 in *soleus* muscles is 5- and 1.5-times greater than EDL muscles in CNT and H30x4 groups, respectively.



**Fig. 2.** Effects of repeated hyperthermia on absolute amount of HSP72 content of EDL and *soleus* muscles. Pure HSP72 (1-20 ng) and total muscle homogenates (20  $\mu$ g protein) from EDL (**A**) and *soleus* (**C**) were run on the same membrane and were quantified as arbitrary units (a.u.). Relative loading amounts were verified by actin as a loading control. HSP72 content of EDL (**B**) and *soleus* (**D**) muscle homogenates (1.5 and 8.9 ng in CNT group and 12.9 and 19.5 in H30x4 group in 20  $\mu$ g protein, respectively) was calculated using a calibration curve, which was obtained by plotting band density for four standards of known amount of purified HSP72 in each membrane.

To our knowledge this is the first study to investigate the effects of repeated bouts of hyperthermia on HSP72 expression in skeletal muscle. Our results show clearly that HSP72 expression in both EDL and *soleus* muscle was significantly increased by repeated bouts of hyperthermia. These data are accordance with the study on hamster fibroblasts showing that thermal stress (43  $^{\circ}$ C for 15 min) applied every alternate day for four weeks induced a greater expression of HSP72 compared to those for two weeks (Banerjee Mustafi *et al.* 2009). Moreover, our data showed that mRNA expression of HSP72 after repeated bouts of hyperthermia

in both EDL and *soleus* muscle is significantly higher than control. These data demonstrate that the increased expression of HSP72 after multiple bouts of hyperthermia is induced, at least in part, by transcriptional activation.

The reason for the reduced HSP72 mRNA levels after repetitive bouts of hyperthermia compared to that found after a single application in *soleus* muscle is unknown, but could be due to an autoregulatory loop by which cells regulate the activation of heat shock transcription factor (HSF) (Mosser *et al.* 1993). During thermal or other forms of stress, HSP72 is separated from HSF, allowing the inactive non-DNA binding monomers

to form active DNA-binding trimmers that bind to the heat shock element (HSE) and subsequently increase HSP72 content (Morimoto 1993). In contrast, HSP72 has been implicated in negatively regulating the activation of the HSF and high constitutive levels of HSP72 are inversely correlated with HSF activation (Mosser *et al.* 1993). In the present study, although the degree of increase in HSP72 protein expression after repeated hyperthermia was remarkably higher in EDL than *soleus* muscles (8.5- and 3.6-fold, respectively), the absolute amount of HSP72 protein was 1.5-fold higher in *soleus* than EDL muscles. These data therefore suggested that HSF activation would be inhibited more strongly in *soleus* muscles following repeated bouts of hyperthermia.

In conclusion, the present study shows that there is a preserved transcriptional activation of heat shock response for HSP72 protein following repeated bouts of hyperthermia in both EDL and *soleus* muscles, which is differentially regulated in a muscle type-specific manner.

### Conflict of Interest

There is no conflict of interest.

### Acknowledgements

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