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HSP70 Expression in Skeletal Muscle of Patients with Peripheral Arterial Occlusive Disease

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Objectives: heat shock protein (HSP70) has been studied in the ischaemic myocardium and proven to provide protection against ischaemia. However, HSP70 in ischaemic skeletal muscle in patients with peripheral arterial occlusive disease (PAOD) has not been reported.

Methods: thirty-four patients with PAOD (Fontaine's criteria: stage II: 15; III: 9 and IV: 10, respectively) and ten non-PAOD controls were enrolled in the study. Calf muscle samples were taken. HSP70 was quantitated by SDS-PAGE using ultrasensitive silver staining with reference to a series of standard HSP70, and HSP70 mRNA was estimated using RT-PCR.

Results: in comparison with the controls [median with range: 24.8 (14.1–35.6) ng in 2.5 µg total protein], HSP70 was increased significantly in PAOD [stage II: 93.1 (62.7–114.3); stage III: 110.1 (89.7–134.5) and stage IV: 77.4 (67.3–101.1)]. Similar results were obtained with HSP70 mRNA.

Conclusions: HSP70 is increased in the ischaemic skeletal muscle in patients with PAOD, and HSP70 expression is different with regard to clinical stages, and the upregulation of HSP70 mRNA implies that the expression of HSP70 seems to be regulated at transcriptional level.

Key Words: Heat shock protein; Ischaemia; Skeletal muscle; Arterial occlusive disease; Human.

Introduction

In response to a variety of stresses, cells produce a series of proteins, i.e. stress proteins. One family of these stress proteins is so-called heat shock protein (HSP), and the most prominent HSP is that with molecular mass 70 kD (HSP70).^{1,2} To date, induction of HSP has been demonstrated in all examined eukaryotic cells and is considered a universal and highly conserved characteristic.³ HSP70 has been proven to play an important role in surviving a critical stress. In protection against ischaemia, HSP70 has reportedly an important impact on myocardium.^{4,5} The induction of HSP70 can not only reduce injury to ischaemic myocardium but also improve post-ischaemic myocardial recovery.^{6–8} Another interesting fact is that the so-called “preconditioning” in which myocardium develops

resistance against ischaemia can be achieved not only by pre-ischaemia, but also by pre-hyperthermia. This may indicate a “cross-protection” or “cross-tolerance” against ischaemia.^{5,9}

There are many factors or stressors which cause the induction of HSP70 including free radical production, decreased cellular pH, glycogen depletion.^{3,10} Although the mechanisms of HSP70 induction are not completely understood, ischaemia and reperfusion can both cause HSP70 production.^{11–13} However, most of the studies dealt with myocardial ischaemia, and few studies addressed the issue in skeletal muscles. Recently, we have reported that HSP70 could be induced in skeletal muscle during physical training¹⁴ and that HSP70 expression seemed to be dependent upon the exercise intensity.¹⁵ To our knowledge, there is no report on HSP70 expression in ischaemic human skeletal muscle.

This study was designed to investigate whether HSP70 induction takes place in the skeletal muscle in patients with PAOD and if any, whether the HSP70 expression is different between clinical stages of PAOD.

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Methods

HSP70 quantitation

Subjects

Forty-four subjects (male 31, female 13), aged 62 (50–72) years, were studied (Table 1).¹⁶ There was no significant difference with regard to age, sex, body mass and smoking history. Diabetes was excluded. The age-matched controls were the patients from traffic accidents who were operated within two hours and PAOD excluded by clinical examination and Doppler ankle pressures. PAOD patients were diagnosed by clinical examination, Doppler ankle pressure and arterial angiography. Three patients with PAOD II and all patients with PAOD III underwent a percutaneous transluminal angioplasty (PTA) while those with PAOD IV got an amputation at the knee level. The other twelve patients with PAOD II did not undergo any PTA or vascular surgery procedure. This study was approved by the ethics committee of the University of Ulm (Ulm, Germany) and informed consent of all subjects were obtained.

Muscle sampling

From the twelve patients with PAOD II who did not undergo any PTA or vascular surgery, muscle samples were obtained by fine needle biopsy.^{14,15} Muscle samples from the other subjects were obtained during the percutaneous transluminal angioplasty or vascular surgery procedure or operation on trauma (for controls) under sterile conditions (open biopsy). The muscle samples were taken prior to the planned PTA or surgical procedure. All muscle samples were taken from the m. gastrocnemius, and in the controls muscle samples were taken from the not-traumatic side. About 0.5 g (by open biopsy) or 15 mg (by fine needle biopsy) muscle tissue was taken and frozen immediately in liquid nitrogen, and then transferred to -80°C for subsequent analysis.

The quantitation of HSP70 was done on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using silver staining with referring to a series of standard HSP70, the details were described elsewhere.¹⁵ In brief, about 5 mg muscle tissue was removed from the muscle samples and homogenised in protein-extraction buffer,¹⁷ and total protein concentration of the homogenates was determined according to Lowry *et al.*¹⁸ Total protein measuring 2.5 μg was loaded on SDS-PAGE for the protein separation.¹⁹ HSP70 bands of muscle samples were detected using ultrasensitive silver staining,²⁰ identified according to the standard HSP70 run parallel to the samples (Fig. 1). The certainty of HSP70 bands detected by silver staining was verified with Western blot using specific antibody (Clone 2A4, Affinity Bio-Reagents, Golden, CO, U.S.A.).¹⁴

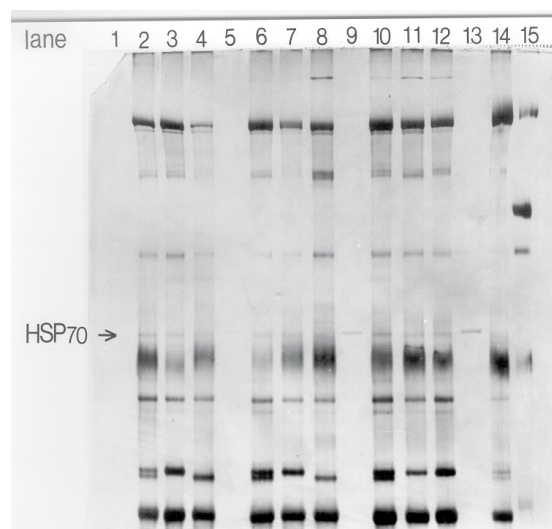


Fig. 1. An example of a silver-stained SDS-PAGE-gel. Lanes 1, 5, 9 and 13 were loaded with known amount of HSP70 0 ng, 25 ng, 50 ng and 75 ng, respectively. Lanes 2–4, 6–8 and 10–12 represent proteins derived from patients with PAOD II, IV and III, respectively. Lanes 14 and 15 for controls.

Table 1. Subjects included in the study (median with range).

	Control	PAOD II	PAOD III	PAOD IV
Number of subjects	10	15	9	10
Age (years)	62 (54–71)	63 (54–68)	60 (50–70)	63 (55–72)
Body mass (kg)	71 (63–81)	73 (63–85)	72 (59–86)	73 (64–79)
Clinical classification	no PAOD	Intermittent claudication	Resting leg pain	PAOD III + necrotic changes of the involved tissues
ABI*	1.15 (1.02–1.23)	0.82 (0.72–0.96)	0.57 (0.50–0.69)	0.38 (0.29–0.55)

ABI, Ankle-brachial index for systolic blood pressure.

* $p < 0.01$ in comparison between any two of the groups.

RT-PCR

About 5 mg muscle tissue were taken to extract total RNA using phenol extraction (RNAClean™ System, AGS Co., Heidelberg, Germany), and total RNA was dissolved in a final volume of 5 µl for each 1 mg muscle tissue. Oligo (dT) primed synthesis of cDNA was performed using MuLV reverse transcriptase (Perkin Elmer, Roche Molecular System, Inc., Branchburg, NJ U.S.A.) according to the standard protocol of the provider. Amplification of cDNA for HSP70 was performed according to the method reported by Taggart *et al.*²¹ In brief, the total reaction volume for each sample was 50 µl containing 25 pmol of each primer, 100 µM of each dNTP, 2 mM MgCl₂, and 0.5 unit of Tag polymerase. Thirty-five cycles of 45 s at 94°C, 60 s at 56°C, and 30 s at 72°C were performed in a microprocessor controlled incubation system (Crocodiell III, Appligene Co., F-67402 Illkirch, France). Simultaneously, PCR amplification for α-actin of skeletal muscle was done according to the method reported by Peuker & Pette.²² Product of RT-PCR for α-actin served as an internal reference for HSP70 mRNA. The expected products of RT-PCR for HSP70 and α-actin are 200 bp and 367 bp segments, respectively. The RT-PCR products were densitometrically

measured on 3% agarose-gel containing ethidium bromide (Fig. 2).

Data analysis

Samples were run in replicate. The calculation of HSP70 amount followed the linear regression between densitometric values and known standard HSP70 amounts.^{14,15} HSP70 mRNA is expressed by its RT-PCR product in relation to the corresponding RT-PCR product for α-actin (i.e. ratio of RT-PCR products for HSP70/α-actin). Data are expressed as median with range, and the difference was tested with nonparametric methods (the Mann-Whitney *U*-test), the difference was assumed to be significant if $p < 0.05$.

Results

All samples were successfully analysed. In comparison with that of the controls, HSP70 increased significantly in patients with PAOD II ($p < 0.01$), and HSP70 in patients with PAOD III had a further increase reaching about five-folds that of the controls ($p < 0.01$). Interestingly, HSP70 in PAOD IV did not increase further, but decreased significantly in comparison with that of PAOD III ($p < 0.01$), although it remained higher than that of the controls (Fig. 3). The difference of HSP70 levels was statistically significant between each two groups.

Similarly, RT-PCR results showed that in comparison with controls, HSP70 mRNA is increased significantly in patients with PAOD ($p < 0.01$); (Fig. 4), and reached the highest level in PAOD III. Again, HSP70 mRNA

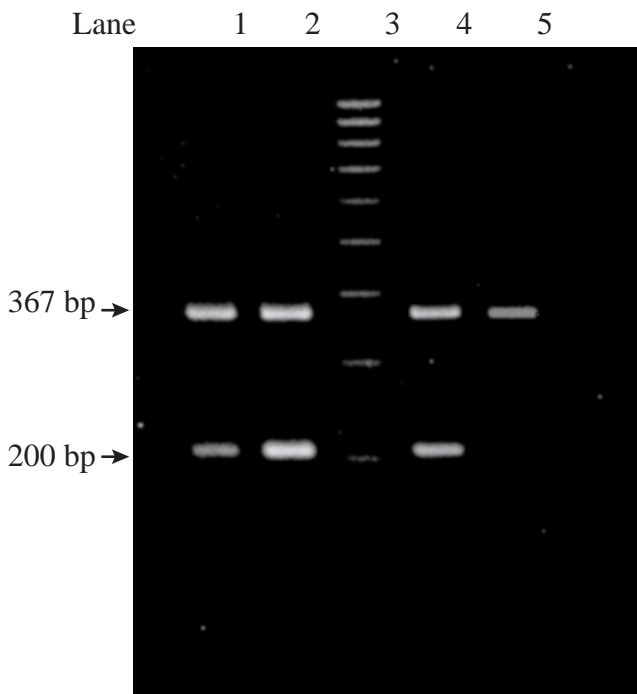


Fig. 2. An example of RT-PCR products for HSP70 and α-actin. Lane 3 is the molecular size lane. Lanes 1, 2, 4 and 5 were loaded with samples from PAOD IV, PAOD III, PAOD II and controls. The PCR products for HSP70 (200 bp) and α-actin (367 bp) are indicated.

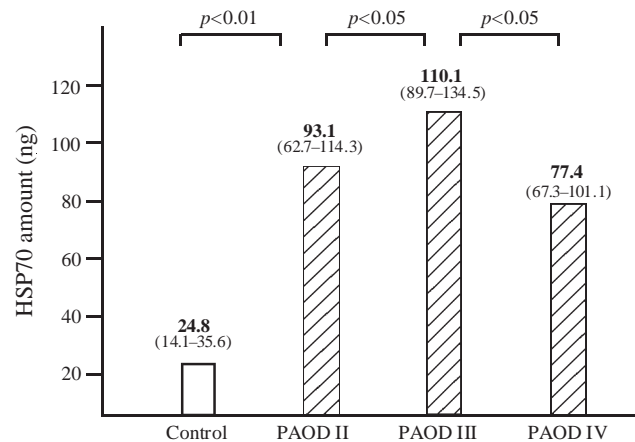


Fig. 3. HSP70 (ng) was determined in 2.5 µg loaded total protein from skeletal muscle samples (median with range). In comparison with controls, HSP70 increased in each of stage of PAOD ($p < 0.01$), and HSP70 level is different between clinical stages ($p < 0.01$).

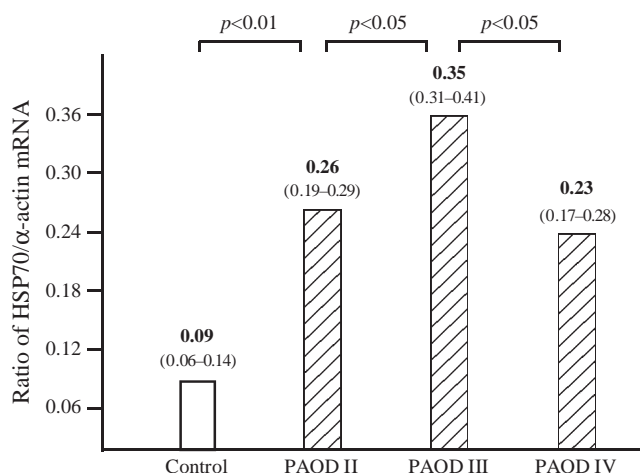


Fig. 4. HSP70 mRNA is represented by the ratio of HSP70/α-actin RT-PCR products (median with range), it is significantly increased in PAOD, and the highest level of HSP70 mRNA was observed in muscle samples of PAOD III. In comparison with that of PAOD III, HSP70 mRNA decreased distinctly in PAOD IV though it remained elevated compared to that of controls.

did not increase further but decrease in PAOD IV compared to that in PAOD III ($p < 0.05$).

Discussion

Studies dealing with HSP70 in ischaemic skeletal muscle in human have not yet been reported. In the present study we have measured HSP70 (both at protein and mRNA level) in the relevant skeletal muscle and found that in comparison with the controls, HSP70 increased in the patients with PAOD, and that HSP70 expression differed between clinical stages of the disease.

This implies that HSP70 induction is associated with the degree of the disease. We have previously investigated the distribution of myosin heavy chain (MHC) isoforms in PAOD patients and found that with advancing disease the percentage of MHC I increases.²⁴ HSP70 may be associated with certain MHC isoforms.^{25,26} The induction of HSP70 in skeletal muscle may also be affected by the change of MHC isoforms in PAOD patients.

The results of HSP70 at the protein level are further supported by HSP70 mRNA determined by RT-PCR, which suggests that HSP70 in ischaemic skeletal muscle is essentially upregulated, and the regulation of HSP70 expression may take place at the transcriptional level.

Interestingly, the highest level of HSP70 in patients with PAOD was observed in stage III. Several factors might be responsible for this unexpected result. First,

in PAOD IV the substrates as well as energy for metabolism needed may be limited^{23,27} so that the synthesis of proteins including HSP70 may be reduced. Against the result of HSP70 mRNA was the similar to that of HSP70 at the protein level, indicating that the regulation of HSP70 expression may also take place at transcriptional level. Second, PAOD IV patient may be unable to exercise, and exercise-induced physiological and biochemical changes may induce HSP70. Finally, reperfusion has been reported to have impact on HSP70 induction, and in PAOD IV reperfusion might not occur or might not play so important role.

It is generally accepted that HSP70 can confer protection against critical ischaemia.^{4,28,29} It would be important to know whether the ischaemic skeletal muscle can also be preconditioned by expressing HSP70 in PAODIV. It is not clear whether the failure to produce higher HSP70 in PAOD IV might be responsible for the irreversible tissue damage. Further studies are required.

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