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Transfection-mediated expression of human Hsp70i protects rat dorsal root ganglian neurones and glia from severe heat stress

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Considerable evidence suggests that the expression of heat shock proteins prior to a toxic insult (e.g. ischaemia, excitoxins, heat) can confer protection to neurones and glia. It is not certain which hsp(s) are involved in conveying these neuroprotective effects. Here we show that calcium phosphate-mediated transfection of dorsal root ganglia with an EF-1α promoter-hsp70i expression vector significantly increased the survival of neurones and glia exposed to a severe heat stress. These data suggest that overexpression of hsp70i plays an important role in protecting neurones and glia from the denaturing effects of severe thermal stress. Inducing the expression of specific hsps may lead to the development of novel treatment strategies for CNS diseases.

Transfection; Heat shock protein 70; Neuroprotection; Primary neuronal culture

1. INTRODUCTION

Cells respond to a heat shock by inducing the synthesis of stress or heat shock proteins (hsp) and by suppressing transcription of cellular proteins [1,2]. Hsp's are characterised by their molecular weights with the main families being 20, 70 and 90 kDa. The 70 kDa family are distinguished by being the most abundantly produced following stress, the most conserved throughout evolution and the most extensively investigated [1,2]. Members of the hsp70 family are essential for the translation, folding [3,4] and degradation of proteins [5] and their expression has been correlated with protection of cells from severe heat stress [6].

In the CNS, the induction of heat shock proteins by exposure of cells to a sub-lethal elevation in temperature has been shown to protect cells against glutamate toxicity [7,8]. Correlative evidence suggests that a heat shock response may also be protective against ischaemic stress [9,10] and free radical damage [11]. In non-neuronal tissues the inducible hsp70 protein (hsp70i) has been shown to play a role in mediating protection from heat stress [12,13]. However, it is not clear whether expression of hsp70i plays a functional role in protecting neuronal cells from stress. In this study we transfected rat dorsal root ganglion neurones and glia with the human hsp70i gene using the powerful elongation factor-1α promoter (EF-1α) to drive expression and examined the effect of a severe heat shock on the survival of transfected and control cells.

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2. MATERIALS AND METHODS

2.1. *Preparation of primary cultures*

Dorsal root ganglia (DRG) were dissected from 6–8 rat embryos (E15–16), placed in Hank's balanced salt solution (HBSS) and then dissaggregated by incubation in Hank's without Ca^{2+} and Mg^{2+} containing 0.1% trypsin for 30 min at 37°C. The cells were washed three times in Hank's with Ca^{2+} and Mg^{2+} and triturated in 500 μl of Dulbecco's modified Eagles medium (DMEM) supplemented with glutamine (100 mM), antibiotic/antimycotic (1 μl/ml Sigma), NGF (50 ng/ml) and containing 5% foetal calf serum (FCS) (this medium plus supplements will be referred to as DMEMS). After trituration 2 ml of DMEMs was added and this suspension was aliquoted (50 μl) onto glass cover slips coated with poly-L-lysine (0.1 mg/ml) which had been placed in multiwell (24) dishes. The culture suspension was incubated for 40 min at 37°C in a CO_2 (5%) incubator and after this period a further 0.5 ml of DMEMs was added.

2.2. *DNA transfection*

Cells were transfected by calcium co-precipitation essentially as described by Gabellini [14]. Twelve to eighteen hours after plating cells were washed twice with DMEM and fed with DMEMS containing 5% FCS. Plasmid DNA (10 μg/μl) was mixed with 250 mM $CaCl_2$ and this solution added dropwise to an equal volume of 5 mM HEPES pH 7.0, 1.5 mM Na_2HPO_4, 280 mM NaCl, 10 mM KCl, and 12 mM glucose. This solution was left to stand for 15 min and 50 μl then added to each well. After 7 h the cells were washed twice and refed with DMEMS containing 5% FCS. The cells were then incubated overnight to allow protein to be expressed and then stained for β-galactosidase activity or subjected to a severe and potentially lethal heat stress (46°C for 20 min).

2.3. β-*Galactosidase histochemistry*

Cultures were fixed with 0.2% glutaraldehyde for 30 min, washed three times in 0.1 M phosphate-buffered saline (PBS) and incubated in 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM magnesium chloride containing 1 mg/ml 4-Cl,5-bromo,3-indolyl-β-galactosidase for 24 h at 37°C [15].

2.4. *Preparation of plasmids*

Hsp70i (plasmid pH 2.3, a gift from Dr. R. Morimoto [16], and a

1.2 kb coding fragment of the LacZ gene (a gift from Dr Terry Rabbitts) was sub-cloned into pEF-BOS, a powerful expression vector which uses the promoter of human elongation factor 1α (EF-1α) chromosomal gene [17]. In addition to the pEF-Bos promoter, the CMV and SV40 promoters were also used to drive expression of lacZ.

2.5. *Morphometric analysis*

Cells staining blue, indicating the presence of β-galactosidase resulting from the expression of lacZ, were counted at ×160 magnification. The whole cover slip area was counted for each calcium phosphate transfected culture and the results compared by *t*-test.

3. RESULTS

DRG cultures were transfected 8 h after plating with the Bos lacZ plasmid to assess the efficiency of the calcium phosphate precipitation technique. β-galactosidase activity (evidence of lacZ expression) was detected histochemically (Fig. 1) and in preliminary experiments we found that expression of lac-Z could be seen in cells 8 h–5 days after transfection, and that the procedure resulted in approximately 10–15 times more glia than neurones being transfected. The overall efficiency of the procedure was obtained by calculating the ratio of LacZ expressing neurones and glia from transfected DRG cultures to neurones and glia present in non-transfected cultures (cells were counted under low power microscopy). Gabellini et al. [14] reported transfection rates of 90% into neurones, however, we were unable to achieve this rate of transfection, and found neurones were routinally transfected at approximately 1% efficiency and glia at approximately 50% efficiency. Carrying out the transfections in 0.5% as opposed to 5% FCS (as suggested by Gabbellini et al. [14] did not improve the rate of transfection but led to fewer neurones surviving the procedure. Using the cytomegalo virus promoter (known to be a powerful promoter in neurones) instead of the EF-1a promoter to drive lacZ expression did not improve the neuronal transfection rates. When the SV40 promoter was used to drive lacZ expression very few blue staining cells were seen.

To study the neuroprotective effects of hsp70i we co-transfected rat DRG with the Bos-lacZ and Bos-hsp70i plasmids. The plasmid vectors were heated to 65°C just prior to transfection so that upon cooling plasmids would associate randomly, forming homoge-

Fig. 1. DRG cultures stained histochemically for β-galactosidase resulting from the expression of lacZ. a and b are DRG cultures transfected respectively with the bos lacZ plasmid alone, or with both the Bos lacZ and Bos hsp70i plasmids, they were not exposed to a heat shock. c and d are DRG cultures transfected respectively with the Bos lacZ plasmid alone, or with both the Bos lacZ and Bos hsp70i plasmids, and then exposed to a severe heat shock (46°C for 20 min). These results show that cells transfected with the Bos hsp70i plasmid are more resistant to the toxic effects of severe heat shock.

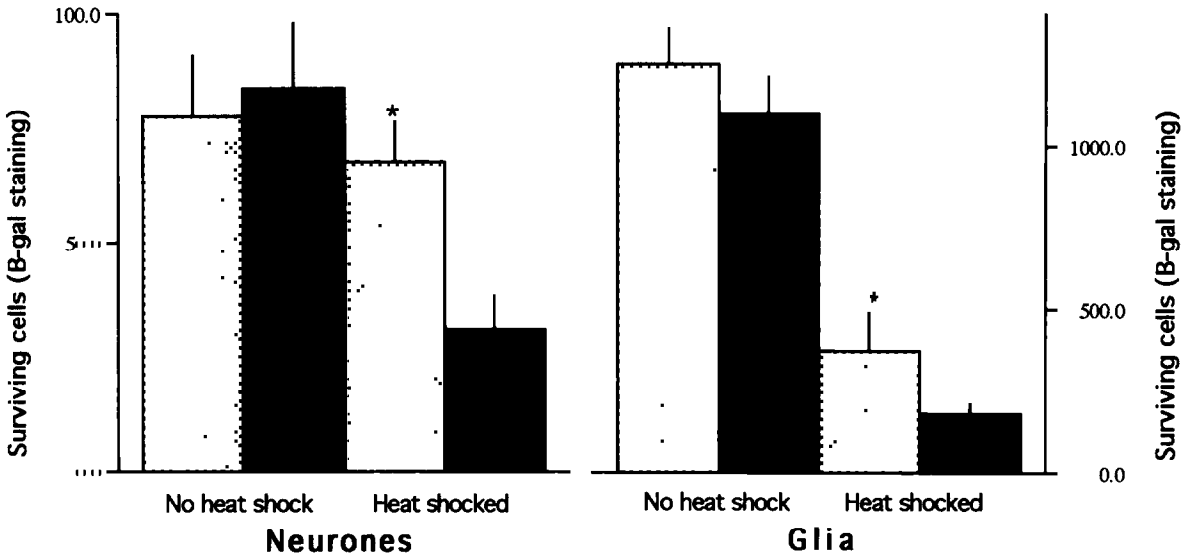

Fig. 2. The effect of severe heat shock on the survival of neurones and glia transfected with the Bos hsp70i plasmid. Cells were transfected with either Bos lacZ alone or with Bos lacZ and Bos hsp70i. The cells were then exposed to a severe heat shock (46°C for 20 min) and the surviving cells stained blue (indicating lacZ expression) were counted. The values are the mean ± S.E.M. of 12 experiments. $*P < 0.005$ (by t-test) when cells transfected with Bos lacZ alone and heat shocked are compared to cells transfected with Bos LacZ and Bos hsp70i and heat shocked. Stippled bars represent DRG cultures transfected with both the Bos hsp70i and Bos LacZ plasmids; filled bars represent DRG cultures transfected with the Bos lacZ plasmid alone.

neous concatemers. We assumed that the probability of cells being transfected with both plasmids would be very high, and that the majority of cells staining blue for lacZ expression would also represent cells which had been transfected with hsp70i. Using this strategy we transfected cultures of DRG with the bos-lacZ and/or Bos hsp70i vectors, exposed them to a heat stress (46°C for 20 min) and then counted the surviving blue cells (24 h later). Light microscopic analysis revealed that more cells survived the heat shock if they were transfected with both the Bos-hsp70i + Bos-lacZ plasmids than if transfected with the Bos-lacZ plasmid alone (Fig. 1).

These experiments were repeated several times and Fig. 2 shows the mean number of surviving neurones and glia (12 experiments). Significantly more neurones and glia survived the heat stress if they had been transfected with both the Bos-lacZ and Bos-hsp70i plasmids than if they had been transfected with the Bos-lacZ plasmid alone (Fig. 2). When these experiments were repeated using a Bos-hsp70i antisense DNA plasmid (hsp70i inserted in the antisense orientation into Bos) no protection was seen.

4. DISCUSSION

Previous studies have shown that pre-induction of hsp's in neuronal cells is protective [7,8], however, there has been no clear data to show which protein of the stress response is involved in conferring protection. The data obtained in this study show that the prior expression of hsp70i can enhance the survival of both neurones and glia following exposure to severe heat stress. This supports findings obtained on non-neuronal tissues and suggests that hsp70i plays a functional role in conferring thermotolerance to neurones and glia. Although this relationship has been argued previously from correlative data, this is the first direct evidence to suggest that expression of hsp70i by neurones and glia may be protective against a denaturing insult.

The physiological function of hsp70i is not known, however it does share 80% protein homology with hsp70c (the constitutively expressed form of hsp70i). Hsp70c has been studied extensively, and the data suggest that it acts to prevent inappropriate protein interactions [18], is involved in the transport of precursor proteins across membranes [3,4] (acting as an unfoldase or chaperone protein) and is involved in protein catabolism as it is the ATPase responsible for clathrin uncoating [5,19]. These actions suggest that in the CNS, hsp70i may protect cells against stress by allowing them to, (a) stabilise partially denatured proteins, which otherwise may aggregate and/or bind non-specifically to cellular proteins and interfere with their normal cellular function; (b) quickly replace and remove proteins irreversibly damaged following stress.

In many human neurodegenerative diseases there is a characteristic slow degeneration of neurones (with their eventual death) and accompanying production of abnormal intracellular inclusions derived from once normal cellular proteins (e.g. Lewy bodies in Parkinson's Disease and neurofibrillary tangles in Alzheimer's Disease). Also common to most neurodegenerative diseases, is the finding that inclusion bodies can be labelled by antibodies which recognise hsp's [20]. Furthermore,

the expression of hsp70i is increased following, experimentally induced status epilepticus [9], ischaemia [10], and excitotoxic damage [21]. These observations suggest that, neuronal cells detect abberrant proteins produced during the diseases progress and elicit a stress response in order to rectify abnormal protein metabolism, and/or that abberrant proteins are produced which become irreversibly bound to heat shock proteins. However, these observations may also be seen to suggest that a malfunctioning hsp protein may underly certain degenerative mechanisms.

Overall this study suggests that increasing hsp70i levels by transfection protects intracellular proteins from the denaturing effects of severe thermal stress. Inducing the expression of specific hsps's may lead to the development of novel treatment strategies for neurodegenerative conditions which result in the acute or chronic denaturation of cellular proteins.

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