Summary. Aluminium (Al) is the third most widespread metal in the environment. It is toxic for the brain, bone and haematological system but unfortunately very little data exist for other organs. Stress proteins are induced or enhanced against metal toxicity with an essential role in the recovery of organules and other cellular proteins. This immunohistochemical study was performed to analyze the distribution of three stress proteins (HSP25, HSP72, GRP75) in rat kidney and liver orally exposed to Al sulphate daily for 3 and 6 months. Al-induced alterations were further studied by histopathology (H&E, PAS, Perl’s, Masson) and ultrastructural morphology. In the kidney: HSP25 was enhanced in proximal tubules after 6 months Al-exposure when abnormal brush borders were observed; HSP72 was induced in proximal tubules only after long Al-treatment; GRP75 was raised in midcortical area sometimes within nuclei. Furthermore, lysosomal and lipofuscin densities increased in the juxtamедullary tubules after 3 months Al exposure with respect to controls. In the liver: Perl’s-positive deposits and fibrosis became evident after Al treatment. HSP25 was very weak; HSP72 focal in pericentral hepatocytes at 3 months and induced also in Kupffer cells at 6 months; GRP75 diffuse in periportal hepatocytes and non parenchymal cells at 6 months. Prolonged Al exposure stimulated stress proteins strictly organ-dependently in the rat. Their distribution in kidney and liver seems related to cumulative sublethal effects induced by metal and could be a sensitive index of Al susceptibility of these organs.

Key words: Aluminium, Stress proteins, Kidney, Liver, Immunohistochemistry
filtration rate or other global functions. Prolonged side-effects of Al were reported mainly in the renal cortex that showed tubular degeneration (Roy et al., 1991). In regard to the liver, this organ possibly plays a role in the metabolism and deposition of Al. Both in vivo and in vitro studies described Al–hepatotoxicity as a consequence of long exposures (Ebina et al., 1984; Klein et al., 1988).

Cells and tissues adopt different strategies, for example, the induction or enhancing of stress proteins to protect themselves from adverse conditions such as toxic metal exposure and therefore in this study we analyzed stress proteins reaction against Al.

Stress proteins are a superfamily of proteins, called heat-shock proteins (HSP) and glucose-regulated proteins (GRP), highly conserved from procarotid to mammals. Major members are commonly expressed in the kidney and liver under physiological conditions but play a cytoprotective role during adverse conditions. They act as chaperones capable of repairing other proteins and organelles affected by stress (Beck et al., 2000). Heat-shock proteins are classified according to their molecular size into small, medium and large. HSP25 in rodents, alias HSP27 in mammals, is a small constitutive HSP, located in the kidney, associated with actin, but enhanced in several adverse conditions, such as ischemia, cancer, drug or metal-induced nephrotoxicity (Takashi et al., 1998; Smoyer et al., 2000; Stacchiotti et al., 2002, 2004). Inside liver cells HSP27 overexpression promotes survival against xenobiotic chemicals (Katsuki et al., 2004). By contrast HSP72 is an inducible chaperone, almost undetectable physiologically, but induced in response to stressors (Schiaffonati and Tiberio, 1997; Komatsuda et al., 1999; Barisic et al., 2002). Glucose-regulated proteins (GRP) are stress proteins related to glucose metabolism and calcium flux in the mammalian cells (Lee, 2001). GRP75 (called also mortalain) may participate in mitochondrial protein assembling, transport and maintenance during oxidative damage and ATP loss (Mitsumoto et al., 2002). Furthermore it is involved in cell proliferation in both normal and immortal cells (Wadhwa et al., 1994) and is a marker of senescence (Kaul et al., 2003).

At present no histopathological studies have yet addressed whether and which type of cytoprotective stress protein is involved in non-nervous organs upon prolonged Al-exposure. The main objective of this study was to analyse the localization and temporal expression of HSP25, HSP72 and GRP75 in kidneys and liver of rat exposed to Al-sulphate for 3 and 6 months by histochemical and immunohistochemical methods. Since very recently the lysosomes were shown to be affected in kidneys by Al intake (Haynes et al., 2004), we also carried out an ultrastructural morphometric analysis of lysosomal compartment of kidney. Al exposure is a cofactor of aging (Miu et al., 2004), so in adult rats treated with Al peculiar morphological hallmarks of senescence, i.e. lipofuscin deposition and lysosomal derangement, should be present. Therefore an additional senile group (3 years old rats) was examined to compare ultrastructural morphometric data better.

Materials and methods

Animals and experimental treatments

Thirty Sprague-Dawley male 2 months old rats (Harlan Italy, Milan, Italy) were used at the beginning. They were housed in a controlled environment, at regular 12 h light and 12 h dark cycles/day and fed with standard rodent food and water ad libitum. Animal care was in compliance with Italian regulations on the protection of laboratory animals (D.M. 116192), as well as with EC regulations (L 358/1 12/18/1986). The animals were divided into three groups(8 rats/group) that received: (1) Aluminium sulphate (Al, Sigma-Aldrich Chemicals, Milan, Italy) 2.5% in drinking tap water daily for 3 months; (2) Aluminium sulphate 2.5% in drinking tap water daily for 6 months; (3) Tap water ad libitum as controls. Compared to adult controls, treated animals consumed 17±6 ml of water supplemented with 2.5% Al-sulphate corresponding to 33 mg of Al per rat per day (Rodella et al., 2001). Additionally 6 age-matched SD male rats (3 years old) received tap water ad libitum. At the end of treatment, all animals were deeply anesthetized with halothane and perfused transcardially with 100ml of saline, 300ml of 4% paraformaldehyde in phosphate buffer (pH 7.4). The kidneys and liver were excised and then fixed in 4% paraformaldehyde for 48h at 4°C then dehydrated in progressive ethanol concentration and finally embedded in paraffin wax (BDH).

Histopathological analysis

On 4 µm-thick paraffin sections differential stainings were performed to assess the effects of treatment: H&E to overall definition of toxicity and possible regeneration, PAS to define brush-border and membranes in renal proximal tubules, Masson trichrome to detect fibrosis and Perl’s Prussian blue to detect iron. The mitotic index determined as percentage of mitotic tubular epithelial cells or hepatocytes per the total number of cells in 30 high-power microscopic fields (x100) was evaluated. Digitally acquired images were analysed under a light microscope equipped with an image analyzer.

Immunohistochemical analysis

Sequential paraffin sections were collected on slides coated with poly-L-lysine (Sigma) and dried overnight at 37°C. The next day, sections were deparaffined, rehydrated and immersed in 3% H2O2 in methanol for 30 min at room temperature to block endogenous peroxidase activity. To avoid non-specific binding, some sections were incubated for 60 min in PBS with normal
goat or horse serum (1:5) at room temperature, then incubated overnight at 4°C with the following antibodies: a rabbit polyclonal antibody against mouse-HSP25 which cross reacts with rat protein (1:200); a mouse monoclonal antibody against human Hsp70 which cross reacts with mouse and rat Hsp70 (1:400); a mouse monoclonal antibody against human Grp75 which cross reacts with mouse and rat proteins (1:400). All antibodies were purchased from StressGen Biotechnologies, Canada. Following several washings in phosphate-buffered saline (PBS), slides were incubated with appropriate secondary biotinylated antibodies (anti-rabbit or anti-mouse IgG) and finally with the avidin-biotin peroxidase complex (Vector Laboratories). The reaction product was visualised using 0.3% H2O2 and 3',3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma). Control of immunohistochemistry was performed: omitting of the primary antibody or incubating serial sections with non immune rabbit or mouse serum respectively. None of these controls showed any brown staining.

**Morphometric and ultrastructural analysis**

Kidneys were fixed by immersion in 2.5% glutaraldehyde in phosphate buffer (pH 7.4), post-fixed in osmium tetroxide and embedded in araldite epoxy resin as previously reported (Stacchiotti et al., 2002). Semithin sections (1 µm-thick) were stained with toluidine blue and dense bodies were computed on computer-assisted histomorphometry using Image-Pro Plus program (Media Cybernetics Inc.). To evaluate blue stained vacuolar area, within the proximal tubules, ten midcortical tubules for each section, randomly selected, were estimated at the same magnification (x100). For ultrastructural morphometry, ultrathin sections (80 nm thick) were collected on copper formvar–coated grids, double stained with uranyl acetate and lead citrate and observed under a Philips TEM CM10 at 80 kV. High-power micrographs (x 21,000) of lysosomal compartment in different experimental groups were analysed by a point-counting method to accurately evaluate, with the best definition, numerical density of different subtypes of organules, i.e. lysosomes and lipofuscins.

**Statistical analysis**

Each experiment was performed at least in triplicate for each animal. Slides were blindly analysed by different investigators. Data are presented as means ± SD. Differences were evaluated by analysis of variance (ANOVA) corrected by the Bonferroni test and set at p<0.05.

**Results**

By histopathological analysis no overt necrosis was observed after 3 and 6 months Al exposure in the rat kidney and liver. At 6 months, focal nuclear pyknosis, brush-border detachment, fibrosis and occasional hepatic fat degeneration were detected. The mitotic index was not statistically different in kidney and liver exposed to Al versus control groups.

Semi-quantitative expression of three stress proteins, HSP25, HSP72, GRP75, in rat kidney and liver treated with aluminium sulphate compared to controls was resumed in Table 1.

**Kidney**

Histochemistry and Immunohistochemistry

After 3 months Al exposure, cortical proximal tubules revealed a regularly defined PAS-positive brush-border and also distal tubules were normal (Fig. 1A). By

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>Tap water</th>
<th>Al Sulphate 2.5% 3 MONTHS</th>
<th>Al Sulphate 2.5% 6 MONTHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROLS</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
</tr>
<tr>
<td>HSP25</td>
<td>Kidney C +/-</td>
<td>Kidney C +/-</td>
<td>Kidney C 2+</td>
</tr>
<tr>
<td></td>
<td>Kidney M +</td>
<td>Kidney M +</td>
<td>Kidney M 2+</td>
</tr>
<tr>
<td></td>
<td>Liver -</td>
<td>Liver -</td>
<td></td>
</tr>
<tr>
<td>HSP72</td>
<td>Kidney C -</td>
<td>Kidney C -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney M -</td>
<td>Kidney M -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver -</td>
<td>Liver -</td>
<td></td>
</tr>
<tr>
<td>GRP75</td>
<td>Kidney C +/-</td>
<td>Kidney C +/-</td>
<td>Kidney C +</td>
</tr>
<tr>
<td></td>
<td>Kidney M +/-</td>
<td>Kidney M 2+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver +</td>
<td>Liver +</td>
<td></td>
</tr>
</tbody>
</table>

Undetectable (-), weak staining (+/-), moderate staining (+), intense staining (2+); C: Cortex; M: Medulla; n: number of rats.

**Table 2.** Lysosomal subtypes in rat proximal tubules exposed to aluminium sulphate for 3 months vs aging.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tap water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 months old</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
</tr>
<tr>
<td>Al-sulphate 2.5%</td>
<td>13.4±9.6</td>
<td>27.4±10.5</td>
<td>16.8±9.7</td>
</tr>
<tr>
<td>3 years old</td>
<td>n=6</td>
<td>n=6</td>
<td>n=6</td>
</tr>
<tr>
<td>Cortical lysosomes</td>
<td>3.4±1.5</td>
<td>8.3±5.2</td>
<td>10.8±5.3</td>
</tr>
<tr>
<td>Cortical lipofuscin</td>
<td>18.6±10.3</td>
<td>32.1±12.8</td>
<td>25.4±11.2</td>
</tr>
<tr>
<td>Juxtamedullary lysosomes</td>
<td>3.8±1.6</td>
<td>10.8±5.2</td>
<td>13.6±10.1</td>
</tr>
<tr>
<td>Juxtamedullary lipofuscin</td>
<td>3.8±1.6</td>
<td>10.8±5.2</td>
<td>13.6±10.1</td>
</tr>
</tbody>
</table>

Numerical density is expressed as mean ± SD; A, B, C superimposed letters indicate significant value vs respective group (p<0.05); n: number of rats.
contrast, at 6 months Al sulphate treatment in rat kidney, abnormal proximal tubules with irregular PAS-positive brush-border but regular distal tubules were detected (Fig. 1B). Perl’s staining was restricted to a few proximal tubules in the renal cortex and glomeruli were unstained. HSP25 signal in the renal cortex was faint after 3 months Al treatment and similar to controls. The staining was restricted to brush-border lining the proximal tubules whereas distal tubules were unstained (Fig. 2A). After 6 months Al treatment, HSP25 staining became intense in cortical proximal tubules, restricted to altered brush-border (Fig. 2B).

Inducible HSP72 was undetectable in controls and 3
months Al treated rats (Fig. 3A) but appeared, after long treatment, in both proximal and distal tubules. Its signal was moderate within the cytoplasm of cortical proximal tubules whereas nuclei were unstained (Fig. 3B).

GRP75 was weak in proximal tubules associated with basolateral membranes in controls (Fig. 4A); at 3 months sometimes translocated in the nuclei (Fig. 4B) and at 6 months progressively enhanced in the corticomedullary junction (Fig. 4C). In the medulla, Henle loops were also heavily stained.

**Morphometry and Ultrastructure**

Using light microscopic morphometry, we evaluated on semithin sections a significant increase in the mean percentage of dense bodies/proximal tubule since 3 months Al treated with respect to the control kidneys (2% in controls vs 12% in Al group). This trend was maintained also after 6 months of exposure (data not shown). By electron microscopy, dense bodies were well characterized as primary lysosomes and secondary lipofuscins (Fig. 5). Furthermore in the senescent group we described similar polymeric lipofuscins (Fig. 6). The density of lysosomal subtypes in Al-treated versus senescent groups is resumed in Table 2. In the proximal tubules after 3 months Al treatment, both lysosomes and lipofuscins were significantly increased with respect to adult controls (lysosomal density about twice, lipofuscins triplicated). Furthermore the trend of lipofuscins in 3 months Al treated group was similar to aged rats, whereas lysosomes were significantly more in Al exposed than in senescent rats particularly in the juxtamедullary area.

**Liver**

**Histochemistry and immunohistochemistry**

After 3 months Al intake, a clear Perl’s positive iron-deposition was detected in pericentral parenchymal and Kupffer cells compared to control group. (Fig. 7A, 7B). Evident fibrosis by Masson’s method was described (Fig. 8).

HSP25 reaction was very low in hepatic parenchyma and did not change during Al-treatments (data not shown).

HSP72 was undetectable in the control liver (Fig. 9A) but in 3 months Al treated liver, was focal in the cytoplasm of pericentral hepatocytes (Fig. 9B) and after 6 months was also expressed in Kupffer cells (Fig. 9C).

GRP75 staining was moderate and associated to cell-membrane in inner pericentral hepatocytes but nuclei were negative at 3 months Al-exposure, almost similar to controls (Figs. 10A,B). After 6 months, GRP75-signal became diffuse also in perportal hepatocytes associated with high cytoplasmic vacuolation and intense in Kupffer cells (Fig. 10C).

**Discussion**

Despite its environmental abundance, for many years aluminium (Al) has been considered an indifferent metal from a toxicological point of view. This was due to its poor intestinal absorption (Druke, 2002) and probably to the difficulty in measuring its deposition inside organs (Priest, 2004). Indeed restricted Al ultrafilterability is one of the factors which limits renal
excretion together with its reabsorption by the renal tubules (Lote et al., 1995). When Al enters the cell, it is not metabolised but easily bound to phosphate groups of cellular structures (Martin, 1992). Thus this metal perturbs membranes and organules resulting in altered cellular homeostasis. Stress proteins are molecular chaperones essential in the maintenance and recovery of organules and other proteins altered by metals (Delmas et al., 1996). This immunohistochemical study shows the peculiar localization of three stress proteins (HSP25, HSP72, GRP75) in rat kidney and liver after chronic exposure to Al-sulphate. A clear induction of HSP72 after 3 months Al exposure was detected in liver whereas it was absent in kidneys as in controls. In contrast, over the same period, HSP25 was clearly expressed in the kidney but barely detectable in the liver. After long term Al exposure, stress proteins increased in specific regions and in peculiar cell-types. We further

Fig. 7. Perl’s staining in liver. Controls (A): Scarce signal; at 3 months Al (B) evident blue iron-deposition in the parenchyma. x 400.

Fig. 8. Masson’s staining in Al-treated liver. At 3 months periportal fibrosis. x 200.

Fig. 9. HSP72 immunostaining in rat liver. Controls (A): Undetectable in the hepatocytes, (V) pericentral vein; at 3 months Al (B): Focal cytoplasmic signal in the hepatocytes (arrows); at 6 months Al (C): Extensive signals in hepatocytes (H) and in Kupffer cells (squares). x 400.

Fig. 10. GRP75 immunostaining in rat liver. Controls (A): Faint in hepatocytes (H) and Kupffer cells (squares); at 3 months Al (B): Moderate basolateral in the parenchyma; at 6 months Al (C): Enhanced in hepatocytes (H) and Kupffer cells (squares). x 400.
demonstrated that in the kidney, tubular membranes and the lysosomal compartment in the juxtamedulla were affected by Al. By contrast, in the liver, abnormal iron deposition, periporal fibrosis and Kupffer cells involvement were the most relevant Al side-effects.

Among pathogenic mechanisms of Al, free-radicals deposition and altered iron homeostasis have been recently suggested in different experimental studies that used an antioxidant supply to ameliorate side-effects. The administration of melatonin in rats exposed to Al or vitamin E in liver cell-cultures did not reduce directly Al-uptake, but indirectly influenced lipid peroxidation of membranes and biochemical parameters of oxidative enzymes activities (Abubakar et al., 2003; Abreo et al., 2004; El-Demerdash, 2004; Yousef, 2004).

Oxidative damage was able to stimulate specific stress proteins. In particular HSP25, a cytoskeletal chaperone, strictly involved in F-actin assembly, was increased in cortical proximal tubules after 6 months Al-exposure. This is consistent with in vitro data by Sargazi et al. (2001) that used Al lactate for shorter periods. We previously reported HSP25 in strict association with the brush-border and its altered pattern as a reaction against cytoskeletal damage (Stacchiotti et al., 2002). However HSP25 expression was almost undetectable in the liver parenchyma exposed to Al. This protein usually increased inside the liver during inflammation and its level has been recently considered as a crucial sign against toxicity induced by acetaminophen in mice (Sumioka et al., 2004). This could be due to the absence in the hepatocytes, compared to the kidney, of selective sites of cytoskeletal proteins, i.e. the brush-border or to the difficulty of studying in vivo non parenchymal cells such as Kupffer cells. Indeed Kupffer cells are tissutal macrophages with a crucial role in hepatotoxicity (Ding et al., 2003). Various studies by X-ray microanalysis reported large Al deposition inside the lysosomes in this cell-type (Fiejka et al., 1996; Daimon et al., 2000).

In the HSP70 family, there are multiple members with essential roles in cytoprotection. The most studied HSP70 (HSP70i or HSP72), is virtually absent in a physiological state but induced in the cytoplasm often translocates inside nucleus upon stress (Ellis et al., 2000). Here we found that HSP72 distribution and their time course were different in the two examined organs. Inside the liver, HSP72 staining was faint in the cytoplasm of pericentral hepatocytes after 3 months-Al exposure whereas in the kidney it was undetectable as in controls. After prolonged Al exposure, we detected Kupffer cells that were heavily HSP72-positive. Using histochemistry and morphometry at light and ultrastructural level, we characterized the cellular effects of Al. By Perls’ Prussian blue staining, we found diffuse iron-deposits at 3 months of Al exposure within pericentral lobular hepatocytes whereas the kidney seemed only occasionally involved. This could be related to altered cellular homeostasis as a consequence of iron deposits caused by Al overloading in different organs. In addition, a similar signal in senescent liver agrees with altered metabolism induced by Al as reported in the hippocampus of rat exposed to aluminium gluconate (Miu et al., 2004). Al is associated with abnormal iron deposition and cellular oxidation (Zatta et al., 2003). The major iron storage protein, i.e. ferritin, was localized in the liver together with transferrin receptors. Ward et al. (2001) reported that, even if less then 1% of Al was associated with ferritin, iron deposition paralleled alterations induced by Al-gluconate in brain and other organs. Therefore many neurotoxicological studies, even if performed with different Al-salts and administration routes, had a common feature: i.e. Al alters iron-metabolism and contributes to neurodegeneration. Here we adopted a dose of 33 mg Al/animal/day, much lower compared to previous studies that used Al-citrate or Al-maltol corresponding to 1g Al-salts/Kg (Florence et al., 1994). Nevertheless our group reported that in rats orally exposed to 2.5% Al-sulphate for 5 weeks there were alterations in the NO pathway in the cerebellum and in the cerebral somatosensory cortex (Rodella et al., 2001). Here we analysed the effects of a similar Al dose on two non-nervous organs, the kidney and liver at different periods. Al ions cannot directly affect cell membranes and organelles but they stimulate Fe2+-dependent lipid peroxidation leading also to lysosomal fragility (Britton et al., 2002). Lysosomal internal acidic pH is needed to activate enzymes that digest engulfed proteins. Low pH is maintained by an ATPase proton pump that translocated H+ ions inside the lysosome. Zatta et al. (2000) demonstrated that Al sulphate and fluoride are the most effective salts that inhibited the proton pump so altering lysosomal function. In this study we clearly demonstrated, by ultrastructural morphometry, that since 3 months Al-sulphate exposure affects qualitatively and quantitatively the lysosomal system (lysosomes and lipofuscins) inside proximal tubules mainly in the juxtamedullary area. The abundance and morphology of lipofuscins were similar to senescent control rat kidney. These findings agree with Chagnac et al. (1987) who analysed ultrastructural changes in the kidney of rats receiving i.p. Al chloride for 3 months and with lipofuscin distribution reported in aged rat kidney by Melk et al. (2003). Haynes et al. (2004) recently described in the koala, fed with eucalyptus leaves contaminated with Al, the onset of renal failure and decreased lysosomal count in the cortical tubules. In contrast, another environmental study on the bivalve “Anodonta cygnea”, showed that Al increased the number of lysosomes in the kidney and that it remained high even after transfer to clean water (Kadar et al., 2001). Both different environmental and experimental in vivo studies stressed that renal lysosomes may be a specific Al target.

GRP75/mortalin, is a constitutive member of HSP70 family, localized in the cytoplasm and in organelles like mitochondria, lysosomes (Wadhwa et al., 2002) and endoplasmic reticulum (Ran et al., 2000). Recently in an experimental nephrotoxic study using mercury we
reported GRP75 overexpression in rat proximal tubules (Stacchiotti et al., 2004). Here this chaperone enhanced time-dependently both in the kidney and liver treated with Al with respect to controls and sometimes changed location. Indeed after Al-exposure, GRP75 sometimes was nuclear in the proximal tubules, but only cytoplasmic in liver hepatocytes. Nuclear presence of mortalin has been related to cell proliferation and recovery (Kaul et al., 1997).

Previous experimental studies with mercury and cadmium demonstrated that stress proteins localization in the organ–target preceds the onset of nephrotoxicity or hepatotoxicity (Goering et al., 1992, 1993). In contrast, Tolson et al. (2005) reported that stress proteins in preconditioned kidney are not directly related to uranium nephrotoxicity but represent an epiphenomenon due to tubular regeneration. However in our study, lacking overt histopathological features of Al-induced damage in liver and kidney at 3 and 6 months exposure, a regenerative progression could not be activated yet. Taken together, our data demonstrated that prolonged exposure to Al affects peculiar cellular sites causing cumulative sublethal effects that activate different chaperones in the rat kidney and liver.

The differential expression of stress proteins detected in kidney and liver exposed to Al could be due to different organ abilities, different absorption and deposition of the metal, interference with constitutive and developmentally-regulated stress proteins machinery (D’Souza and Brown, 1998). In conclusion, we suggest here that stress proteins may constitute a sensitive index of in vivo Al susceptibility of these two essential organs.

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