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Mercuric chloride-induced alterations in stress protein distribution in rat kidney

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Summary. Mercuric chloride ($HgCl_2$) induces acute renal failure associated to tubular impairment in experimental animals and humans. Stress proteins are a superfamily of proteins, comprising heat- shock proteins (HSP) and glucose-regulated proteins (GRP), enhanced or induced in the kidney in response to stress. They act as molecular chaperones that protect organelles and repair essential proteins which have been denatured during adverse conditions. The involvement of stress proteins in mercury-nephrotoxicity has not yet been well clarified. This study was undertaken to detect the tubular distribution of four stress proteins (HSP25, HSP60, GRP75, HSP72) in the rat kidney injected with $HgCl_2$ and to quantify lysosomal and mitochondrial changes in straight proximal tubules, the main mercury target. Sprague-Dawley rats were administered i.p. with progressive sublethal doses of $HgCl_2$ (0.25 mg/kg, 0.5 mg/kg, 1 mg/kg and 3.5 mg/kg) or saline (as controls) and sacrificed after 24 h. In dosages over 0.50 mg/kg, stress proteins increased and changed localization in a dose-dependent manner. HSP25 was focally expressed in altered proximal tubules at 1 mg/kg but in the macula densa it was at 3.5 mg/kg. HSP60 and GRP75 were intense in the nucleus and cytoplasm of proximal tubules but moderate in distal tubules. HSP72 was induced in distal tubules after low exposures but in proximal tubules it happened at the highest dose. Moreover, a significant increase in lysosomal and total mitochondria (normal and with broken cristae) area and density were progressively found after $HgCl_2$ treatments. Stress proteins could represent sensitive biomarkers that strongly correlate with the degree of oxidative injury induced by $HgCl_2$ in the rat proximal tubules.

Keywords: Stress proteins, Mercury, Nephrotoxicity, Rat

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Introduction

Mercury and its compounds are enviromental and industrial agents able to induce severe nephrotoxicity both in man and animals. Mercuric chloride ($HgCl_2$) administration is a classic model for the study of the pathogenesis of inorganic mercury toxicity in both in vitro and in vivo systems as rewieved by Zalups (2000). $Hg\,Cl_2$ affects the oxidative function because of its high affinity for cellular cysteine thiols (Clarkson, 1997). Mercury nephrotoxicity is characterized by altered antioxidant enzymes (Shimojo et al., 2002), lipid peroxidation (Nava et al., 2000), reduced ATP content (Mahboob et al., 2001) and it leads to tubular epithelium necrosis even after a single exposure (Diamond and Zalups, 1998). $HgCl_2$-induced damage is strictly dependent on the route of administration, time and dose (Nielsen et al., 1994). The toxic dose of inorganic mercury is significantly different in various renal systems. Moreover, in humans it is fatal in the range of 10-42 mg/kg (Kostial et al., 1978), while in mice the oral lethal dose 50 (LD50) is higher, between 25.9-77.7 mg/kg (Nielsen and Andersen, 1990). Mc Dowell et al. (1976) reported progressive morphological and functional alterations from 6 h on in the straight portion of rat proximal tubules after a dose of 4 mg/kg $HgCl_2$. Administering 1.5 mg/kg $HgCl_2$, Girardi and Elias (1995) reported an increase in glutathione reductase and peroxidase and reduced urinary volume, but severe tubular necrosis was detected only after 16 h in rat proximal tubules and after 48 h in the entire cortical area.

Some molecular mechanisms are generally used from the cells with the purpose of protecting them from a toxic substance, such as a heavy metal. Nevertheless, if the toxic agent is not removed, the cells begin an irreversible process of degeneration. Cellular recovery from acute renal injury involves the expression of early "stress genes" such as epidermal growth factor (Coimbra et al., 1990), c-jun, c-fos (Bardella and Comolli, 1994) and stress proteins (Cowley and Gudapaty, 1995). Stress

proteins represent a highly conserved superfamily including heat-shock proteins (HSP) and glucose-regulated proteins (GRP). Major members are expressed in the renal parenchyma even under physiological conditions but play an essential role in damaged kidney as molecular chaperones that protect or repair other cellular proteins and specific organules (Beck et al., 2000). Heat-shock proteins are classified according to their molecular size into small, medium and large HSP. HSP25 in rodents, alias HSP27 in mammals, is constitutively expressed in the kidney, associated to the actin cytoskeleton, but is enhanced in adverse conditions, such as ischemia, cancer or drug toxicity (Takashi et al., 1998; Smoyer et al., 2000; Stacchiotti et al., 2002). HSP60 is a constitutive mitochondrial chaperone located in the mitochondrial matrix, where it regulates correct import and folding of oxidative enzymes (Soltys and Gupta, 1996). D'Souza and Brown (1998) reported a strict coincidence between developmental expression of the subunit IV of cytochrome oxidase and HSP60 in rat tissues. HSP72 is a well-known inducible chaperone, almost undetectable in the normal kidney, but largely induced in response to stressors, such as high osmotic flux (Neuhofer et al., 1999), ischemia/reperfusion (Komatsuda et al., 1999) and nephrotoxic drugs (Barisic et al., 2002). Moreover HSP72 preinduction has been shown to be beneficial against oxidative damage, and when ATP levels decrease its overexpression maintains both cellular homeostasis and integrity (Mallouk et al., 1999).

Glucose-regulated proteins (GRP) are different stress proteins related to glucose consumption and altered calcium flux in the mammalian cells (Lee, 2001). GRP75 (also called mtHSP70 or mortalin) is a member of the HSP70 family but cloned also as glucose-regulated protein (Wadhwa et al., 2002). It is involved in protein assembling, transport and refolding during oxidative damage and ATP loss. GRP75/mortalin is associated with the mitochondrial inner membrane, but is present also in the endoplasmic reticulum or is free in the cytoplasm (Ran et al., 2000). To our knowledge only Goering et al. (1992) have reported a direct relationship between enhancement of stress proteins and mercuric chloride-induced nephrotoxicity in the rat kidney. Moreover in a recent study, they showed that $HgCl_2$, administered in rats at sublethal doses (0.25 mg/kg to 1 mg/kg), induced regional and cell-specific stress proteins in the kidney at 24 h (Goering et al., 2000). HSP72 was induced in the cortex and GRP94 in the medulla, while constitutive HSP73 and HSP90 were unchanged after mercury exposure. However, since other stress proteins are also potentially involved in $HgCl_2$ nephrotoxicity, the present study was aimed at immunohistochemically determining the effect of sublethal mercury doses on the intensity and tubular distribution of HSP25, HSP60 and GRP75 in comparison with HSP72 at 24 h. Moreover, a morphoquantitative ultrastructural analysis was performed on both mitochondrial and lysosomal compartments in the straight proximal tubule, the main mercury target, as recently characterized by our group using almost similar exposures (Stacchiotti et al., 2003). The final goal of our study was to demonstrate a relationship between the distribution of specific cytoprotective stress proteins and oxidative alterations induced in rat proximal tubular cells by mercury. If this is true, HSP25, HSP60, GRP75 and HSP72 could be used as reliable pathogenic fingerprints in this in vivo nephrotoxic model.

Materials and methods

Animals

Thirty male Sprague Dawley rats (weight 250± 10 g) (Charles River, Italia) were used in this study. The animals were housed in a controlled enviroment, at a regular 12 h light and 12 h dark cycle at 22 °C, relative humidity 45 - 55% and fed with standard food and water ad libitum. They were housed in metal wire cages with alderwood beddings in groups of three. Experiments started after almost 1 week of stability from purchase. Animal care was realized according to the Italian law on the protection of laboratory animals (D.M.116192), as well as with the EC regulations (L 358/112/18/1986) and all procedures were approved by the Italian Ministry of Health.

Experimental procedures

Animals were divided into five experimental groups (6 rats per group) and treated according to the following protocols: group 1 (control): a single i.p. injection of sterile saline; group 2: a single i.p. injection of mercuric chloride, $HgCl_2$ (Sigma-Aldrich, Milan) at 0.25 mg/kg (T0.25); group 3: a single i.p.injection of $HgCl_2$ at 0.50 mg/kg (T0.50); group 4: a single i.p.injection of $HgCl_2$ at 1 mg/kg (T1); and group 5: a single i.p. injection of $HgCl_2$ at 3.5 mg/kg (T3.5). All the injections were made at 11 a.m. to avoid circadian variations of mercury delivery as reported by Cal et al. (1990). After 24h all rats were killed by cervical dislocation. One kidney was rapidly removed and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) 0.1 M, pH 7.4, overnight at 4 °C for histopathology and immuno-histochemistry and the other kidney was fixed in 2.5% glutaraldehyde in PBS and then postfixed in 1% osmium tetroxide in PBS for ultrastructural analysis and morphometry. Samples were subsequently dehydrated and embedded in paraffin wax or in araldite resin, respectively. For ultrastructural analysis, ultrathin (70 nm) sections, double-stained with uranyl acetate and lead citrate, were observed under a Philips TEM CM 10 at 80 kV.

Histopathological analysis

Routine histopathological analysis was performed on serial paraffin sections stained by hematoxylin and eosin

and observed under a light microscope at x400. The extent of tubular damage was estimated for each group on 100 proximal and 100 distal midcortical tubules randomly observed by different investigators and expressed in a range from 0 to 4 according to Goering et al. (2000).

Immunohistochemistry

4-µm paraffin sections were cut and collected on poly-L-lysine-(Sigma, St Louis, MI, USA) coated glass slides and dried overnight at 37 °C. Sections were deparaffinized, rehydrated and immersed in 3% hydrogen peroxide in methanol for 30 min to block the endogenous peroxixdase activity, whereas non-specific binding of primary antibodies was blocked with normal goat serum or horse serum (Vector Laboratories), diluted 1:5 in Tris-buffered 10 mM saline, pH 7.6, containing 0.1% Triton (TBS) for 1 h at room temperature. The slides were then incubated with polyclonal anti-HSP25 (1:200) and monoclonal anti-HSP60 (1:300), anti-GRP75 (1:400) and anti-HSP72 (1:400) antibodies overnight at 4 °C. All primary antibodies were purchased from StressGen Biotechnologies. After several washings in TBS, sections were incubated with secondary biotinylated anti-rabbit or anti-mouse IgG antibodies followed by the Vectastain Elite-peroxidase kit using diaminobenzidine staining, as previously reported (Stacchiotti et al., 2001). Different immunohisto-chemical controls were performed: 1) by omitting the primary antibody which was replaced by TBS buffer; or 2) by pre-absorption of the primary antibody with its respective recombinant or natural stress protein. All slides, except immunohistochemical controls, were counterstained with haematoxylin, dehydrated and mounted with DPEX (BDH). For a better comparison of stress protein immunostaining under different treatments, a semiquantitative grading system was adopted. In longitudinal sections containing a whole kidney, 100 midcortical proximal tubules and 100 distal tubules for each experimental group were analyzed in 20 randomly-chosen fields at x250 in three sets of experiments.

Morphometric ultrastructural analysis

Data were collected from 50 randomly-chosen electron micrographs of the straight portion of the proximal tubule for each group at a final enlargement of X11,700. All measurements were obtained by point-counting stereology as previously reported (Stacchiotti et al., 1995). We calculated both sectional area (or volume) and numerical density (number per $100\mu m^2$) of mitochondria and lysosomes in control and in mercuric chloride treated groups. In particular, because mitochondria are organules which are highly susceptible to oxygen impairment, in order to exclude alterations simply induced by morphological artifacts such as inadequate fixation, both intact and broken cristae mitochondria were examined. Therefore, here we have indicated as "total mitochondria", the sum of both normal and altered organules compared with intact mitochondria only. All counts were made by three different investigators unaware of the experimental treatment observed.

Statistical analysis

Data are presented as means ± SD. Statistical comparison between groups was made by analysis of variance (ANOVA) corrected by the Bonferroni test and set at $P<0.05$.

Results

Histopathological analysis

All animals survived the experimental treatments.

Table 1. Intensity of stress protein expression in saline and mercury-treated rat renal tubules (n=6).

	SALINE	MERCURIC CHLORIDE			
		0.25 mg/Kg	0.50 mg/Kg	1 mg/Kg	3.5 mg/Kg
HSP25	PT +/- DT +	PT +/- DT +	PT +/- DT +	PT + DT +	PT ++ DT ++
HSP72	PT – DT –	PT – DT –	PT – DT +/–	PT + DT +	PT ++ DT +/-
HSP60	PT +/- DT +	PT+/- DT +	PT+ DT+	PT + DT +	PT ++ DT ++
GRP75	PT +/- DT +/-	PT +/- DT +/-	PT + DT +/-	PT ++ DT +	PT +++ DT +
Nephrosis grade (Scale 0 to 4)	0 NONE	0 NONE	2 MILD	3 MODERATE	4 SEVERE

PT: Proximal Tubule; DT: Distal Tubule; (-): absence of staining; (+/-): very weak staining; (+): moderate staining; (++): intense staining; (+++): striking staining.

However, in the T3.5 group administered with 3.5 mg/kg $HgCl_2$, 5/6 rats showed ascites, a dull-appearance, bristly fur and trembling. The kidney weight was similar in all groups (1.0±0.02 g). Macroscopical analysis of the whole kidney evidenced in the T3.5 group a pale cortico-medullary junction, often full of blood clots. All these signs were absent in the other experimental groups. In controls, microscopical analysis showed that both glomerular and tubular atrophy were negligible. In contrast, after mercuric chloride treatment, progressive tubular alterations ranging from focal to complete tubular damage were seen in renal midcortical tubules. In the T1 group, more than 20% of the proximal tubules were damaged and distal tubules were occasionally destroyed. Finally, in the T3.5 group, more than 45% of the proximal tubules and 15% of the distal tubules were greatly affected. Glomeruli were always morphologically normal. The nephrosis degree is indicated in Table 1.

Immunohistochemical analysis

The immunohistochemical pattern of the four stress proteins, HSP25, HSP72, HSP60, GRP75, in both saline and mercury-treated renal tubules, was focused on the midcortical area. The staining intensity is semiquantitatively resumed in Table 1. Non-specific staining was undetectable with any of the primary monoclonal and polyclonal antibodies used.

Constitutive HSP25 and Inducible HSP72

In control animals, HSP25 was faint in the mid-cortical proximal tubules and mainly associated with the brush border epithelium whereas it was moderate in the distal tubules and vessels (data not shown). After $HgCl_2$ exposure, HSP25 intensity increased and tubular localization changed from a single cell to the complete tubule, according to nephrosis degree. HSP25 staining

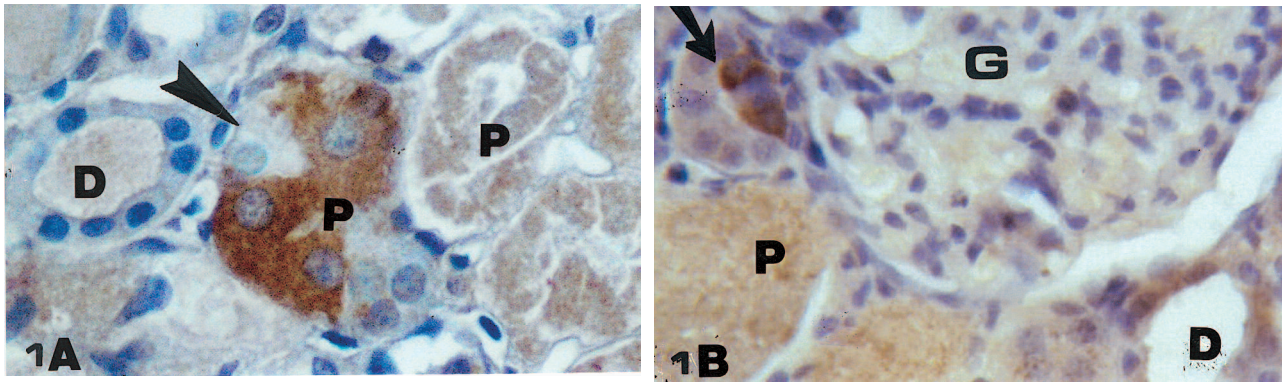

Fig. 1. HSP25 immunostaining in $HgCl_2$-treated kidney. **A.** (1 mg/kg): Moderate focal reaction in proximal tubules (P) and faint in distal tubules (D). Arrowhead indicates an unstained normal epithelial cell. **B.** (3.5 mg/kg): Moderate reaction in glomerulus (G) and intense in proximal (P) and distal tubules (D). Arrow: positive macula densa cells. x 400

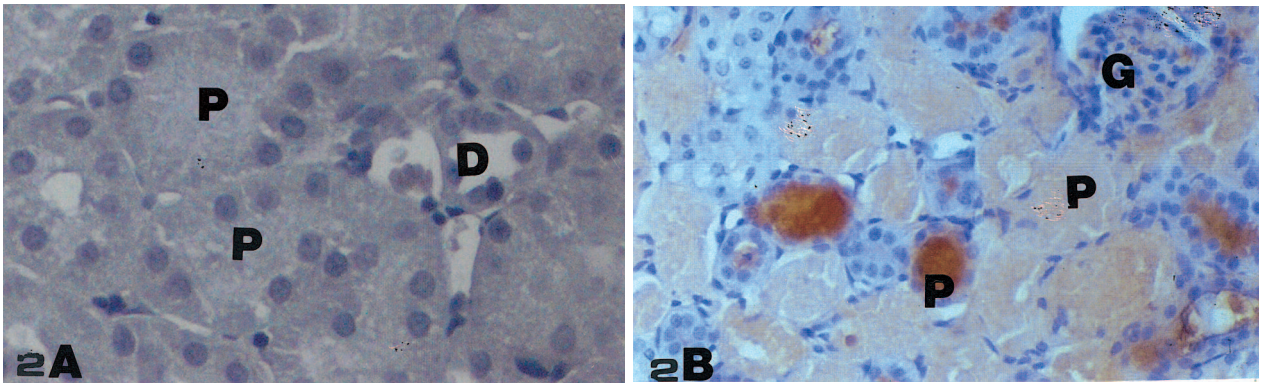

Fig. 2. HSP70 immunostaining in $HgCl_2$-treated kidney. **A.** (0.25 mg/kg): Undetectable reaction in proximal (P) and distal (D) tubules. x 400. **B.** (3.5 mg/kg): Faint in glomerulus (G) and intense in proximal (P) tubules. x 200

was intense but focal in the damaged proximal tubules in the T1 group (Fig. 1A). In contrast, in the T3.5 group, characterized by severe nephrosis, it was intense in both convoluted and straight proximal tubules and was detected within the cytoplasm of the macula densa cells (Fig. 1B). Inducible HSP72 was almost undetectable in the kidney of both the control and the T0.25 groups (Fig. 2A). After $HgCl_2$ treatment at concentrations greater than 0.5 mg/kg, an evident staining was detected in the cytoplasm of distal tubules. After the highest mercury exposure, HSP72 was induced in affected proximal tubules but was weak in distal tubules in the T3.5 group (Fig. 2B).

Mitochondrial chaperones HSP60 and GRP75

Constitutive HSP60 was located in a faint basolateral pattern in the cytoplasm of both the proximal and distal tubules in the control and T 0.25 groups (Fig. 3A). Starting from 0.50 mg/kg $HgCl_2$, immunoreaction was enhanced and was scattered in the cytoplasm of proximal tubules and distal tubules, in a dose-dependent manner. HSP60 staining was moderate in some proximal and distal tubules of the T1 group (Fig. 3B) but became diffuse, often inside the lumen, in proximal tubules in the T3.5 group. GRP75 immunostaining was faint and similar to the previous chaperone both in the control and T0.25 groups. In the T1 group, the GRP75 signal was more intense in cortical proximal tubules, associated to brush-border detachment or in the luminal side (Fig. 4A). Finally, in the T3.5 group, GRP75 staining was very strong both in the nuclei and cytoplasm of altered proximal tubules and often found along the luminal side of some tubules devoid of a basolateral cytoplasmic reaction (Fig. 4B).

Ultrastructural and morphometric analysis

Ultrastructural features of the straight portion of midcortical proximal tubules treated with 0.25 mg/kg

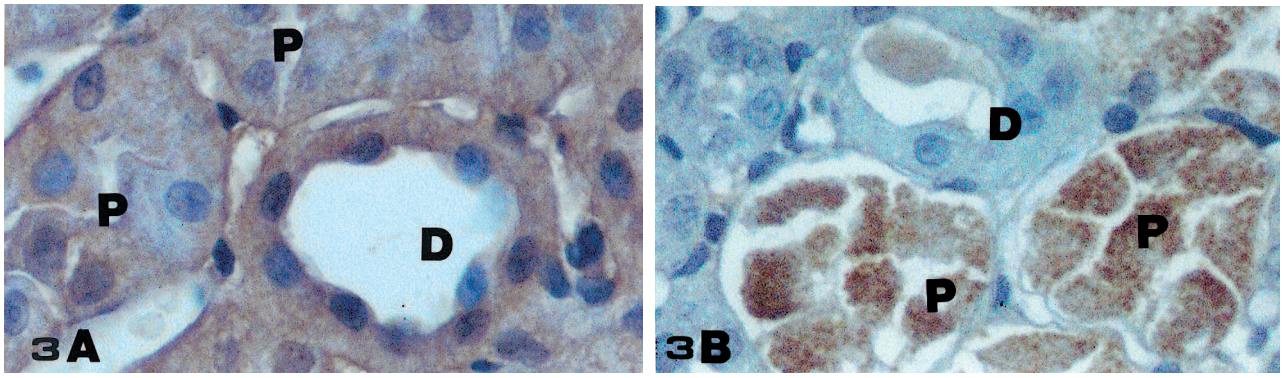

Fig. 3. HSP60 immunostaining in $HgCl_2$-treated kidney. **A.** (0.25 mg/kg): Faint basolateral reaction in proximal (P) and distal (D) tubules. **B.** (1 mg/kg): Moderate signal in proximal (P) tubules and in the luminal side of distal (D) tubules. x 400

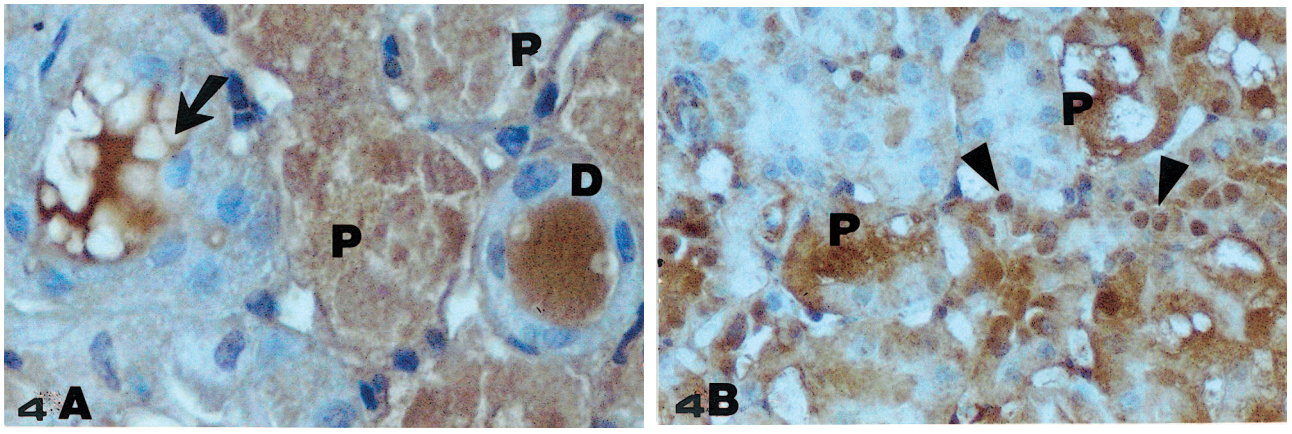

Fig. 4. GRP75 immunostaining in $HgCl_2$-treated kidney. **A.** (1 mg/kg): Intense reaction in proximal (P) tubules and in the lumen of distal (D) tubules. Arrow: positive detatched brush border. x 400. **B.** (3.5 mg/kg): Scattered striking reaction in cytoplasm and nuclei (arrowheads) of proximal (P) tubules. x 200

$HgCl_2$ at 24h were similar to the unaffected control group. Only few scattered lysosomes and vacuoles, sometimes with a target-like appearance, were detected in the cytoplasm (Fig. 5A). At higher magnification, mitochondria were regular, elongated, with a homogeneous matrix, regular cristae and a normal outer and inner membrane (Fig. 5B). In contrast, in dosages of over 0.5 mg/kg $HgCl_2$, progressive tubular changes were detected. In particular, in the T3.5 group, a collapsed brush border, a highly vacuolated cytoplasm and disrupted mitochondria were observed (Fig. 6A). At higher magnification, mitochondria showed a heterogeneous size, swollen and irregular cristae, a dense matrix and were often completely disrupted (Fig. 6B).

Morphometric data on both lysosomal and mitochondrial compartments are resumed in Table 2. When compared to controls no significant changes were detected in the T0.25 group except for a significant increase in lysosome density (about 44%). Lysosomal area increased only in the T1 and T3.5 groups (more than 10%). In contrast, starting from the T0.5 group,

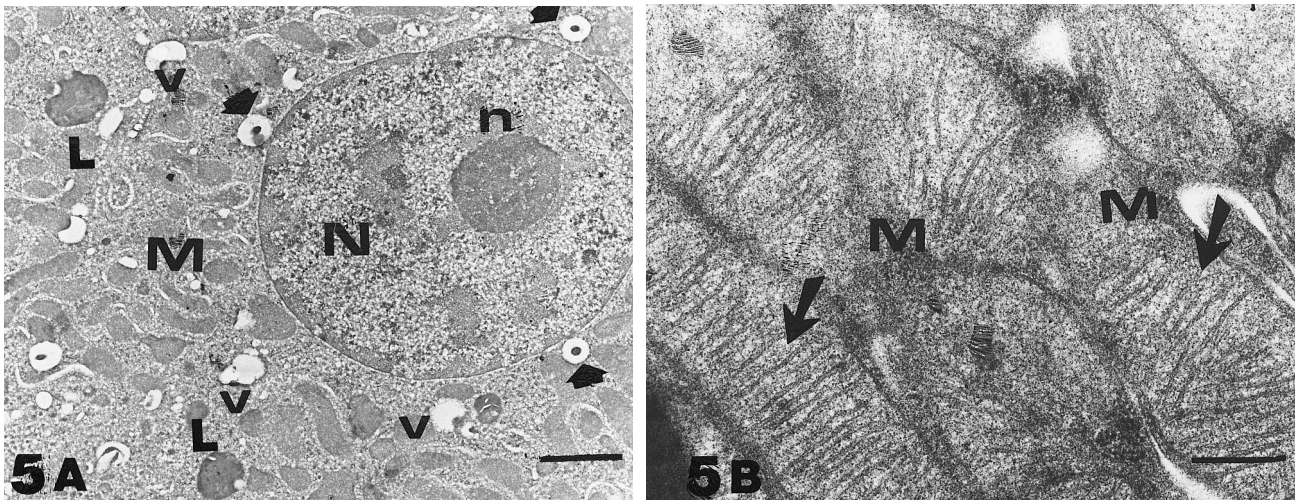

Fig. 5. Ultrastructure of straight proximal tubule treated with 0.25 mg/kg $HgCl_2$. **A.** Normal nucleus (N) and nucleolus (n), cytoplasm with lysosomes (L) and vacuoles (v), some target-like inclusions (thick arrow). Bar: 1 µm. **B.** At higher magnification, normal elongated mitochondria (M) showing regular cristae (arrows). Bar: 0.5 µm.

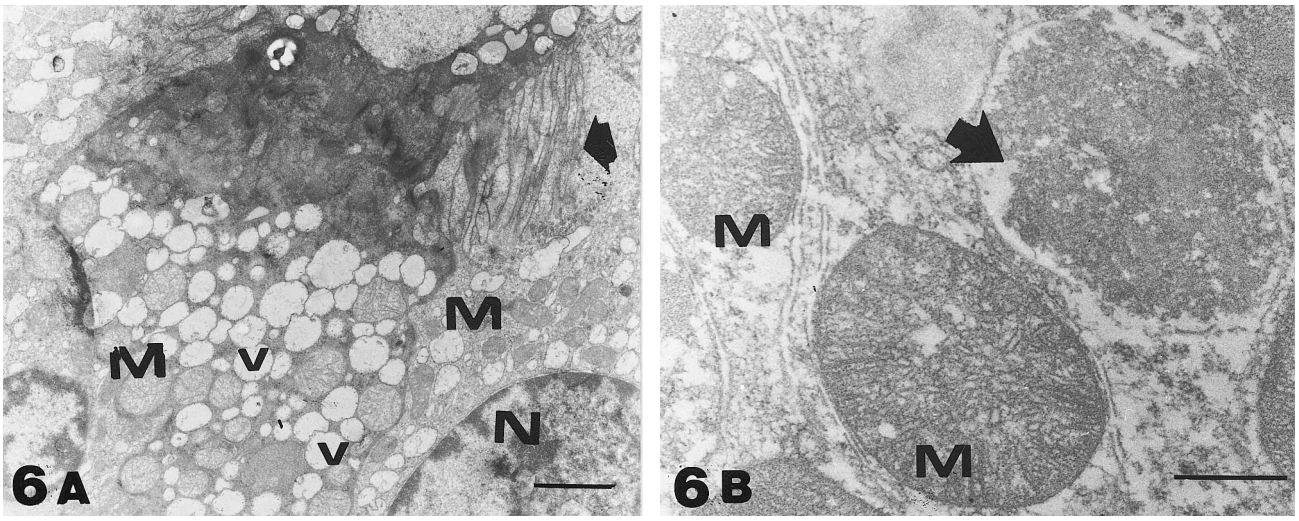

Fig. 6. Ultrastructure of straight proximal tubule treated with 3.5 mg/kg $HgCl_2$. **A.** Collapsed microvilli of the brush-border (thick arrow), cytoplasm filled with vacuoles (v). Mitochondria (M), nucleus (N). Bar: 1 µm. **B.** At higher magnification, round mitochondria (M) with irregular cristae, sometimes completely disrupted (arrow). Bar: 0.5 µm.

both total mitochondria area (around 60%) and density (more than 6%) were significantly increased. This trend was also kept in other treated groups but was higher in the T3.5 group where, with respect to controls, the total mitochondria area doubled and mitochondrial density was maximal (more than 13%).

Discussion

In this in vivo toxicological study we have shown that $HgCl_2$ enhances and changes the tubular distribution of four stress proteins (HSP25, HSP72, HSP60 and GRP75) in rat kidney in a dose-dependent manner. Moreover, the over-expression of stress proteins is concurrent with histological nephrosis and progressive morpho-quantitative lysosomal and mitochondrial alterations detected in proximal tubules after sublethal mercury exposures at 24 h.

$HgCl_2$ directly or indirectly destabilizes intracellular proteins and organules, mainly cytoskeletal proteins (Elliget et al., 1991) and mitochondria (Santos et al., 1997). Its main target is the straight portion of proximal tubules, as has been previously reported in both in vivo (Stacchiotti et al., 2003) and in in vitro studies (Bohets et al., 1995). The high tubular sensitivity to mercury is due to selective enzymes involved in the uptake of this metal into the proximal tubules (Zalups and Barfuss, 2002). Distal tubules also accumulate mercury, even if to a lesser extent (Girolami et al., 1990; Lash et al., 1998), and show a decrease in transepithelial potential difference that greatly contributes to alter electrolyte excretion (Jungwirth et al., 1991). Stress proteins, comprising both heat-shock proteins (HSP) and glucose-regulated proteins (GRP), are molecular chaperones that play essential homeostatic functions in physiological conditions (Beck et al., 2000) but enhance the protection of kidneys against adverse conditions such as diabetes (Dunlop and Muggli, 2000), nephrotoxic drugs (Komatsuda et al., 1999; Stacchiotti et al., 2001) and osmotic stress (Van Why and Siegel, 1998). HSP25 is a small-size heat-shock protein that stabilizes cytoskeletal proteins, mainly actin, in normal rat proximal tubules where it stains brush-border epithelium well (Stacchiotti et al., 2002). In the present study we reveal that HSP25 is detached from the brush border and is focally expressed in the cytoplasm of proximal tubular epithelial cells according to mercury dose. In addition, our ultrastructural observations confirmed brush-border collapse and altered basolateral membranes at higher mercury exposure. $HgCl_2$ modulates the conformational status of the Na/K-ATPase molecule and inhibits its activity (Anner et al., 1992). Bidmond et al. (2002) reported that HSP25 stabilized sodium pump and tubular cytoskeleton in rat renal cortex after ischemia. Recently, Van Why et al. (2003) showed that the association of HSP27 with actin in LLC-PK1 renal cells limited the detachment of Na/K-ATPase from the cytoskeleton in response to experimental energy deprivation. Moreover, in the kidney of mice treated with a high $HgCl_2$ dose (8.3 mg/kg) Bartosiewicz et al. (2001) reported, by the DNA-array technique, a significant increase in m-RNA HSP25 expression. This finding is in line with the present immunohistochemical data on enhanced protein expression in rat kidney. Moreover Hg^{2+} affects the glomerular-tubular feedback mechanism (Yanagisawa et al., 2002). Macula densa cells within distal tubules represent the site that reveals urinary flux composition and transmits signals to glomerular vascular components (Bell et al., 2003). Therefore, the intense HSP25 reaction found in the macula densa cells after the highest $HgCl_2$ exposure could counteract glomerular impairment and reduce tubulo-glomerular feedback.

HSP72, a member of the mammalian HSP70 family, is greatly induced in the kidney in numerous toxicological in vitro (Ait-Aissa et al., 2000; Gennari et al., 2003) and in vivo studies (Hernandez-Pando et al., 1995). Our immunohistochemical data on HSP72 induction in rat cortical tubules are in line with Goering et al. (2000) who used almost similar doses, but not the 3.5 mg/kg $HgCl_2$ dose. Moreover, in rat liver consecutively administered with $HgCl_2$ for three days, Sanchez Reus et al. (2003) detected a rapid HSP72 induction as a cell strategy against mercury-induced oxidative stress. HSP72 is also an anti-apoptotic marker

Table 2. Morphometric data on rat proximal tubules after mercuric chloride exposure.

TREATMENTS n=rats	A SALINE n=6	B MERCURIC CHLORIDE (0.25mg/kg) n=6	C MERCURIC CHLORIDE (0.50mg/kg) n=6	D MERCURIC CHLORIDE (1 mg/kg) n=6	E MERCURIC CHLORIDE (3.5mg/kg) n=6
Lysosome Area	1.28±0.04	1.30±0.05	1.35±0.05	1.43±0.06[a,b,c]	1.47±0.04[a,b,c]
Lysosome density	18.3±1.8	26.3±2.7[a]	29.7±2.2[a]	25.4±3.7[a,b,c]	23.6±2.1[a,b,c]
Intact mitochondria area	0.08±0.02	0.08±0.02	0.06±0.01	0.08±0.01	0.06±0.01
Total mitochondria area*	0.08±0.02	0.08±0.03	0.13±0.02[a,b,e]	0.13±0.02[a,b,e]	0.16±0.02[a,b,c,d]
Total mitochondria density*	74.4±3.2	75.6±3.3	79.8±3.4[a, b]	81.6±3.6[a, b]	84.3±3.4[a,b,c]

All data are expressed as mean ± SD; [a], [b], [c], [d], [e]: indicate statistically significant value vs respective group ($p < 0.05$); *: total mitochondria comprises both intact and broken cristae mitochondria.

that limits cell death by nephrotoxic drugs such as cisplatin (Zhou et al., 2003) and its liposome delivery in renal tubular cells blocks nuclear factor-kappa B activation and apoptosis (Meldrum et al., 2003). Besides tubular necrosis, $HgCl_2$ induces apoptosis in vitro (Woods et al., 2002) and in vivo (Homma-Takeda et al., 1999) at sublethal exposure.

HSP60 and GRP75/mortalin represent cytoplasmic chaperones that control both refolding and transport of oxidative enzymes (Martin, 1997). Their fundamental role inside mitochondria has been demonstrated in different mammalian cell lines during nephrotoxic (Bruschi et al., 1998) and oxidative damage (Mitsumoto et al., 2002). In the present study these mitochondrial chaperones are present in the basolateral side within tubular epithelial cells in controls. In contrast, after sublethal mercury exposures, they spread in the cytoplasm of both distal and proximal tubules and sometimes also in the nuclei. Even if speculative, the nuclear distribution of GRP75/mortalin could indicate a role in cell proliferation and recovery as suggested in different tissues by Kaul et al. (1997).

Mitochondrial morphology both in normal cells and during apoptosis has recently been reviewed by Karbowski and Youle (2003). Here we have shown by ultrastructural morphometry that both mitochondrial size and density are progressively affected by $HgCl_2$ concentrations greater than 0.5 mg/kg. Our data are in line with a previous functional study by Santos et al. (1997). In particular, the prevalence of round mitochondria with swollen cristae after the highest mercury exposure could be due to abnormal calcium release (Chavez and Holguin, 1988) and reduced transmembrane potential (Königsberg et al., 2001) induced by Hg^{2+}. Finally, we have shown that $HgCl_2$ treatments change the lysosomal compartment in midcortical proximal tubules. In particular, we have detected a significant increase in lysosome area but a reduced density, indicating the presence of large autophagic vacuoles. Besides the nucleus and mitochondria, lysosomes have also been described among intracellular sites of mercury accumulation (Madsen, 1980). Moreover, Nath et al (1996) reported that $HgCl_2$ affected the lysosomal transmembrane proton gradient. In an experimental in vitro study using cadmium, which damages tubular sodium pump and mitochondria in a similar way as mercury, both lysosomal proteases and endocytosis have been involved (Thevenod and Friedmann, 1999). Intracellular proteolytic activity requires specific cochaperones associated to the lysosomal membrane that transport proteins inside lysosomes (Agarraberes and Dice, 2001). Therefore, altered stress protein localization could reflect abnormal lysosomal activity induced by mercuric chloride in the straight segment of renal tubules.

In conclusion, mercuric chloride administration resulted in a clear direct oxidative damage on midcortical proximal tubules where we demonstrated the higher expression of four specific stress proteins in a strict dose-dependent manner. Therefore HSP25, HSP72, HSP60 and GRP75 could play a fundamental role in the recognition of intracellular proteins or organules altered by mercury and work together as molecular chaperones to repair damage (Stohs and Bagchi, 1995) and to maintain tubular survival. Finally we suggest that these stress proteins could be used as sensitive toxicological biomarkers for monitoring progressive proximal tubule impairment in mercuric chloride-induced nephrotoxicity in the rat.

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