

Mitochondrial DNA variability modulates mRNA and intra-mitochondrial protein levels of HSP60 and HSP75: experimental evidence from cybrid lines

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Abstract To explore possible relationships between mitochondrial DNA (mtDNA) polymorphism and the expression levels of stress-responder nuclear genes we assembled five cybrid cell lines by repopulating 143B.TK⁻ cells, depleted of their own mtDNA (Rho⁰ cells), with foreign mitochondria with different mtDNA sequences (lines H, J, T, U, X). We evaluated, at both basal and under heat stress conditions, gene expression (mRNA) and intra-mitochondrial protein levels of HSP60 and HSP75, two key components in cellular stress response. At basal conditions, the levels of HSP60 and HSP75 mRNA were lower in one cybrid (H) than in the others ($p=0.005$ and $p=0.001$, respectively). Under stress conditions, the H line over-expressed both genes, so that the inter-cybrid difference was abolished. Moreover, the HSP60 intra-mitochondrial protein levels differed among the cybrid lines ($p=0.001$), with levels higher in H than in the other cybrid lines. On the whole, our results provide further experimental evidence that mtDNA variability influences the cell response to stressful conditions by modulating components involved in this response. Sentence summary of the article: the results reported in the present study provide important experimental evidence that in human cells mtDNA variability is able to influence the cellular response to heat stress by modulating both the transcription of genes involved in this response and their intra-mitochondrial protein levels.

Keywords Cybrid cell lines · HSP60 · HSP75 · mtDNA variability · Stress response

Abbreviations

ROS reactive oxygen species
RFLP restriction fragment length polymorphism
RT-PCR reverse-transcriptase-polymerase chain reaction

Introduction

Recent data indicate that polymorphisms of the mitochondrial genome (mtDNA) are not neutral and evidence of an association between mtDNA variability and complex traits is increasing (Wallace 2005; Crispim et al. 2006; Bai et al. 2007). Furthermore, numerous evidences suggest that the different mtDNA lineages are qualitatively different from each other, bearing mutations that can modulate mitochondrial function and consequently influence complex phenotypes (Mishmar et al. 2003). This modulation is carried out either directly by influencing energy production efficiency (Baudouin et al. 2005), or indirectly by interaction with nuclear genes (Ryan and Hoogenraad 2007). In humans, the involvement of nuclear-mitochondrial interactions in modulating complex phenotypes is supported by the observation of non-random associations between mtDNA and nuclear variability (De Benedictis et al. 2000; Carrieri et al. 2001; Maruszak et al. 2008). In mice, by means of conplastic strains expressing different combinations of mitochondrial/nuclear genomes, it has been unequivocally demonstrated that mtDNA variability affects complex phenotypes, such as hearing loss (Johnson et al. 2001), cognitive function (Robertoux et al. 2003) and risk of type 2 diabetes (Pravenec et al. 2007). Finally, in vitro transmitochondrial hybrids have shown that nuclear-mitochondrial interactions

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may modulate nuclear gene expression (Jahangir Tafrechi et al. 2005), mitochondrial reactive oxygen species (ROS) production (Vives-Bauza 2006) and intracellular calcium dynamics (Kazuno et al. 2008). Recently, we have provided experimental evidence of such interactions by analyzing human cybrid cell lines that share the same nuclear genome but have different mtDNA (Bellizzi et al. 2006). In these cells, mtDNA variability is associated with expression levels of genes encoding cytokines and cytokine-receptors. In particular, the existence of mitochondrial-specific effects on the expression of interleukin-1 β (IL-1 β), interleukin 6 (IL-6) and tumor necrosis factor receptor 2 (TNFR2) genes has been observed at both basal and oxidative stress conditions.

Considering these observations, the aim of the present study was to investigate whether the modulation of stress-responder nuclear genes by mtDNA variability is a general phenomenon concerning not only cytokines and oxidative stress, but also other stress-responder systems, such as heat shock proteins (HSPs) and heat stress.

To this purpose, we developed cybrid cell lines by repopulating osteosarcoma Rho⁰ cells with foreign mitochondria having different mtDNA sequences. In these cells we analyzed the expression of two heat shock protein genes, HSP60 and HSP75, at both basal and heat stress conditions. HSP60 (Cpn60) and HSP75 (TRAP1) are mitochondrial chaperones that assist, in both stressed and non-stressed cells, in the folding, unfolding, or disaggregating of proteins either imported from the cytosol or synthesized within mitochondria (HSP60) (Cheng et al. 1989; Frydman 2001; Itoh et al. 2002; Saibil 2008) and in the reallocation of cytosolic protein into mitochondria (HSP75) (Felts et al. 2000; Mokranjac and Neupert 2005). In addition to its chaperone activity, HSP60 has well-documented anti- or pro-apoptotic roles (Arya et al. 2007; Chandra et al. 2007) as well as immunoregulatory properties (Habich and Burkart 2007; Pockley et al. 2008). HSP75 acts as an antagonist of ROS and exhibits anti-apoptotic activity (Hua et al. 2007; Pridgeon et al. 2007).

The results reported here show that the levels of HSP60 and HSP75 mRNAs, and the intra-mitochondrial protein level of HSP60, are correlated to mtDNA variability thereby providing additional evidence for the role played by such variability in the stress response.

Materials and methods

Cell lines and culture conditions

143B.TK⁻ osteosarcoma cells and cybrid cell lines were grown in Dulbecco's modified eagle medium (DMEM,

Invitrogen) containing 25 mM glucose and 1 mM sodium pyruvate, supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 0.1 mM gentamycin (Invitrogen). Rho⁰ cells were grown in the above medium supplemented with 0.2 mM uridine (Sigma). 143B.TK⁻, cybrid cell lines, and Rho⁰ cells were cultured in a water-humidified incubator at 37°C in 5% CO₂/95% air.

Heat stress treatment

143B.TK⁻, Rho⁰, and cybrid cells, 1 \times 10⁵, were seeded in 24 well/plates. In the exponential growth phase, the cells were incubated at 42°C for 2, 4 and 6 h. Untreated cells were also used as control.

Cell viability assay

Treated and untreated cell lines were assayed for viability by Trypan blue exclusion assay. Floating and adherent cells were collected and 200 μ l of cellular suspension were added to an equal volume of 0.4% Trypan Blue solution (Sigma). Then, viable and non-viable cells were counted on a hemocytometer with an inverted light microscope using a \times 20 magnification.

RT-PCR of HSP genes

Total RNA was extracted from control and heat-treated cells with RNeasy kit (Qiagen). The reverse-transcriptase-polymerase chain reactions (RT-PCR) were carried out by using the ImPromII Kit (Promega). An RT mix including 500 ng of total RNA and 0.5 μ g of oligo-dT primers was pre-heated at 70°C for 5 min. The reaction was carried out in a 40 μ l final volume containing 1X RT buffer, 0.5 mM of each dNTP, 3 mM MgCl₂, 20 U RNase inhibitor, and 5 U reverse transcriptase. The mix was incubated at 25°C for 5 min, then 37°C for 1 h and, successively, at 95°C for 10 min to inactivate the reverse transcriptase.

The primers used in gene expression analyses were the following:

| | | |
|-------|----------------|----------------------------|
| HSP72 | Forward primer | 5' AAGTTGCAATGAACCCACC 3' |
| | Reverse primer | 5' TTGCGCTTAAACTCAGCAA 3' |
| HSP60 | Forward primer | 5' ATCCAGCAATGACCATTGC 3' |
| | Reverse primer | 5' GAGTTAGAACATGCCACCTC 3' |
| HSP75 | Forward primer | 5' TGGCAGTTATGGAAGGTA 3' |
| | Reverse primer | 5' AGCAATGACTTTGTCTTCTG 3' |
| GAPDH | Forward primer | 5' GACAACCTTGGTATCGTGGA 3' |
| | Reverse primer | 5' TACCAGGAAATGAGCTTGAC 3' |

The PCR mixture (30 μ l) contained 1.5 μ l of cDNA, 1X buffer RB, 0.5 mM of each dNTP, 3.5 mM $MgCl_2$, 0.6 μ M of each primer, and 10 U DNA polymerase (EuroTaq). After an initial denaturation step at 94°C for 1 min, the PCR was carried out for 25 cycles at 92°C for 1 min, followed by 56°C for 1 min and 72°C for 1 min. The final step was an incubation at 72°C for 10 min. Then, PCR products were analyzed on 2.5% agarose gel containing 0.5 mg/ml ethidium bromide. Fluorescence intensity of each band was calculated using densitometric analyses (Kodak Electrophoresis Documentation and Analysis System 290, EDAS 290) and then normalized to glyceraldehyde phosphate dehydrogenase gene (GAPDH) band intensity.

Isolation of mitochondrial protein fractions

Mitochondrial extracts were prepared by using Mitochondrial Fractionation Kit (Active Motif). 2×10^7 heat-treated and untreated cells were scraped on ice after the addition of 10 ml of ice-cold 1X phosphate-buffered saline (PBS) and then centrifuged at $600 \times g$ for 5 min at 4°C. Cell pellets were re-suspended in 5 ml of ice-cold PBS and centrifuged at $600 \times g$ for 5 min at 4°C. Then, cell pellets were resuspended in 250 μ l of 1X cytosolic buffer included in the kit and then incubated on ice for 15 min. Successively, cell pellets were homogenized with a homogenizer and the resulting supernatant was centrifuged at $800 \times g$ for 20 min at 4°C. Then, the supernatant, containing the cytosol and the mitochondria, was removed and centrifuged a second time at $800 \times g$ for 10 min at 4°C. Then, the supernatant was newly removed and centrifuged at $10,000 \times g$ for 20 min at 4°C to pellet the mitochondria. Mitochondrial pellets were washed with 100 μ l of 1X cytosolic buffer and then centrifuged at $10,000 \times g$ for 10 min at 4°C. Finally, mitochondrial pellets were lysed by adding 35 μ l of complete mitochondria buffer, prepared by adding mitochondria buffer, protease inhibitor cocktail, and dithiothreitol included in the kit, and incubating on ice for 15 min.

Western blot analyses

Fifteen microgram of mitochondrial extracts were separated on 10% SDS-PAGE and transferred into Hibond-P membranes at 60 V for 1 h at 4°C. Membranes were washed with TBST 1X (0.3 mM Tris-HCl, pH 7.5, 2.5 mM NaCl, 0.05% Tween 20) for 10 min and then incubated overnight at room temperature with 5% non-fat dried milk in TBST 1X. Then blots were washed three times with TBST 1X for 10 min and incubated in TBST containing 1% milk with anti-HSP60 polyclonal mouse antibodies (1:1,000) (Stressgen) and anti-HSP75 polyclonal rabbit antibodies (1:50) (SantaCruz). Then, anti-mouse (1:5,000) or anti-rabbit

(1:2,000) antibodies conjugated with horseradish peroxidase (HRP, Amersham) were used as secondary antibodies. Immunoreactivity was determined by means of the ECL chemiluminescence reaction (Amersham). Anti-CoxIV antibody (1:500, Molecular Probes) was used as an internal control of the mitochondrial fraction. Quantitative evaluation of the blots was carried out by using densitometric analyses of immunoreactive band intensities (Kodak Electrophoresis Documentation and Analysis System 290, EDAS 290) and then normalized to the internal control.

Statistical analyses

Statistical analyses were performed using SPSS 15.0 statistical software. We adopted one-way analysis of variance for multiple comparisons and student *t*-test for pair-wise comparisons. Significance level was defined as $\alpha=0.05$.

Results

Cell lines

We analyzed the 143B.TK⁻ native cell line, its derivative Rho⁰ line, and the H and J cybrid cell lines which we have previously described (Bellizzi et al. 2006). In addition, we produced ex-novo three cybrid cell lines by fusing Rho⁰ cells with platelets isolated from young donors. According to the variability at diagnostic positions (RFLP analyses), mtDNAs of the donor platelets were classified as belonging to U, X, and T haplogroups (Torroni et al. 1996). Therefore, we named the newly produced cybrid cell lines according to the name of the respective mtDNA haplogroup. By analyzing the *AvaII*8249 polymorphic site, we found that the mtDNA of the X cybrid line and that of the 143B.TK⁻ native line, although both belonging to the X haplogroup, were of different haplotype (Table S1 in Supplementary Material).

Then, we assessed the cellular state of the newly produced U, X, and T cybrid lines by carrying out control experiments (proliferation assays, quantification of mtDNA, and mitochondrial membrane potential—MMP—assay) as previously described (Bellizzi et al. 2006). Proliferation rate, number of copies of mtDNA per cell and MMP values of U, X, and T cybrid lines did not differ significantly from those of the native cell line (data not shown).

Heat stress

In order to determine the optimal conditions necessary to induce heat stress in our cell lines, we treated the seven cell

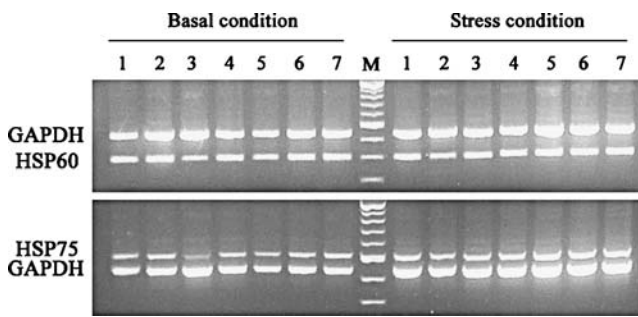


Fig. 1 Representative RT-PCR electrophoresis patterns of HSP60 and HSP75 mRNAs at basal and under stress conditions (42°C for 4 h) in the following cell lines: 143B.TK⁻ (1), Rho⁰ (2), H (3), J (4), U (5), X (6) and T (7). HSP60 heat shock protein 60, HSP75 heat shock protein 75, GAPDH glyceraldehyde phosphate dehydrogenase, M molecular weight 100 bp ladder

lines at 42°C for 2, 4 and 6 h, and then we checked the cellular state of the seven lines by cell viability assay. In all the lines, the treatment did not induce excessive cell death, as the percentage of living cells was 90% about in all the cases. Slight differences in the percentage of living cells between basal and stress condition were observed at 2 h (Fig. S1 in Supplementary Material), probably because of casual cell fluctuations or experimental manipulation. These differences were minimum at 4 h. Then, since HSP60 and HSP75 are reported to be constitutively expressed genes we measured the cell response to heat stress by looking at the expression pattern of the major heat shock-inducible gene, Heat shock protein 72 (HSP72). In Fig. S2 (Supplementary Material) a representative HSP72 gene expression pattern is shown, while Table S2 (Supplementary Material) reports the densitometric analyses of three independent experiments. We observed that the HSP72 gene is significantly up-regulated by heat, and such up-regulation is maximum at 4 h. Therefore, considering cell viability assays and HSP72 gene expression analysis we choose 4 h of heat treatment as the ideal experimental condition.

HSP60 and HSP75 mRNA level analysis

The levels of HSP60 and HSP75 mRNAs were evaluated in the seven cell lines at basal and stress conditions by semi-quantitative RT-PCRs. We ruled out the possibility that PCRs reached the stationary level by assembling saturation curves for each gene including the glyceraldehyde phosphate dehydrogenase gene, used as an internal control (data not shown).

In Fig. 1, a representative RT-PCR electrophoresis pattern of HSP60 and HSP75 gene expression is shown, while Table 1 summarizes the densitometric analyses for three experiments. Considering the fluctuating values of mRNA levels of HSP60 and HSP75 genes in our cell lines, we pointed our attention only to differences of gene expressions where one cell line consistently had a gene expression level at least twofold higher with respect to another cell line.

At basal conditions, comparing 143B.TK⁻ and Rho⁰ cell lines no significant difference was observed for either HSP60 or HSP75 ($p=0.871$ and $p=0.523$, respectively), thus indicating that the expression of the two genes is independent of the presence of active mitochondria. In contrast, under stress conditions HSP60 mRNA levels were significantly lower in Rho⁰ cells than in the native line ($p=0.003$).

As for the cybrid lines, at basal conditions the mRNA levels of both HSP60 and HSP75 differed among the lines ($p=0.005$ and $p=0.001$, respectively), with the H cybrid showing mRNA levels lower than those of the other lines (Table 1). This result was confirmed by RT-PCRs carried out on an independent H clone (data not shown). Since mtDNA is the sole variant among the cybrid lines, we can conclude that a correlation exists between mtDNA variability and mRNA levels of HSP60 or HSP75. Interestingly, under heat stress the mRNA levels of both HSP60 and HSP75 in H cybrid increase up to the levels of the other cybrid lines ($p=0.924$ and $p=0.744$, respectively). This

Table 1 Densitometric analysis of HSP60 and HSP75 mRNA levels, normalized to GAPDH mRNA levels, in 143B.TK⁻, Rho⁰, H, J, U, X and T cell lines at basal and under stress conditions (42°C for 4 h)

| Densitometric analysis | | | | | | | |
|------------------------|----------------------|------------------|---------------|---------------|---------------|---------------|---------------|
| Basal condition | | | | | | | |
| | 143B.TK ⁻ | Rho ⁰ | H | J | U | X | T |
| HSP60 | 0.573 (0.080) | 0.550 (0.110) | 0.245 (0.089) | 0.675 (0.086) | 0.532 (0.056) | 0.640 (0.065) | 0.555 (0.051) |
| HSP75 | 0.465 (0.045) | 0.416 (0.056) | 0.211 (0.042) | 0.342 (0.024) | 0.365 (0.017) | 0.416 (0.048) | 0.461 (0.062) |
| Stress condition | | | | | | | |
| | 143B.TK ⁻ | Rho ⁰ | H | J | U | X | T |
| HSP60 | 0.847 (0.040) | 0.489 (0.062) | 0.734 (0.065) | 0.680 (0.058) | 0.764 (0.039) | 0.751 (0.065) | 0.742 (0.104) |
| HSP75 | 0.369 (0.041) | 0.272 (0.050) | 0.400 (0.032) | 0.328 (0.057) | 0.413 (0.050) | 0.386 (0.050) | 0.352 (0.057) |

Average values over three experiments are reported with standard error mean in parentheses

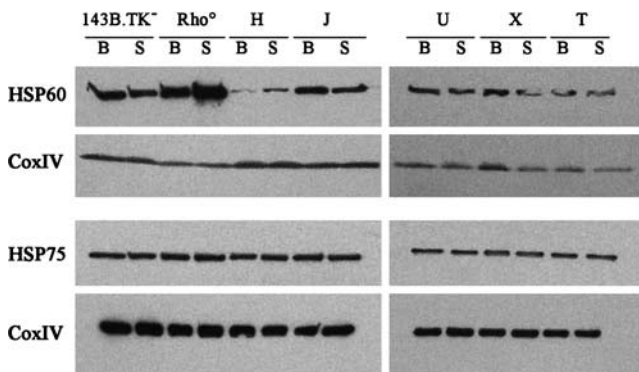


Fig. 2 Representative Western blot electrophoresis patterns of HSP60 and HSP75 intra-mitochondrial proteins in 143B.TK⁻, Rho⁰, H, J, U, X and T cell lines at basal (B) and stress conditions (S 42°C for 4 h). CoxIV cytochrome c oxidase subunit IV

observation suggests that the H line reacted to heat stress by up-regulating the expression of the two genes.

Western blot analysis

Considering that both HSP60 and HSP75 are mitochondrial proteins, we evaluated whether mitochondrial protein levels were correlated with mtDNA variability. By Western blot we analyzed the amount of HSP60 and HSP75 present in mitochondria in all the cell lines. In Fig. 2, a representative Western blot pattern of the HSP60 and HSP75 intra-mitochondrial protein levels is shown, while Table 2 summarizes the densitometric analyses of three experiments. As referred in the previous paragraph, we pointed our attention only to differences of protein levels where one cell line consistently had levels at least twofold higher with respect to another cell line.

At basal conditions, the HSP60 and HSP75 intra-mitochondrial protein levels were higher in Rho⁰ cells than in the native line ($p=0.046$ and $p=0.036$, respectively). Under stress conditions, only the HSP60 protein level increased in Rho⁰ cells compared to the native line ($p=0.015$). As for HSP75, although the same trend was

observed, the difference between basal and stress conditions was not statistically significant ($p=0.258$).

By comparing cybrid cell lines at basal conditions, no difference was found either in HSP60 or in HSP75 intra-mitochondrial protein levels ($p=0.419$ and $p=0.064$, respectively) thus indicating that these levels are independent of mtDNA variability. On the contrary, under stress conditions the HSP60 protein level differed among the lines ($p=0.001$), with the H cybrid showing higher levels compared to the other cybrid lines (Table 2). These results were also confirmed by Western blot carried out on an independent H clone (results not shown).

Discussion

The aim of the present study was to investigate whether mtDNA variability affects gene expression levels and/or intra-mitochondrial protein concentration, of HSP60 and HSP75, two key components of the mitochondrial stress response machinery. The question is of general interest due to the well-documented role of HSPs in maintaining cellular homeostasis in response to stress.

The cybrid technology used in our model to answer the question is largely debated in regards to the effect of cybridization on transcription patterns (Danielson et al. 2005). In the present case, however, we are confident of the reliability of our results for two reasons. First, observing Table 1, we see that under the heat stress condition, the cybridization process does not affect either HSP60 or HSP75 mRNA levels with respect to the native line, while these levels are lower in the cells depleted of active mitochondria (Rho⁰ cells). Second, the down-regulation of HSP60 and HSP75 genes observed in the H line in basal conditions, as well as the up-regulation observed in this line under stress conditions (Table 1), were confirmed in an independent clone.

To our knowledge, the present study identifies a correlation between mtDNA variability and mRNA levels of HSP genes for the first time. We note that as for the H

Table 2 Densitometric analysis of intra-mitochondrial protein levels of HSP60 and HSP75, normalized to CoxIV protein levels, in 143B.TK⁻, Rho⁰, H, J, U, X and T cell lines at basal and under stress conditions (42°C for 4 h)

| Densitometric analysis | | | | | | | |
|------------------------|----------------------|------------------|---------------|---------------|---------------|---------------|---------------|
| Basal condition | | | | | | | |
| | 143B.TK ⁻ | Rho ⁰ | H | J | U | X | T |
| HSP60 | 2.078 (0.444) | 4.303 (0.880) | 2.071 (0.439) | 2.023 (0.559) | 1.761 (0.277) | 1.832 (0.329) | 1.524 (0.314) |
| HSP75 | 0.171 (0.029) | 0.363 (0.060) | 0.152 (0.012) | 0.151 (0.035) | 0.178 (0.010) | 0.164 (0.029) | 0.159 (0.020) |
| Stress condition | | | | | | | |
| | 143B.TK ⁻ | Rho ⁰ | H | J | U | X | T |
| HSP60 | 2.748 (0.445) | 5.259 (0.425) | 3.939 (0.222) | 1.688 (0.185) | 1.108 (0.090) | 1.264 (0.208) | 1.620 (0.473) |
| HSP75 | 0.254 (0.092) | 0.502 (0.244) | 0.132 (0.005) | 0.130 (0.019) | 0.145 (0.016) | 0.115 (0.016) | 0.146 (0.018) |

Average values over three experiments are reported with standard error mean in parentheses

cybrid, the heat induction of HSP60 gene is in line with literature data, while the up-regulation of the HSP75 gene is unexpected, as it had been reported to be up-regulated by stresses different from heat (Hadari et al. 1997; Ryan et al. 1997; Carette et al. 2002; Zhao et al. 2002; Tokalov et al. 2003; Murray et al. 2004; Voloboueva et al. 2008). Considering the role of the two HSPs in the processes of protein import into mitochondria and subsequent protein folding (Frydman 2001; Mokranjac and Neupert 2005; Saibil 2008), we could hypothesize that mtDNA variability modulates mRNA levels of HSP60 and HSP75 through transcription factors that coordinate the activity of the two genes. In fact, numerous evidence indicate that the mitochondrial genome is able to regulate a series of nuclear target genes by transcription factors, such as NF κ B and CEBP, that acts as mediators of the well known cross-talk nucleus-mitochondrion (Biswas et al. 2005). The above hypothesis is also in line with literature data showing that promoters of HSP genes contain common and highly conserved binding sites for transcription factors some of which are specifically required for the heat shock response (Amin et al. 1988; Trinklein et al. 2004). We are currently investigating whether the above transcription factors are able to regulate also genes encoding for heat shock proteins localized in the cytoplasm and not only for those localized in the mitochondria.

The cybrid-specific response to stress (line H in Table 1) is in agreement with the results for cytokines and cytokine-receptors we described previously (Bellizzi et al. 2006). Therefore, the correlation between mtDNA variability and expression levels of stress-responder nuclear genes could be a general phenomenon, even if further studies are needed to generalize this assumption.

We obtained a confirmation of the cybrid-specificity in the response to stress by Western blot data (Table 2): the H line is the sole cybrid line that accumulated HSP60 within mitochondria. This result is very interesting because it suggests a correlation between mtDNA variability and accumulation within mitochondria of a protein which has a crucial role in coping with stress damage. In line with this role, the Rho⁰ cells, which are in very stressful conditions due to mitochondrial dysfunction, show an increase in intra-mitochondrial protein levels of both HSP60 and HSP75. The cell mechanisms through which the accumulation of both proteins within mitochondria occurs independently of nuclear gene expression is not known, and further studies are needed to clarify this point.

On the whole, the results reported in the present study provide important experimental evidence that in human cells mtDNA variability is able to influence the cellular response to heat stress by modulating both the transcription of genes involved in this response and their intra-mitochondrial protein levels.

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