RAPID REPORT

INDUCTION OF HEAT SHOCK PROTEIN 70 REDUCES THE ALTERATION OF STRIATAL ELECTRICAL ACTIVITY CAUSED BY MITOCHONDRIAL IMPAIRMENT

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Abstract—Since mild thermal stress seems to exert neuroprotection via induction of heat-shock protein 70 kDa (hsp70), we tested whether hsp70 would preserve striatal bioelectrical activity under conditions of mitochondrial impairment. Corticostriatal slices from rats that had undergone mild thermal stress were exposed to either rotenone or 3-nitropropionic acid (3-NP), that selectively inhibits mitochondrial complex I and complex II, respectively. Rotenone is utilized to obtain an experimental model of Parkinson's disease while 3-NP replicates Huntington's disease phenotype in experimental animals. The cerebral hsp70 increase did not alter field potential amplitude of the slices but partially protected them against rotenone-induced neurotoxicity. Similarly, induction of hsp70 had also a partial neuroprotective effect on the neurotoxicity caused by 3-NP on striatal field potential. Since rotenone and 3-NP treatments mimic the mitochondrial impairment and oxidative stress that contribute to development of Parkinson's disease and Huntington's disease, these data suggest that induction of hsp70 might represent a possible neuroprotective mechanism against the pathophysiological chain of events implicated in these neurodegenerative disorders. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: hsp70, mitochondrial impairment, 3-NP, rotenone, striatal neurotoxicity.

In mammals, the heat shock protein 70 kDa family (HSP70) consists of several members some of which, such as constitutive heat shock protein 70 kDa (hsc70; synonymous to hsp73), are constitutively expressed while inducible heat shock protein 70 kDa (hsp70; synonymous to hsp72) is induced by stresses. Both of them facilitate nascent protein

folding, refolding or degradation of abnormal proteins, transport and translocation across membranes of newly-synthesized proteins into intracellular compartments, and inhibit formation of incorrect protein aggregates (Craig, 1993). hsp70 Induction by mild hyperthermia or pharmacological agents seems to exert neuroprotection in cerebral ischemia and in neurodegeneration (Ota et al., 2000; Sittler et al., 2001; Lu et al., 2002; Ren et al., 2004).

In this study, we tested whether overexpression of hsp70, induced by mild heat shock, could prevent electrophysiological alterations in two *in vitro* models of neurotoxicity obtained by administration of rotenone and 3-nitropropionic acid (3-NP), which selectively inhibit mitochondrial complex I (Betarbet et al., 2000; Costa et al., 2008) and complex II (Calabresi et al., 2001; Centonze et al., 2006; Saulle et al., 2004), respectively. Rotenone is utilized to obtain an experimental model of Parkinson's disease (PD) (Betarbet et al., 2000). Moreover, it has also been postulated that rotenone not only acts as a dopaminergic toxin but also generates lesions that are not limited to midbrain dopaminergic neurons. In fact, rotenone intoxication *in vivo* has been suggested to model atypical forms of PD such as progressive supranuclear palsy (Hirsch et al., 2003).

3-NP replicates mitochondrial dysfunction and striatal lesions observed in Huntington's disease (HD) (Brouillet et al., 2005; Di Filippo et al., 2006).

Our findings suggest that mild heat shock and resulting hsp70 induction do not alter basal electrophysiological properties of corticostriatal slices while they partially protect the electrical activity recorded from slices exposed to either rotenone or 3-NP.

EXPERIMENTAL PROCEDURES

Animals and heat shock preconditioning

Male Wistar rats ($n=18$, 4–6 weeks, Charles River, Calco, Italy) were randomly assigned to either heat-shocked (HS; $n=10$) or control ($n=8$) groups. To induce heat stress, the rats were exposed to a temperature of 43 °C until body temperature reached 41.5–42 °C. Then they were allowed to recover their normal body temperature at room temperature. Four hours later, they were sacrificed by decapitation under chloral hydrate (400 mg/kg) anesthesia.

Brains were quickly removed and corticostriatal coronal slices (270 μm thick) were cut according to a method previously described (Calabresi et al., 1998, 2001; Centonze et al., 2006; Tozzi et al., 2007; Costa et al., 2008). Control animals underwent the same experimental procedure without being HS.

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Abbreviations: HS, heat shocked; hsp70 (synonymous to Hsp72), inducible heat shock protein 70 kDa; PD, Parkinson's disease; 3-NP, 3-nitropropionic acid.

The study was approved by the Bioethical Committee of the University of Perugia and was conducted according to the European Communities Council Directive of November 1986 (86/609/EEC). Every effort was made to minimize the number of animals used and their suffering.

Electrophysiological study

The brain slices were transferred to a maintenance chamber and submerged in a continuously flowing Krebs's solution (pH 7.4) containing (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl₂, 1.2 NaH₂PO₄, 2.4 CaCl₂, 10 glucose, and 25 NaHCO₃ and in which a mixture of 95% O₂ and 5% CO₂ bubbled. Each slice was transferred to a recording chamber and submerged in the flowing Krebs's solution (32–33 °C; 2.4–2.6 ml/min) bubbled with 95% O₂ and 5% CO₂. Rotenone (Sigma-Aldrich, Milan, Italy) was first dissolved in dimethylsulfoxide (DMSO), (Merck KGaA, Darmstadt, Germany) and then diluted in Krebs's solution to a final concentration of 1 μ M. Instead, 3-NP (Sigma-Aldrich, Milan, Italy) was directly dissolved in a Mg²⁺-free Krebs's solution to a final concentration of 10 mM. Drugs were bath-applied by switching the solution to one containing a known concentration of drugs (Tozzi et al., 2007). For patch-clamp recordings we used a method previously described (Costa et al., 2008).

Extracellular recordings of striatal neurons were carried out by using microelectrodes pulled from borosilicate glass pipettes backfilled with 2 M NaCl (15–20 M Ω). An Axoclamp 2B amplifier (Axon Instruments, Foster City, CA, USA) was used for extracellular recordings.

Field potentials were evoked every 10 s by means of bipolar electrodes connected to a stimulator unit (Grass Telefactor, West Warwick, RI, USA). The stimulating electrodes were located in the corpus callosum area to activate corticostriatal fibers. Recording

electrodes were placed within the striatum, located 1–2 mm from the white matter. The field potential amplitude was defined as the average of the amplitude from the first positive peak to the negative one and the amplitude from the negative peak to the second positive one.

Quantitative data were expressed as a percentage of the field potential amplitudes with respect to the basal control amplitude values, the latter representing the mean of responses recorded during a stable period (15 to 20 min). Off-line analysis was performed using Clampfit (Axon Instruments, Foster City, CA, USA) and Microcal Origin (Northampton, MA, USA) software. For the input–output curve, a stimulus of increasing voltage intensity and constant duration (200 μ s) was delivered every 5 s.

Western blot analysis

Pools of corticostriatal slices from each HS and each control animal were homogenized to determine levels of hsp70 by Western blot analysis using a mouse monoclonal anti-hsp70 (SPA-810, StressGen Biotechnologies, Victoria, Canada) according to a method previously described (Mariucci et al., 2007).

Statistical analysis

Data are presented as means $\pm$ SEM. Two-way ANOVA and Bonferroni's post hoc test were used for statistical analysis of electrophysiological data. Statistical differences between densitometric data were analyzed by Mann–Whitney *U*-test.

RESULTS

Medium spiny neurons were recorded from control (*n*=8) and from HS (*n*=8) slices by conventional whole-cell patch-clamp

Fig. 1. Hsp70 overexpression does not affect basal electrophysiological properties recorded from corticostriatal slice preparations. (A) Voltage traces (upper) show firing discharge induced by positive current steps (lower) and recorded from two medium spiny neurons during whole cell patch clamp recordings in slices from control (left) and HS rats (right). (B) Current voltage plots obtained from several medium spiny neurons recorded from control and HS rats. (C) Input–output curves representing extracellularly recorded field potential amplitude at different intensities of stimulation both in control and HS animals.

recordings (Costa et al., 2008) in order to explore possible differences in the electrophysiological properties of these neurons. We performed current–voltage relationship and, as shown in Fig. 1A, B, the plots represent similar curves in the two groups suggesting no differences in their intrinsic electrical properties ($P>0.05$). As shown in the input–output curves presented in Fig. 1C, field potential amplitudes were similar when the slices, obtained either from control animals or from HS rats, were stimulated with increasing stimulus intensities (control vs. HS, $P>0.05$, $n=9$ and $n=10$, respectively).

The addition of rotenone ($1 \mu\text{M}$) to the perfusion solution caused a rapid continuous reduction of corticostriatal field potential amplitude which was almost completely abolished ($\sim 90\%$) within 25 min in the controls ($n=8$) (Fig. 2A, B). The field potential reduction, relative to the HS slices, showed a different time course (Fig. 2A, B). Indeed, 15 min after the onset of rotenone application, the slices ($n=10$) from HS animals still presented a field potential amplitude of about 90% while in the control ones it was about 57% ($P<0.01$). Moreover, in the HS slices, the amplitude of field potentials at 20 and 25 min was still higher than controls ($P<0.01$ and $P<0.05$, respectively). As expected, cerebral slices from HS rats expressed higher levels of hsp70, with respect to euthermic controls (Fig. 2C, D).

In the presence of 3-NP, within 10 min, the control slices ($n=10$) showed a reduction of about 50% of the basal value of the field potential amplitude, which reached its maximum reduction ($\sim 93\%$ in mean) within 35 min (Fig.

3A, B). Conversely, HS slices ($n=10$) showed a field potential amplitude that was significantly higher at every recording time. In particular, 35 min after the addition of 3-NP, when the field potential of the controls was completely abolished, the amplitude of field potential of the HS slices was still about 50% of its basal value. Also the 3-NP-treated slices from HS rats had levels of hsp70 that were higher than those from the euthermic controls (Fig. 3C, D).

Taken together our data indicate that while overexpression of hsp70 did not modify the electrophysiological properties of the corticostriatal slices, it seemed to confer resistance to inhibition of both complex I and complex II of mitochondrial respiratory chain.

DISCUSSION

Numerous studies demonstrate that mild heat shock confers cerebral cytoprotection against different stressors via induction of heat shock proteins (HSPs). Actually, mild heat shock leads to induction of stress proteins other than hsp70, such as hsp25/27 and hsp90. These proteins are expressed at different time scales and they also have a specific localization, suggesting that they have distinct roles during stress response and they may cooperatively contribute to the protection of neuronal cells from stressful insult (Kabakov and Gabai, 1995; Nishizawa and Nagata, 1999).

A possible neuroprotective role of hsp70 has never been addressed by utilizing an electrophysiological approach. In

Fig. 2. Hsp70 overexpression reduces the irreversible field potential changes induced by rotenone. (A) Traces of a field potential recorded before, at 20 min, and at 35 min after application of $1 \mu\text{M}$ rotenone in a corticostriatal slice preparation from control or HS rats. (B) Time course of the field potential amplitude in presence of $1 \mu\text{M}$ rotenone in corticostriatal slice preparations from control and HS rats; * $P<0.05$, ** $P<0.01$. (C) Representative Western blot showing Hsp70 overexpression in corticostriatal slice homogenate of HS rats versus euthermic controls. Expression of β -actin was also analyzed as loading control. (D) Bar graph of Hsp70 densitometric data in pooled corticostriatal slice homogenates from control ($n=3$) or HS ($n=4$) rats. Data are mean $\pm$ SEM * $P<0.05$.

Fig. 3. Hsp70 overexpression reduces the irreversible field potential changes induced by 3-NP. (A) Traces of a field potential recorded before, at 20 min, and at 35 min after application of 10 mM 3-NP in a corticostriatal slice preparation from control or HS rats. (B) Time course of the field potential amplitude in presence of 10 mM 3-NP in corticostriatal slice preparations from control and HS rats; ** $P < .01$. (C) Representative Western blot showing Hsp70 overexpression in corticostriatal slice homogenate of HS rats versus euthermic controls. Expression of β -actin was also analyzed as loading control. (D) Bar graph of Hsp70 densitometric data in pooled corticostriatal slice homogenates from control ($n=3$) or HS ($n=4$) rats. Data are mean $\pm$ SEM * $P < 0.05$.

the present study we carried out the electrophysiological analysis 4 h after the heat shock. In fact, a significant increase in hsp70 has been detected between 1.5 and 6 h after hyperthermia (Krueger et al., 1999). Conversely, hsp25/27 was expressed only 24 h following such stress (Krueger-Naug et al., 2000; Franklin et al., 2005).

To explain the molecular mechanisms underlying hsp70-mediated cellular protection against rotenone and 3-NP neurotoxicity, several possibilities can be envisaged, considering that a complex combination of molecular events mediates cell death when mitochondrial energy metabolism is impaired. In both cases, energetic deficit and oxidative stress result in disrupted ionic homeostasis, irreversible membrane depolarization, increased intracellular calcium, lowered intracellular pH, kinase activation, increased free radicals, and misfolding and aggregation of many proteins including enzymes and constituents of cytoskeletal and contractile structures.

Hsp70 protects ATP-depleted cells from rapid necrotic death by inhibiting the aggregation of cytoskeletal proteins. This idea is supported by the results of studies on the phenomenon of cell resistance to prolonged energy deprivation after mild thermal stress that were carried out in murine tumor cells treated with rotenone to block ATP generation (Kabakov et al., 2002). In these cells, in which ATP rapidly decreased to 3–6% of the initial level, a rapid necrotic death was directly correlated with aggregation of cytoskeletal proteins and membrane damage. After mild thermal stress diminution in aggregated insoluble proteins

was coupled with the delay of rotenone-induced necrotic death in these cells overexpressing hsp70.

Resistance to both mitochondrial toxins of slices overexpressing hsp70 could be ascribed in great part to the protective action exerted by the chaperone against the oxidative stress secondary to inhibition of mitochondrial complex I or complex II (Betarbet et al., 2000). This hypothesis is corroborated by several studies focused on the role of hsp70 in reducing mitochondria impairment-dependent oxidative stress (Lee et al., 2001). On the other hand, cases exist in which hsp70 overexpression does not protect against mitochondrial neurotoxins. For example, in hippocampal cell cultures, while overexpression of hsp70 protected neurons against a heat shock, it did not protect against 3-NP neurotoxicity (Fink et al., 1997).

Astrocytes from hsp70 transgenic mice were protected from oxidative stress produced by hydrogen peroxide in a dose-dependent manner and, to a lesser extent, by glucose or combined glucose–oxygen deprivation, confirming that the protective effects of the overexpressed protein are related to the nature and severity of the insults (Lee et al., 2001). In line with the aforementioned data, it has been demonstrated that hsp70 transfection can significantly prevent rotenone-mediated mitochondrial inhibition and oxidative stress and rotenone-mediated α -synuclein aggregation in mesencephalic/neuroblastoma hybrid cells (MES) cells (Zhou et al., 2004).

An additional function of hsp70 in protecting cells from rotenone and 3-NP toxicity can be expressed in terms of facilitation of the intracellular trafficking of polypeptide

chains destined for post-translational insertion into organelle membranes by preventing them from aggregation and/or by transporting them to the translocation pore. In particular, considering that part of the subunits of mammalian complexes I, III, IV and V and all the subunits of complex II, cytochrome c_1 , and citrate synthase are encoded for by nuclear DNA (Johns, 1996), an hsp70-mediated protection/enhancement of mitochondrial respiratory chain may be exerted primarily by positively influencing both the translation and import of nuclear-encoded protein in the mitochondria. This hypothesis was first advanced on the basis of findings from studies in which myocardial tolerance to prolonged ischemia and reperfusion was investigated in both heat-stressed and hsp70-transfected animal hearts (Sammut and Harrison, 2003). On the whole, this hypothesis fits with our data showing hsp70-mediated resistance against rotenone and 3-NP cytotoxicity in corticostriatal slices and could explain the greater resistance of corticostriatal slices to 3-NP with respect to rotenone. The 3-NP, as inactivator of succinate dehydrogenase, inhibits both the Krebs cycle and complex II of the mitochondrial electron transport chain (Coles et al., 1979). Therefore, the high levels of cytosolic hsp70 could have enhanced the energetic capacity of the slices by facilitating both translation and maintenance of the proper folding of both succinate dehydrogenase and subunits of complex II (which are codified by nuclear DNA).

CONCLUSION

In conclusion, this electrophysiological study, using *in vitro* application of neurotoxins that model neurodegenerative disorders of the basal ganglia, shows that the induction of hsp70 partially preserves electrical activity during mitochondrial dysfunctions. Interestingly, it has been previously postulated that preserving electrical activity may be crucial in itself in preventing neurons from initiating a cell death program (Michel et al., 2007).

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