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Stress proteins in experimental nephrotoxicity: a ten year experience

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Summary

Heat shock proteins and glucose-regulated proteins represent an extraordinary mechanism of defense induced in the kidney by chemicals or drugs and essential to survive. Here we resume our experience on the presence and regulation of stress proteins into acute and chronic nephrotoxic models in rodents and *in vitro*.

In acute renal damage, induced in rats by a single injection of inorganic mercury, stress proteins enhanced in a dose-dependent manner to recover cytoskeleton and mitochondria and maintain nuclear activity. When we pre-treated mercury injected-rats with antioxidant melatonin or with bimoclomol, a stress proteins-coinducer, stress proteins expression was modulated together with tubular recovery. Similar data were obtained in ischemia-reperfusion in rats treated with stannous chloride, that provided cytoprotection stimulating heme oxygenase induction. During nephrotoxicity induced by administration of cyclosporine A at therapeutic dosage for 1-2 months, stress protein overexpression well correlated with oxidative and cell death, but decreased if we counteracted renal damage using antioxidants. In aluminium intoxication through drinking water for 3-6 months, we detected a time-dependent stress response in the rat kidney that was organ specific and different from the liver. *In vitro* studies on rat tubular proximal cells exposed to heavy metals demonstrated that stress protein expression was related to peculiar mechanisms of action of each metal. In conclusion, experimental studies on the renal chaperones can greatly contribute to understand their role, and agents able to modulate the stress response might be considered promising therapeutic tools to reduce nephrotoxicity.

Key words

Stress proteins, cyclosporine, mercury, aluminium, kidney

Introduction

Prokaryotes and eukaryotes adopted a highly conserved mechanism against stress, called heat shock response, that consists in the rapid and specific activation of heat shock proteins (HSPs; Kultz, 2005). Even if Hans Selye (1955), the pioneer of stress, defined stress "any sudden change in the cellular environment, to which the cell is not prepared to respond, such as heat shock", actually we know that almost all types of stress induce HSPs. The discovery of HSPs was due to Ferruccio Ritossa (1996), an Italian geneticist, who studying puffs in the salivary glands of *Drosophila melanogaster* evidenced new RNA synthesis after hyperthermia. The main dogma of stress proteins research is that, if there is an increased need of proteins recovery

and a scarce elimination via the proteasome, HSPs help cells as efficient chaperones to mediate proteins assembly and recovery (Ellis, 1996). Indeed their functions are broader and expanding such as anti-apoptotic regulators and endoplasmic reticulum stress signals (Inagi, 2009). Besides HSPs, stress proteins include also glucose-regulated proteins (GRPs) and metallothioneins (MTs), classified according to the molecular weight, with different subcellular locations and functions. Among small proteins, we have studied HSP25 and alpha B-crystallin as early markers of ischemic damage and apoptosis (Arrigo, 2007) and heme oxygenase 1-HO1, known also as HSP32, which is the cytoprotective inducible isoform of this enzyme that converts heme to carbon monoxide and biliverdin/bilirubin with release of iron (da Silva et al., 2001). Among medium-sized proteins, we have analysed several members of HSP70 family: HSC70, a constitutive chaperone, HSP72/HSP70, the inducible member and GRP75/HSP75, a mitochondrial member that recovers respiratory enzymes during oxidative stress (Wadhwa et al., 2002). Other proteins studied have been GRP78/BiP, the main endoplasmic reticulum chaperone involved in Ca^{2+} regulation (Kimura et al., 2008), and inducible metallothioneins (MTs), which participate to detoxification of metals and oxygen free radicals (Florianczyk, 2007).

The kidney is a favourite target to study stress proteins due to its physiological reaction against chemical and physical stresses (Borkan and Gullans, 2002). So, we aimed at best characterizing the set of stress proteins involved in nephrotoxicity and modulating their expression without using genetic manipulations or transgenic animals. The final goal was to find new potential therapeutic opportunities according to Soti et al. (2005).

Materials and methods

In vivo studies

We treated several groups of adult rats (Sprague Dawley or Wistar, purchased from Charles River, Milan), housed in a controlled environment with 12 h light/12 h dark cycle at 20°C, relative humidity 50%, fed with a standard diet and water ad libitum. Rats were cared according to national (D.M. 1161/92) and EU regulations for the protection of laboratory animals. All chemicals were of the highest grade and purchased from Sigma-Aldrich (Milan).

For chronic protocols we treated animals as follows:

a) Daily s.c. injection of cyclosporine A (CsA; Sandimmun, Sandoz, Basel, Switzerland), 15 mg/kg/day in olive oil, for 30-60 days; some rats were administered melatonin at 5-10 mg/kg/day and CsA for the same periods of time; b) aluminium sulphate dissolved in drinking water, 2.5%, for 3-6 months; animal were killed at the end of treatments.

For acute protocols we treated animals as follows:

a) a single intra-peritoneal injection of mercuric chloride, 1-3.5 mg/kg 14 h or 24 h before sacrifice; in one additional experimental group, melatonin, 5 mg/kg subcutaneously, or Bimoclomol (Biorex R&D, Hungary), 50 mg/kg per os, were administered before mercury, animals were sacrificed after 14 h or 24 h;

b) bilateral, 45 min occlusion of renal arteries followed by 30 min-3 h reperfusion; some rats were pretreated with intra-peritoneal injection of stannous chloride, 10 mg/kg, 12 h before ischemia.

In vitro studies

NRK52E, a rat renal cell line from proximal tubules, was routinely cultured in Dulbecco's modified medium supplemented with 10% foetal bovine serum, 0.1 mmol/l non-essential aminoacids, 2 mmol/l L-glutamine and antibiotic-antimycotic solution, at 37°C in a 5% CO_2 humidified atmosphere till the beginning of treatments with mercuric chloride ($HgCl_2$) 1-40 μmol/l, or lead chloride ($PbCl_2$) 60-500 μmol/l, for 24-72 h.

On all the above models we studied the effects of treatments by routine histopathology and electron microscopy analyses and by immunohistochemistry and immunoblotting with a set of commercial antibodies against stress proteins. Statistical analysis of data was performed in a blind fashion by analysis of variance (ANOVA) corrected by Bonferroni test (significant at $p < 0.05$).

Results

Experimental *in vivo* nephrotoxicity

Cyclosporine A enhanced the constitutive expression of HSP25 and alpha-B-crystallin in glomeruli, proximal tubules and collecting ducts, where often it appeared within nuclei. Inducible HSP72 was found in the proximal and distal tubules, in Henle limbs and collecting ducts. In particular the presence of this inducible molecule in the macula densa may be related to increased renin/angiotensin production due to CsA hypertensive effect. Curiously, in rats administered with melatonin plus CsA, the immunostaining for all antigens was decreased and the tubular morphology was preserved to a great extent. Aluminium sulphate stimulated the expression of HSP25 in proximal tubules and that of GRP75 and HSP72 in mid-cortical area, all these effects were time-dependent and more evident after 6 months.

A single mercuric chloride injection induced HSP72 in proximal tubules (S3-segment) that are the main mercury-target. Moreover mitochondrial chaperones and MTs were also overexpressed. By contrast, when we used melatonin, all signals became lower. Remarkably, when we pretreated rats with Bimoclomol before mercury, HSP72 expression was enhanced in proximal tubules and this was concurrent with tubular recovery. In ischemia-reperfusion, inflammation and apoptosis were significantly attenuated by induction of HSP32 by stannous chloride. Cumulative data obtained *in vivo* are resumed in Table 1.

Experimental *in vitro* nephrotoxicity

In the NRK52-E cell line exposed to mercury and lead chloride, the stress proteins activation was specific for each metal tested. In particular, 20 μmol/l $HgCl_2$ was able to induce HSP72 and MTs expression and to enhance that of HSP25, whereas 60-300

μmol/l $PbCl_2$ stimulated the expression of GRP78. Stress proteins response occurred within 24 h, earlier than overt evidence of oxidative damage and apoptosis or necrosis. Cumulative data obtained *in vitro* are resumed in Table 2.

Discussion

Renal cells defend themselves against toxicants by early events that favour survival, the most spectacular being the stress response (Arya et al., 2007).

One major finding of ours on stress proteins, as predictive "biomarkers of damage" in the kidney, was the demonstration of their common presence in several toxic conditions such as sublethal exposure to aluminium (Stacchiotti et al., 2006a), chronic treatment with cyclosporine (Stacchiotti et al., 2001) and acute damage induced by mercury *in vivo* (Stacchiotti et al., 2004) or *in vitro* (Stacchiotti et al., 2009). Second, the subtype of over expressed stress protein was strictly related to the stress type and often the over expression reversed when we used the antioxidant melatonin during stress (Stacchiotti et al., 2002; Stacchiotti et al., 2006c). The third important result was the renal protection obtained by using pharmacological modulators of HSPs. In particular, we tested two drugs (Bimoclomol and stannous chloride) before or concurrent with the nephrotoxic stimulus (Stacchiotti et al., 2006b; Li Volti et al., 2007). Bimoclomol, a HSP72-coinducer which is effective only if associated to stress (Vigh et al., 1997), attenuated acute nephrosis by mercury; stannous chloride, activating heme oxygenase 1 by the hormesis, alleviated ischemia. Despite the beneficial effects of HSPs modulators reported by us and others (Putics et al., 2008), clinical strategies for up-regulation of stress proteins remain elusive. We hope that more experimental studies on this topic might stimulate clinical trials to transfer the results to humans.

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Tables

Table 1 – The stress response to experimental *in vivo* nephrotoxicity

	CsA	CsA + Melatonin	Hg	Hg + Melatonin	Hg + Bimoclomol	Ischemia	Ischemia + SnCl	Al
HSP25	↑↑	↓	↑	↓	↑	↑	↓	↑↑
HSP32	–	–	–	–	–	–	↑↑	–
HSP72	↑	↓	↑	↓	↑↑	↑	↓	↑
GRP75	↑	↓	↑	↓	↑	↑	↓	↑
MTs	↑	↓	↑↑	↓	↑	↑	↓	↑

Legends: (–) absence; (↑) increase; (↑↑) marked increase; (↓) decrease.

Table 2 – The stress response to experimental *in vitro* nephrotoxicity

	NRK52E CTRL	HgCl2 20μM	HgCl2 40μM	PbCl2 60μM	PbCl2 300μM
HSP25	↑	↑↑	↑↑	↑	↑
HSP72	–	↑	↑↑	–	–
GRP75	↑	↑↑	↑↑	↑	↑
GRP78	↑	↑	↑	↑↑	↑↑
MTs	–	↑↑	↑↑	–	–

Legends: (–) absence; (↑) increase; (↑↑) marked increase; (↓) decrease.