Virchows Arch (1995) 427:159–165
© Springer-Verlag 1995

ORIGINAL ARTICLE

Anke Huckriede · Astrid Heikema · Klaas Sjollema
Paz Briones · Etienne Agsteribbe

Morphology of the mitochondria in heat shock protein 60 deficient fibroblasts from mitochondrial myopathy patients. Effects of stress conditions

Received: 6 April 1995 / Accepted: 18 July 1995

Abstract We have described two mitochondrial (mt) myopathy patients with reduced activities of various mt enzymes associated with significantly decreased amounts of heat shock protein 60 (hsp60). Experimental evidence suggested that the lack of hsp60 was the primary defect. Since hsp60 is essential for the proper folding of enzyme subunits in the mt matrix a partial deficiency of this protein can explain the observed defects of the mitochondria. Here we report on morphological studies aimed at obtaining more insight into the relation between lack of hsp60 and pathological changes of the mitochondria. Under standard culture conditions mitochondria in the partially hsp60 deficient fibroblasts showed profound morphological aberrations. In contrast, the mitochondria in fibroblasts from a MELAS patient and a cytochrome *c* oxidase-deficient patient appeared normal. Under stress conditions the integrity of the hsp60 deficient mitochondria declined even further: heat shock induced a temporary collapse of the electrochemical potential across the inner mt membrane, but did not affect the ultrastructure of the mitochondria; prolonged growth in confluent cultures resulted in decrease in mt number. The altered mt morphology in the hsp60 deficient cells is probably indicative of the severely impaired mt metabolism whereas the decreased stress tolerance is likely to be a direct result of paucity of the heat shock protein. Both variables are potentially useful in the diagnosis and molecular characterization of mt disorders with systemic manifestation and multiple enzyme deficiency.

A. Huckriede (✉) · A. Heikema · E. Agsteribbe
Department of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, The Netherlands

K. Sjollema
Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

P. Briones
Institut de Bioquímica Clínica, Consorci Sanitari, Avda Flor de Maig s/n, E-8290 Cerdanyola, Barcelona, Spain

Key words Mitochondrial disorders · Mitochondrial morphology · Heat shock proteins · Stress conditions

Introduction

Mitochondrial (mt) encephalomyopathies are biochemically characterized by dysfunction of the mt metabolism, particularly of oxidative phosphorylation (OXPHOS). One or more of the OXPHOS enzymes may be affected, and occasionally mt enzymes of other biochemical pathways like pyruvate metabolism and/or the citric acid cycle are also deficient. The mt defects can be restricted to certain organs or tissues, usually skeletal muscle, heart muscle, and brain, or can be systemic. The presenting symptoms are quite variable but usually comprise muscle weakness and lactic acidaemia and frequently mental anomaly [5, 13, 20].

Functional defects of the mitochondria are often accompanied by morphological alterations of the organelles. This phenomenon has been intensively studied in muscle biopsies derived from mt myopathy patients. Electron micrographs show enlarged mitochondria with crystalline or dark amorphous inclusions, lipid vacuoles and disarranged cristae which appear unfolded, densely packed or form concentric rings [for reviews see 12, 18]. Fibroblasts derived from patients with systemic manifestation of a mt disorder may also exhibit morphologically altered mitochondria [17]. As these cells can be grown in culture and are easily accessible to both, biochemical and morphological investigations, they are very suitable to study the interrelation between functional and morphological aberrations in more detail.

We have recently described partial deficiency of heat shock protein 60 (hsp60) in skin fibroblasts from two mt myopathy patients with systemic manifestation of the disease [11]. Biochemical investigation of the fibroblasts revealed reduced activities of various mt enzymes in both cases [1, personal communication P. Briones]. One of the cultures was investigated in detail. The low steady state amount of hsp60 appeared to be caused by de-

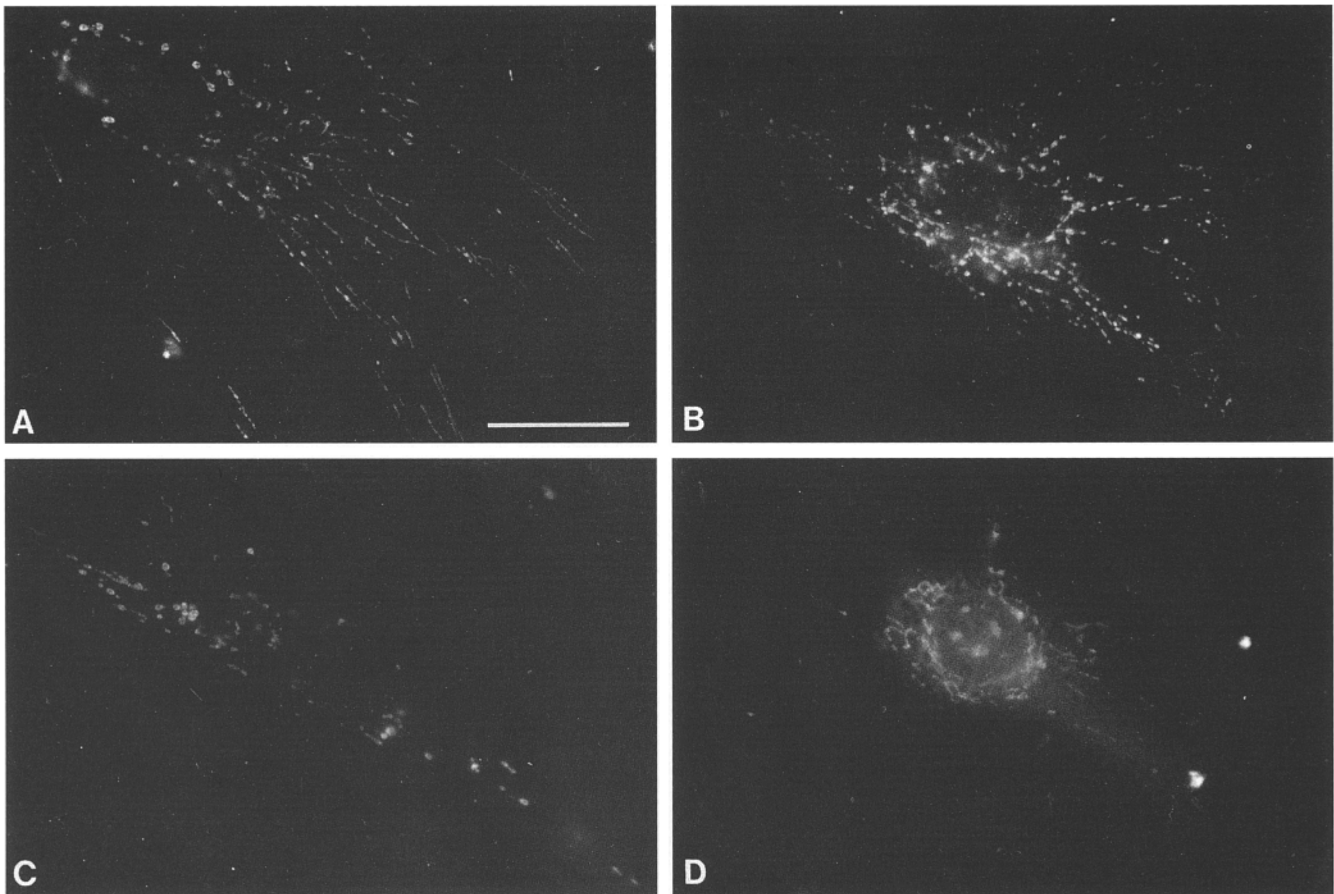


Fig. 1 Immunofluorescence staining of heat shock protein 60 (hsp60) in fibroblasts from a healthy control individual (A), the cytochrome *c* oxidase (COX) deficient patient P2 (B), and the hsp60 deficient patients P1 (C) and P10 (D). Bar=10 μm

creased synthesis of the protein. Moreover, newly translated hsp60 was processed to its mature form at a much slower rate than in control cells [11]. Hsp60 is an essential protein which is involved in the proper folding of enzyme subunits in the mt matrix [2, 15]. In yeast, complete absence of the protein is lethal and partial deficiency leads to accumulation of many proteins as insoluble aggregates [2, 8]. Considering both these data and our own results, we suggested that the insufficient supply of the mitochondria with mature hsp60 results in failure of mt enzyme assembly and is thus the molecular basis of the mt disorder in this patient.

In order to get more insight into the correlation between lack of hsp60 and pathological changes of the mitochondria we studied the mt morphology in the two hsp60 deficient fibroblast cultures in detail. Special emphasis was put on two questions. (1) Do the morphological alterations of hsp60 deficient mitochondria differ from those of defective mitochondria with hsp60-independent pathogenesis? (2) Does shortage of the protecting stress protein hsp60 render the mitochondria more vulnerable to stress conditions? Here we demonstrate the presence of very pronounced changes in the mt morphology in both hsp60 deficient fibroblast cultures. Moreover, the mitochondria in the two cultures appeared to be very sensitive to heat shock or prolonged growth under suboptimal conditions. These results underline that paucity of hsp60 has severe effects on the functional as well as on the structural integrity of mitochondria.

Materials and Methods

Dimethylaminostyryl-1-methyl-pyridinium-iodide (DASPMI) was a generous gift from Prof. J. Bereiter-Hahn. Monoclonal and polyclonal antisera against hsp60 were kindly provided by Dr. R.S. Gupta and have been described elsewhere [7]. Fluorescein isothiocyanate (FITC)-labelled rabbit-anti-mouse or goat-anti-rabbit antisera were obtained from Dako (Glostrup, Denmark).

Fibroblasts were derived from skin biopsies and cultured in Ham's F10 supplemented with 10% fetal calf serum in a 5% carbon dioxide-incubator. For DASPMI-staining or immunofluorescence studies the cells were seeded on glass coverslips in 35 mm Petri dishes.

In vivo staining of mitochondria with DASPMI was performed as described previously [17].

For immunofluorescence cells grown on coverslips were washed once with PBS, fixed in −20° C methanol for 5 min, and washed in PBS again. Incubation with 20 μl of a 1:100 dilution of the primary antibody (monoclonal or polyclonal anti- hsp60) was performed for 45 min at 37° C in a humid atmosphere. The coverslips were washed three times with PBS and incubated with 20 μl of a 1:80 dilution of the appropriate FITC-labelled secondary antibody for another 45 min at 37° C. After extensive washing in PBS coverslips were mounted with fluoromount (Southern Biotechnology Association, Birmingham, USA) containing *n*-propyl gallate to reduce photobleaching [6]. Specimens were investigated with a Leitz Orthoplan microscope equipped with epifluorescence. Pictures were taken on Ilford HP5 film.

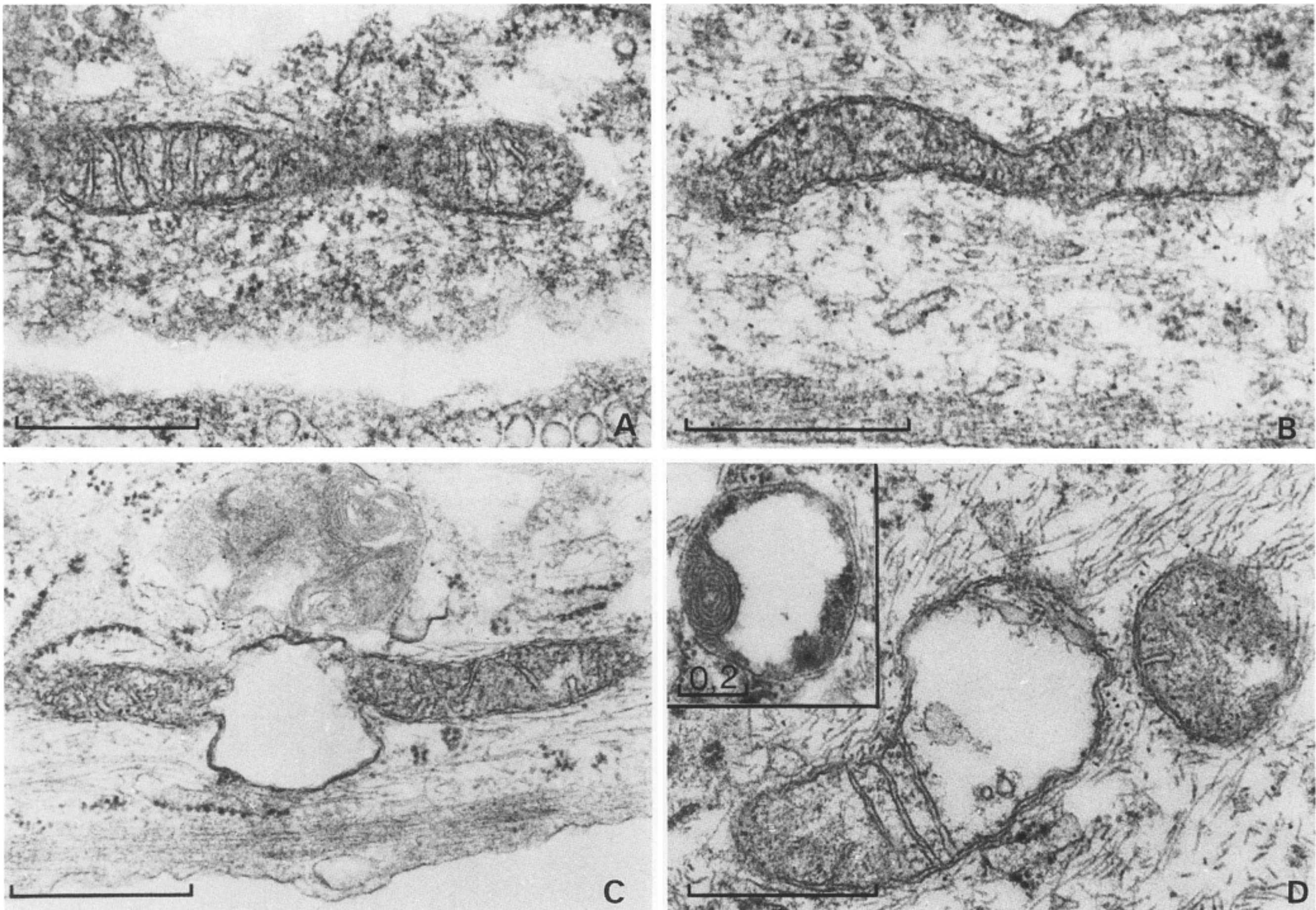


Fig. 2 Electron micrographs of a mitochondrion in the healthy control fibroblasts (A), the COX deficient fibroblasts of patient P2 (B), and the hsp60 deficient fibroblasts of patient P1 (C) and P10 (D), respectively. Bar=0.5 μm, insert 0.2 μm

Fixation, contrast enhancement and embedding of the cells for examination by electronmicroscopy were performed as described previously [1].

To examine the effects of heat shock, fibroblasts were grown on coverslips in 35 mm Petri dishes. For the shock the Petri dishes were sealed with parafilm and placed for 15 min into a water bath which had been prewarmed to 45° C. Covering with a glass plate prevented floating of the dishes. After heat shock the dishes were placed back into the incubator and DASPMI staining was started either directly or at the time points indicated.

Results

In order to elucidate whether hsp60 deficient mitochondria exhibit unique morphological features we investigated the mt morphology in the two partially hsp60 deficient fibroblast cultures (P1 and P10 from [11]), in fibroblasts from two other mt myopathy patients and in fibroblasts from a healthy individual. The 'control' patient fibroblasts with hsp60-independent pathogenesis were derived from a patient with cytochrome *c* oxidase (COX) deficiency as the only biochemical defect (P2 from [11]) and a patient with the MELAS mutation (A to G transition at nt3243 in the mt $tRNA^{Leu(UUR)}$; P8 from [11]), respectively.

Upon immunofluorescence staining of hsp60 the mitochondria in healthy control fibroblasts appear as small beads which are arranged in strings spreading from the perinuclear area to the cell periphery (Fig. 1A). A similar pattern is observed in the COX deficient fibroblasts from patient P2 (Fig. 1B) and in the cells from the MELAS patient P8 (not shown). In the hsp60 deficient fibroblasts from the patients P1 and P10 the total number of mitochondria is decreased and many of the mitochondria have an abnormal appearance. Some are much larger than in the control cells and have a vesicle-like structure with the fluorescence being restricted to the outer layer and the inner matrix seeming empty (Fig. 1C). Others appear like short strings or have formed larger aggregates (Fig. 1D). In the culture derived from patient P1 the proportion of aberrant mitochondria is very high and only few 'normal' mitochondria are left (Fig. 1C). The mitochondria of patient P2 are more heterogenous. In many cells normal and aberrant organelles coexist (Fig. 1D). Other cells contain exclusively aberrant or exclusively normal mitochondria. Often the mitochondria are clustered in the vicinity of the nucleus (Fig. 1D). This abnormal distribution is not due to alterations of the cytoskeleton as the microtubules as well as the microfilaments are perfectly normal in these cells (unpublished observations).

On electron micrographs the mitochondria of the healthy control cells appear as rod-like structures with prominent cristae formed by the extensively folded inner

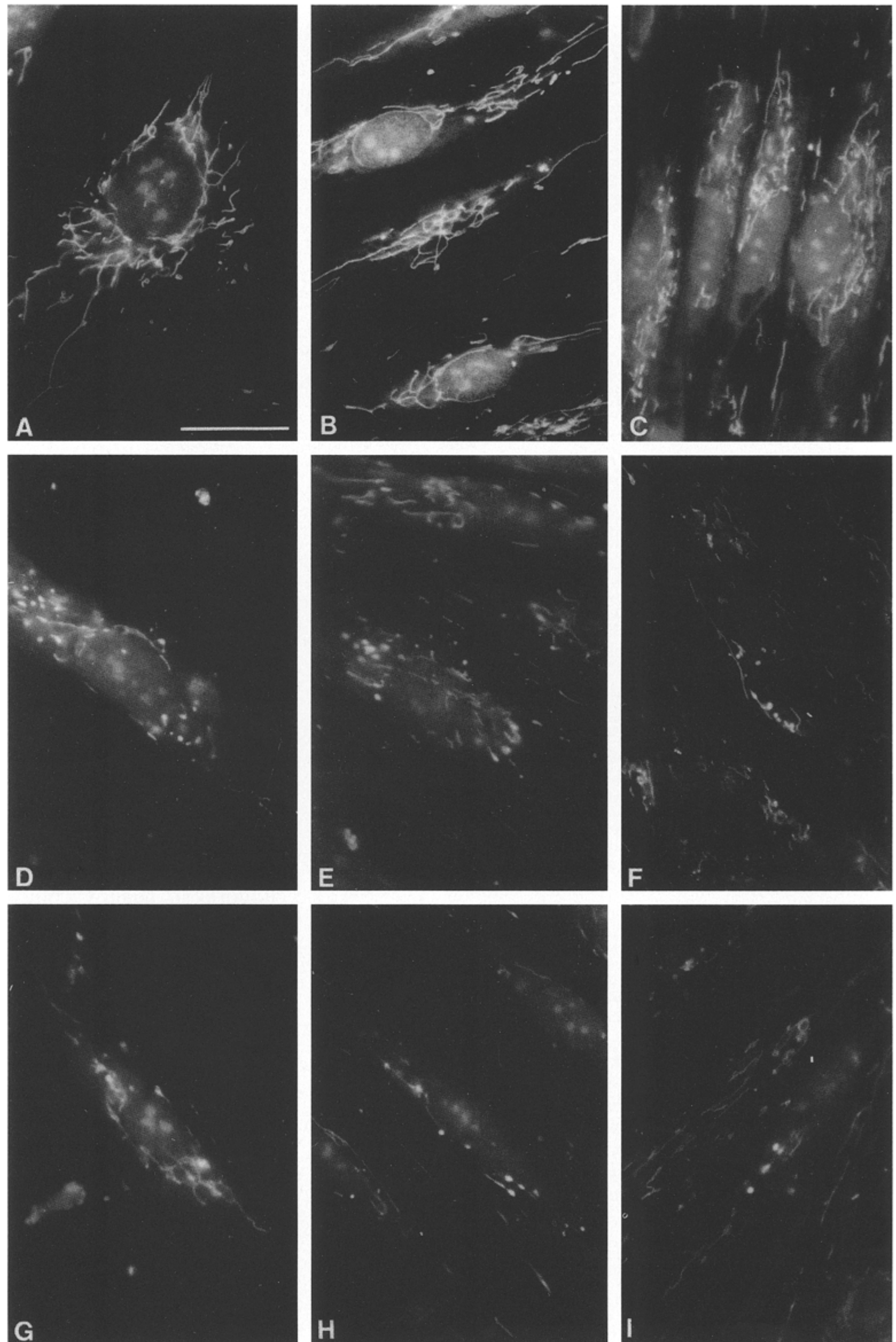
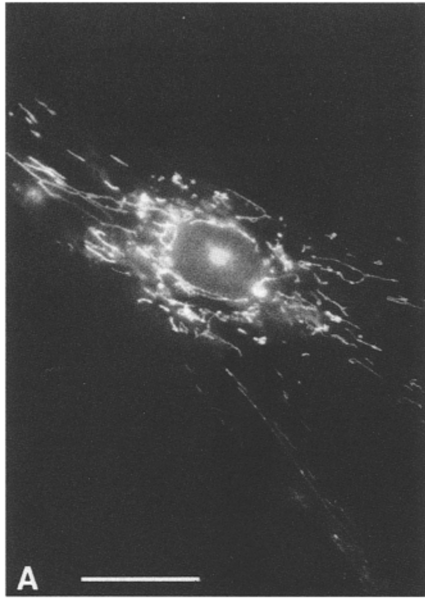
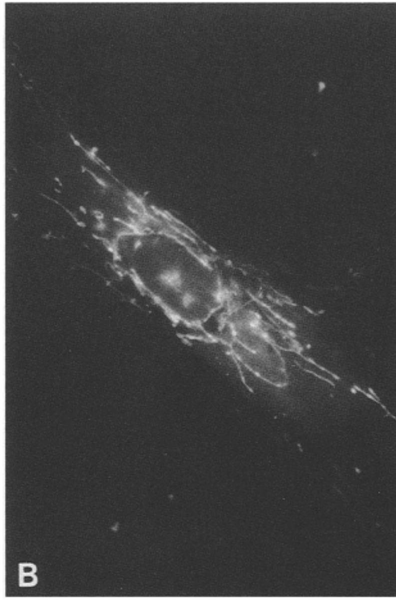


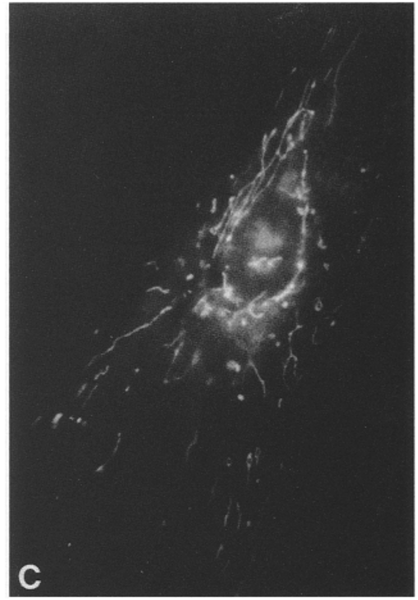
Fig. 3 Dimethylaminostyryl-1-methyl-pyridinium iodide (DASPMI) staining of the mitochondria in sparse cultures (A, D, G), cultures which just reached confluence (B, E, H), and cultures which had been confluent for two weeks (C, F, I). Mitochondria in healthy control cultures (A, B, C) do not change their morphology. Mitochondria in the hsp60 deficient fibroblasts of the patients P1 (D, E, F) and P10 (G, H, I) become more bead-like and decrease in number. Residual thread-like mitochondria are stained only faintly. Bar=10 μm

membrane (Fig. 2A). The mitochondria of the COX deficient cells have the same overall appearance. However, their matrix is more electron-dense so that the cristae are less clearly visible. In contrast, in the hsp60 deficient fibroblast cultures of the patients P1 and P10 the cristae are highly disarranged (Fig. 2C, D). Parts of the mitochondria are completely devoid of folded inner membrane and other electron-dense material. These empty

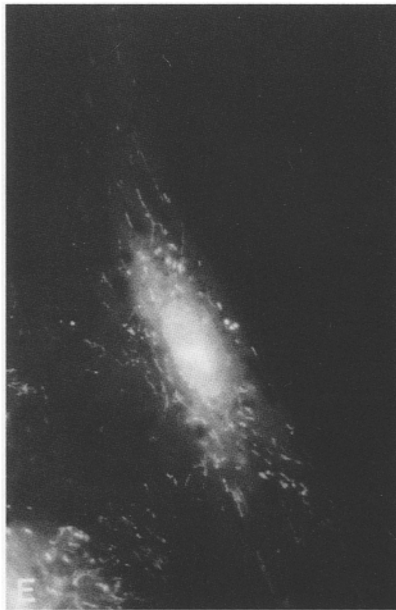
Fig. 4 DASPMI staining of the mitochondria before a 15 min heat shock at 45° C (A, D, G), right after the heat shock (B, E, H), and 1 h after heat shock (C, F, I). Mitochondria of healthy control cells (A, B, C) as well as of the MELAS patient P8 (D, E, F) show no alterations upon heat shock. Mitochondria of the hsp60 deficient fibroblasts (G, H, I) do not incorporate the dye right after heat shock (H) but have recovered partly 1 h later (I). Bar=10 μm

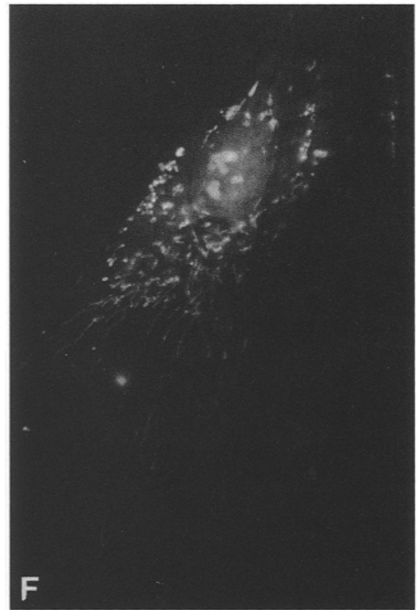

A

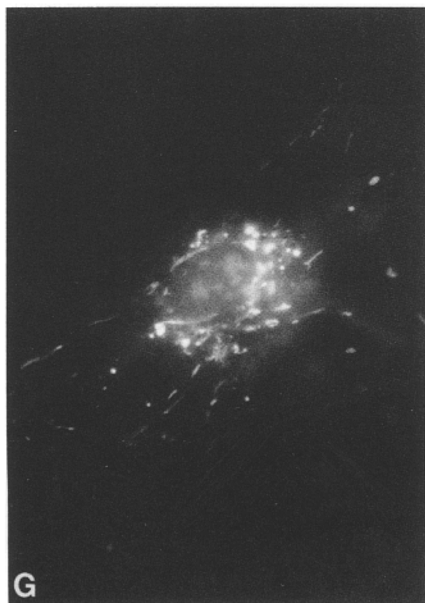

B

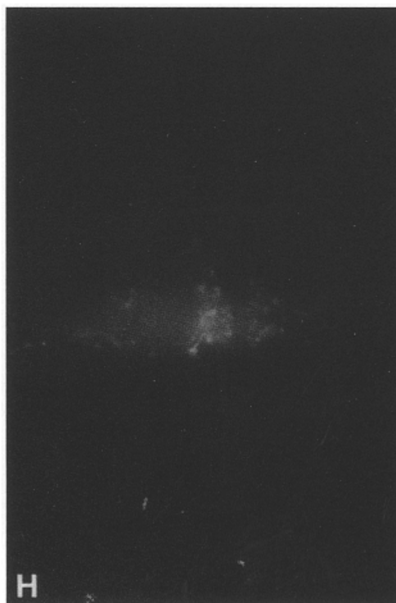

C

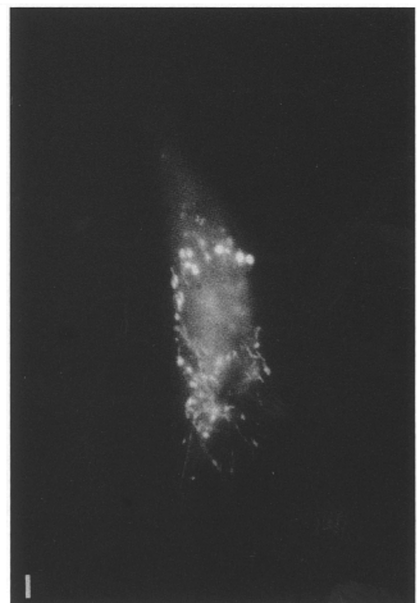
D


E


F


G


H


I

parts are sometimes swollen to vesicle-like structures. In some mitochondria the collapsed inner membrane forms concentric rings (see insert Fig. 2D).

The consequences of hsp60 shortage in stress conditions were tested by subjecting the cells to prolonged confluence or heat shock, respectively. In these experiments the mitochondria were stained in living cells using the fluorescent dye DASPMI. Uptake of this dye by the mitochondria is dependent on the presence of an electrochemical potential across the inner mt membrane which is mainly generated as a proton gradient by the enzymes of the respiratory chain [3]. Thus DASPMI is not only an approach to study the morphology of the mitochondria but is also a tool to analyse their functional integrity: only organelles with an intact inner membrane can take up the dye and become stained.

In healthy fibroblasts DASPMI-stained mitochondria appear as long thread-like structures (Fig. 3A). In the partially hsp60 deficient cells some mitochondria are also thread-like but usually shorter than in the control cells. Many others show up as brightly stained vesicle-like structures as described earlier ([1] and Fig. 3D and G). Upon prolonged growth and concomitant confluence of the cultures, in the healthy control cells the number and morphology of the mitochondria were unaffected (Fig. 3A–C). In contrast, in both hsp60 deficient cultures the number of mitochondria per cell decreased and the proportion of cells with mainly large vesicle-like mitochondria increased significantly. The residual thread-like mitochondria were stained only very faintly (Fig. 3D–F and G–I).

In a second set of stress experiments the cells were subjected to a 15 min heat shock at 45° C. The integrity of the mitochondria was studied either directly after the heat shock or after recovery periods of 1 h and 24 h, respectively. In healthy control fibroblasts (Fig. 4A–C), in the fibroblasts of the MELAS patient P8 (Fig. 4D–F) and in the COX deficient fibroblasts of patient P2 (not shown) the mitochondria tolerated the heat shock very well and appeared normal even when stained right after the treatment. In contrast, in the hsp60 deficient cells the mitochondria were unable to take up the dye right after heat treatment (Fig. 4H). After 1 h the mitochondria could incorporate the dye again (Fig. 4I), but only after 24 h was recovery complete (not shown). As was revealed by electronmicroscopical analysis the ultrastructure of the mitochondria was not affected by heat shock (results not shown). No major changes in morphology of the mitochondria were observed when fibroblasts from healthy controls, the COX deficient or the MELAS patient were subjected to heat shock. Neither did heat shock induce additional distortion of the cristae array in the hsp60 deficient fibroblasts.

Discussion

The first aim of our study was to find out whether partial hsp60 deficiency is associated with unique morphological alterations of the mitochondria. The two hsp60 deficient fibroblast cultures (from patients P1 and P10, respectively) were characterized by uneven distribution of mitochondria and extremely aberrant mt morphology and ultrastructure. The changes observed comprised swelling of the mitochondria, disarrangement or complete absence of cristae and loss of electron-dense material from the mt matrix. In contrast, in fibroblasts derived from a patient with COX deficiency (P2) or a MELAS patient (P8) we detected only minor aberrations of mt morphology [this study and 17].

The morphological changes of the mitochondria in the hsp60 deficient fibroblasts resemble those described for mitochondria in muscle biopsies taken from mt myopathy patients and in myoblasts depleted of mt DNA [10, 12, 18]. Unfortunately, we could not determine whether similar mt aberrations were also present in muscle tissue of the hsp60 deficient patients, since both patients had died before our investigations were started and muscle specimens were not available. Comparison of mt morphology in fibroblasts and muscle tissue of the same patient would be very important as poor condition of mitochondria in fibroblasts might be indicative of highly aberrant mitochondria in muscle tissue.

As far as patient fibroblasts are concerned we are not aware of any literature reports except those of our own group about morphologically abnormal mitochondria. Possibly, mt morphology is not studied routinely in patient fibroblasts although these cells are frequently used for enzymatic analysis [16, 19, 21]. Alternatively, morphological changes of the mitochondria may be less frequent or less spectacular in cultured patient fibroblasts as compared to muscle cells in situ. Indeed we found profoundly altered mitochondria only in the hsp60 deficient fibroblasts with multiple enzyme deficiencies but not in the COX deficient or MELAS fibroblasts.We therefore assume that the presence of morphologically aberrant mitochondria is indicative of particularly severe impairment of mt metabolism as caused by deficiency of hsp60. This assumption is supported by results obtained by Hayashi and co-workers who studied the morphology of mitochondria carrying point mutations in mt tRNAs [9]. They found that mt morphology was altered only in cells with more than 90% mutated mtDNA and that the morphological changes were always correlated with severe metabolic defects of the organelles.

The second object of our study was to test whether the lack of hsp60 renders the cells more vulnerable to stress. In yeast it has been shown that hsp60 is not only involved in protein folding but is also essential to prevent protein denaturation in stress conditions [14]. Two approaches were used to address the problem of mt integrity under stress: firstly, prolonged growth of the cells when confluence of the cultures had been reached and secondly, exposure of the cells to heat shock. In the hsp60 deficient cells the suboptimal growth conditions as encountered in confluent cultures were accompanied by a decrease in the number of mitochondria which could be stained with DASPMI. The proportion of cells with mainly abnormally shaped mitochondria also increased

significantly. Residual 'normal' mitochondria showed only weak fluorescence indicating a decline of the membrane potential. Interestingly, heat exposure of the cells revealed that hsp60 deficient mitochondria were not capable of accumulating DASPMI directly after heat shock. Apparently, the membrane potential across the mt membrane which is essential for uptake of the dye had collapsed due to instability of the inner membrane. This loss of inner membrane function was not accompanied by further changes in ultrastructure as revealed by electronmicroscopic investigation. Moreover, the functional deterioration of the inner membrane was transient since after a recovery period of about 1 h a membrane potential was restored and the mitochondria could be stained again. In both stress experiments control cells behaved differently. Fibroblasts from healthy control individuals showed under conditions of confluence neither loss of mitochondria nor the formation of abnormally shaped organelles. Also, right after heat shock the mitochondria of the control fibroblasts as well as those of fibroblasts from the COX deficient patient and the MELAS patient retained the ability to accumulate DASPMI. Moreover, the mitochondria of these cells did not swell or fuse to large round structures nor did they redistribute to the perinuclear area as described for mitochondria of heat shocked chicken embryo fibroblasts [4]. Also their ultrastructure was unaffected by heat shock.

In summary, the mitochondria in the fibroblast cultures with partial deficiency of hsp60 showed significant morphological alterations which probably reflect the severely altered mt metabolism of these cells. The stress tolerance of the cells was markedly decreased which is possibly a direct result of shortage of the protecting stress protein hsp60. As morphology and stress tolerance of the hsp60 deficient mitochondria differed significantly from mitochondria with other defects these features may be helpful in the diagnosis and characterization of mt disorders of unknown aetiology. Moreover, the low stress tolerance of the fibroblasts in vitro may implicate increased vulnerability of affected patients to stress in form of strenuous exercise, infection or fever. Future studies will concentrate on the effects of stress conditions on mt functions like the activities of various mt enzymes.

Acknowledgements We thank Dr. O. Skjeldal, University of Oslo, Norway for patient fibroblasts and Dr. R. Gupta, University of Hamilton, Canada for antibodies against hsp60. Dr. M. Veenhuis, University of Groningen, The Netherlands is acknowledged for help with the electronmicroscopical investigations and critical reading of the manuscript. This work was supported by the Prinses Beatrix Fonds, The Netherlands.

References

1. Agsteribbe E, Huckriede A, Veenhuis M, Ruiters MHJ, Niezen-Koning KE, Skjeldal OH, Skullerud K, Gupta RS, Hallberg R, Diggelen OP van, Scholte HR (1993) A fatal, systemic mitochondrial disease with decreased mitochondrial enzyme activities, abnormal ultrastructure of the mitochondria and deficiency of heat shock protein 60. Biochem Biophys Res Comm 193:146–154
2. Baker KP, Schatz G (1991) Mitochondrial proteins essential for viability mediate protein import into yeast mitochondria. Nature 349:205–208
3. Bereiter-Hahn J (1976) Dimethylaminostyrylmethylpyridiniumiodine (DASPMI) as a fluorescent probe for mitochondria in situ. Biochim Biophys Acta 423:1–14
4. Collier NC, Sheetz MP, Schlesinger MJ (1993) Concomitant changes in mitochondria and intermediate filaments during heat shock and recovery of chicken embryo fibroblasts. J Cell Biochem 52:297–307
5. DiMauro S, Bonilla E, Zeviani M, Servidei S, DeVivo DC, Schon EA (1987) Mitochondrial myopathies. J Inher Metab Dis 10:113–128
6. Giloh H, Sedat JW (1982) Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by *n*-propyl gallate. Science 217:1252–1255
7. Gupta RS, Austin RC (1987) Mitochondrial matrix localization of a protein altered in mutants resistant to the microtubule inhibitor podophyllotoxin. Eur J Cell Biol 45:170–176
8. Hallberg EM, Shu Y, Hallberg RL (1993) Loss of mitochondrial hsp60 function: nonequivalent effects on matrix-targeted and intermembrane-targeted proteins. Mol Cell Biol 13: 3050–3057
9. Hayashi J-I, Ohta S, Kagawa Y, Takai D, Miyabayashi S, Tada K, Fukushima H, Inui K, Okada S, Goto Y, Nonaka I (1994) Functional and morphological abnormalities of mitochondria in human cells containing mitochondrial DNA with pathogenic point mutations in tRNA genes. J Biol Chem 269:19060–19066
10. Herzberg NH, Middelkoop E, Adorf M, Dekker HL, Van Galen MJM, Van den Berg M, Bolhuis PA, Van den Bogert C (1993) Mitochondria in cultured human muscle cells depleted of mitochondrial DNA. Eur J Cell Biol 61:400–408
11. Huckriede A, Agsteribbe E (1994) Decreased synthesis and inefficient mitochondrial import of hsp60 in a patient with a mitochondrial encephalomyopathy. Biochim Biophys Acta 1227: 200–206
12. Lindal S, Lund I (1992) Mitochondrial diseases and myopathies: a series of muscle biopsy specimens with ultrastructural changes in the mitochondria. Ultrastruct Pathol 16:263–275
13. Lombes A, Bonilla E, DiMauro S (1989) Mitochondrial encephalomyopathies. Rev Neurol 145:671–689
14. Martin J, Horwich AL, Hartl F-U (1992) Prevention of protein denaturation under heat stress by the chaperonin hsp60. Science 258:995–998
15. Ostermann J, Horwich AL, Neupert W, Hartl F-U (1989) Protein folding in mitochondria requires complex formation with hsp60 and ATP hydrolysis. Nature 341:125–130
16. Robinson BH, Chow W, Petrova-Benedict R, Clarke JTR, Van Allen MI, Becker LE, Boulton JE, Ragan I (1992) Fatal combined defects in mitochondrial multienzyme complexes in two siblings. Eur J Pediatr 151:347–352
17. Ruiters MHJ, Van Spronsen EA, Skjeldal OH, Stromme P, Scholte HR, Pzyrembel H, Smit GPA, Ruitenbeek W, Agsteribbe E (1991) Confocal scanning laser microscopy of mitochondria: a possible tool in the diagnosis of mitochondrial disorders. J Inher Metab Dis 14:45–48
18. Stadhouders AM, Sengers RC (1987) Morphological observations in skeletal muscle from patients with a mitochondrial myopathy. J Inher Metab Dis 10 [suppl. 1]:62–80
19. Wanders RJA, Ruiter JPN, Wijburg FA (1993) Studies on mitochondrial oxidative phosphorylation in permeabilized human skin fibroblasts: application to mitochondrial encephalomyopathies. Biochim Biophys Acta 1181:219–222
20. Wijburg FA (1993) Mitochondrial encephalomyopathies: diagnostic and therapeutic studies. Thesis, University of Amsterdam, The Netherlands
21. Wijburg FA, Feller N, Ruitenbeek W, Trijbels JMF, Sengers RCA, Scholte HR, Przyrembel H, Wanders RJA (1990) Detection of respiratory chain dysfunction by measuring lactate and pyruvate production in cultured fibroblasts. J Inher Metab Dis 13:355–358