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Induction of Mitochondrial Chaperonin, Hsp60, by Cadmium in Human Hepatoma Cells

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Summary: Mitochondrial dysfunction is readily induced by a low level of cadmium both *in vivo* and *in vitro* . The gene expression of mitochondrial hsp60 was induced in a dose- and time-dependent manner and also in parallel with the accumulation of cadmium in mitochondria. The levels of hsp60 mRNA increased with time until 18 h, while the induction of heat shock 70 (hsp70) gene peaked at 6 h and then declined. On the other hand, the levels of metallothionein mRNA reached to a plateau at 6 h and were maintained at this level continuously. These results suggest that the regulatory mechanism and/or the signals for hsp60 induction by cadmium are independent from those of hsp70 and metallothionein.

The mitochondrial chaperonin, hsp60, is an essential gene product whose function is required for, based in part on the known functions of the *Escherichia coli* protein GroEL (1), the refolding/stabilization-retransport/assembly of a number of mitochondrial enzyme complexes (2). Mammalian hsp60 show extensive sequence homology to the chaperonin family of bacterial GroEL (3, 4). Hsp60 appears to exist in the cells as a fourteenmer, double rings of seven subunits, and has a lumen in which the folding of newly synthesized mitochondrial proteins will be carried out (5, 6, 7). Since the extensive homology of human hsp60 to the major antigenic proteins of mycobacteria and rickettsiae has been shown (8), the human hsp60 protein may be a likely cellular target for the development of autoimmune response.

In spite of many studies on the functions of hsp60 protein, little attention has been paid to the induction of mitochondrial hsp60. As far as we know, hsp60 proteins exist constitutively in mitochondria and are increased by heat shock (9, 10). However,

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the induction mechanism of hsp60 in relation to the changes of the mitochondrial function has never been examined.

Fluharty and Sanadi (11) found that in rat liver mitochondria, oxidative phosphorylation was uncoupled by cadmium ions. Haugaard *et al.* reported the inhibition of oxidative phosphorylation of mammalian mitochondria by low concentrations of sulfhydryl-inactivating reagents (12). Cadmium has a high affinity for sulfur. It is expected that some toxic effects of cadmium are the result of reactions with essential sulfhydryl groups in proteins (13). These facts prompted us to examine the effects of cadmium on the hsp60 gene expression. The present results suggest that the separate regulatory mechanism may be existing in the cells for the induction of mitochondrial hsp60 and hsp70.

Materials and Methods

Cell culture: HuH-7 cells (JTC-39), derived from human hepatoma were obtained from the Japanese Cancer Research Resources Bank (JCRB). Cells were cultured in a RPMI medium supplemented with 5% fetal calf serum (GIBCO) at 37°C in an atmosphere of 95% air and 5% CO_2. For experiments, cells were plated in a 15 cm-diameter plastic dish (FALCON), 2 x 10^6 cells/plate and used after growing to subconfluent. Four plates were usually employed for one RNA extraction.

Uptake of cadmium: To determine the uptake of cadmium into HuH-7, cells were plated in a 7 cm-diameter plastic dish and treated with 1.5 μM $CdCl_2$ for a given time. After incubation with cadmium, cells were washed by PBS containing 0.1mM EDTA three times and scraped. Cells were homogenized with 1.15% KCl, 10 mM phosphate buffer, pH 7.4, 1 mM EDTA in a Dounce homogenizer. Subfractionation of the homogenate was carried out by differential centrifugation. After the removal of nuclei and cell debris by centrifugation at 800 x g for 5 min, the supernatant was centrifuged at 10, 000 x g for 10 min. The resulting pellets were designated as crude mitochondrial fraction. The cadmium contents in the samples were determined by a flameless atomic absorption spectrophotometry (AAS, Hitachi Polarized Zeeman Atomic Absorption Spectrophotometer Model Z-8000). The cell samples were treated with nitric acid until completely digested (14). The sample solution for AAS analysis was prepared by diluting the digested solution with 0.1 N nitric acid. The most suitable operating conditions for AAS were chosen to determine the amount of cadmium in the samples, and 10 μl of the sample solution was injected into the graphite cup cuvette. The standard solutions and sample solutions were analyzed in triplicate or more often, and the calibration curve was obtained using the least-squares method from the analytical data. The evaluation of cadmium in the samples was made by the calibration curve which was obtained at each run. The protein contents of subcellular fractions were determined using the method described by Bradford (15).

RNA preparation and Northern analysis: Total RNA was extracted by the guanidine thiocyanate procedure (16). Ten μg of the total RNA was electrophoresed in 1.0% agarose gel and transferred to nylon membrane (Hybond N, Amersham). The membranes were prehybridized in 4 x SSC, 50% formamide, 0.5% SDS, 5 x Denhardt's solution, and 20 μg/ml salmon sperm DNA (17). The membranes were hybridized by one of the following ^{32}p-labeled nick-translated probes : hsp 70 (human;Bam HI · EcoRI fragment, 0.8 kb) (18), hsp60 (human; PvuI fragment, 1.5 kb) (9), metallothionein (human MT-IIA; HindIII fragment, 3.0 kb) (19). The hybridization reaction was carried out at 42°C for 16-24h. The membranes were washed in 1 x SSC, 0.1% SDS at room temp. for 20 min once and at 42°C for 30 min, repeated 2-3 times. The hybridized radioactivities were quantitated using the Bio-imaging analyzer BAS 2000 (Fujix, Tokyo).

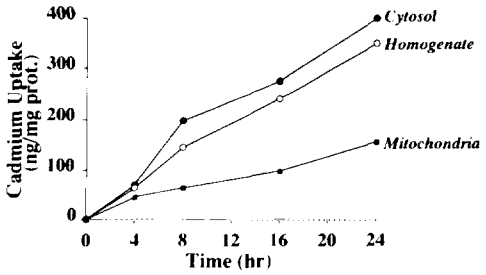

Fig. 1. Time course of cadmium uptake into the subcellular fractions of WISH cells.

Result

It has been reported that a low concentration of cadmium affects on the mitochondrial functions as an early event. We examined the uptake of cadmium into the subcellular fractions while HuH-7 cells were being incubated in the presence of 1.5 μM cadmium chloride. Time-dependent accumulations of cadmium were detectable in both the mitochondria and cytosol (Fig. 1).

Under the same conditions, that is, in the presence of 1.5 μM of cadmium, total RNA was extracted from the cells at a given time and employed for Northern analysis. The levels of hsp60 mRNA increased in a time-dependent manner until 18 h (Fig. 2A). The induction of hsp60 mRNA also occurred dose-dependently (Fig. 2B). It is well known that cadmium is a potent inducer for both metallothionein and hsp70, the most typical heat shock protein. The expression of these two genes were also examined for comparison under the present conditions. In the presence of 1.5 μM cadmium, hsp70 mRNA began to increase at 3 h, peaked around 6 h, and declined to the control levels at 18 h (Fig. 2C). This time course is quite different from that of hsp60. Using 1.5 μM

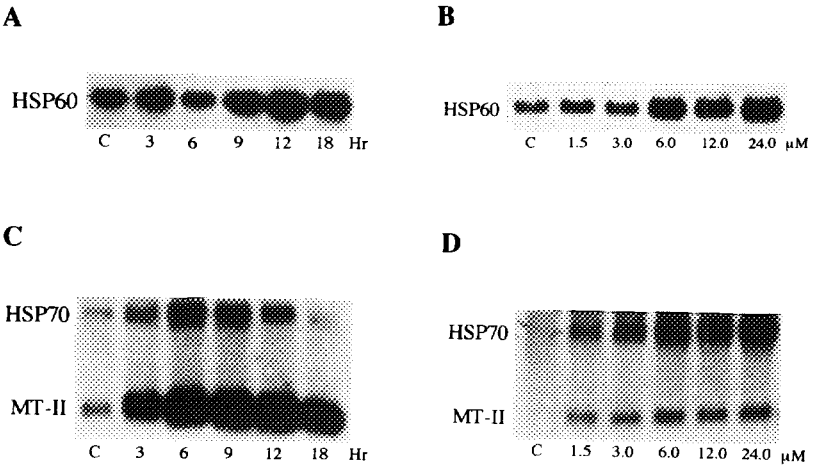

Fig. 2. Changes of mRNA levels of hsp60, hsp70 and metallothionein after cadmium treatment. RNA extraction and hybridization were carried out as described in Materials and Methods. Experiment for panels A and C was carried out with 1.5 μM cadmium. Incubation time for panels B and D was 6 h. RNA samples for panels A and C, and B and D were from the same experiments, respectively.

cadmium, the levels of metallothionein (MT-II) mRNA reached nearly to a plateau at 6 h. A further incubation did not enhance significantly the levels of MT-II mRNA (Fig. 2C). Among these three genes examined in the dose-response study, the increase of hsp70 expression was most dominant at the higher concentrations of cadmium (Fig. 2D). In conclusion, the pattern of hsp60 induction differed from those of hsp70 and metallothionein.

Discussion

The cadmium accumulation in the mitochondria of cultured hepatocytes was demonstrated in the present conditions. Cadmium has been reported to decrease the ATP/ADP ratio and to enhance lipid peroxidation in the cells (20). Mitochondrial respiratory functions are strongly affected at early times by a relatively low cadmium concentration which does not increase in lipid peroxidation. Lipid peroxidation, which is only observed at higher cadmium-concentrations, is not responsible for these early effects of cadmium. Uncoupling of oxidative phosphorylation by low concentrations of cadmium has been reported by Jacobs *et al.* (21). Subsequent studies supported the postulated participation of a dithiol group in oxidative phosphorylation, which may be localized between the electron transport chain and the oligomycin sensitive terminal coupling reaction (22).

Cadmium is considered to produce its toxic effect by binding a dithiol function, because a tendency toward enhanced binding of cadmium is seen in peptides that contain several cysteine residues (13). For example, cadmium inhibits oxygen consumption (21), especially with α-ketoglutarate and pyruvate as substrates, which is perhaps due to the interaction of cadmium on the lipoic acid coenzyme used by these enzymes. A direct demonstration of cadmium binding to the enzymes in the mitochondrial respiratory chain, however, has not been shown.

In the presence of various drugs or toxins, such as electron transport inhibitors or uncouplers, the mitochondrial generation of reactive oxygen species can increase several fold (23). The superoxide anion radical (O_2^-) appears to be the first oxygen reduction product. Two sites of the mitochondrial respiratory chains, that is, complex I and complex III have been identified as sources responsible for the formation of reactive oxygen species (24). Therefore, the superoxide radical can subsequently be converted to hydrogen peroxide. Dismutation of O_2^- and H_2O_2 can result in the production of the more deleterious hydroxyl radical and singlet oxygen. Both •OH and O_2^- are effective inactivators of specific proteins in the respiratory chain (25). These oxygen radicals accelerate further the generation of reactive oxygen species. In contrast, generalized lipid peroxidation, although a simple and widely used indicator of oxidative stress, appears to be unrelated to electon transport chain inactivation (25). Our hypothesis is that the transient induction of hsp70 may be due to the general response to the reduction of the cellular energy generation, but the induction of hsp60

is required to compensate the mitochondrial dysfunction by the increase of mitochondrial enzymes.

At higher cadmium concentrations or such conditions that generate complex mixtures of O_2^-, H_2O_2 and •OH, the defense mechanism against oxidative stress, such as mitochondrial glutathione (26), glutathione peroxidase and Mn-superoxide dismutase, can not scavenge all of the reactive oxygen species. The mitochondrial proteins, lipids, and DNA will be denatured and/or modified and may become the signals for the inductions of heat shock proteins, such as hsp70. Incidentally, little information is available on the localization of metallothionein in the mitochondria.

As far as we know, this is the first demonstration of the induction of mitochondrial hsp60 by an agent which has an effect on the mitochondrial respiration and energy generation. Possible explanations for the mechanism were discussed.

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