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OXYGEN SENSORS AND ENERGY SENSORS ACT SYNERGISTICALLY TO ACHIEVE A GRADED ALTERATION IN GENE EXPRESSION: CONSEQUENCES FOR ASSESSING THE LEVEL OF NEUROPROTECTION IN RESPONSE TO STRESSORS

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1 ABSTRACT

Changes in gene expression are associated with switching to an autoprotected phenotype in response to environmental and physiological stress. Ubiquitous molecular chaperones from the heat shock protein (HSP) superfamily confer neuronal protection that can be blocked by antibodies. Recent research has focused on the interactions between the molecular sensors that affect the increased expression of neuroprotective HSPs above constitutive levels. An examination of the conditions under which the expression of heat shock protein 70 (Hsp70) was up regulated in a hypoxia and anoxia tolerant tropical species, the epaulette shark (*Hemiscyllium ocellatum*), revealed that up-regulation was dependent on exceeding a stimulus threshold for an oxidative stressor.

While hypoxic-preconditioning confers neuroprotective changes, there was no increase in the level of Hsp70 indicating that its increased expression was not associated with achieving a neuroprotected state in response to hypoxia in the epaulette shark. Conversely, there was a significant increase in Hsp70 in response to anoxic-preconditioning, highlighting the presence of a stimulus threshold barrier and raising the possibility that, in this species, Hsp70 contributes to the neuroprotective response to extreme crises, such as oxidative stress. Interestingly, there was a synergistic effect of coincident stressors on Hsp70 expression, which was revealed when metabolic stress was superimposed upon oxidative stress. Brain energy charge was significantly lower when adenosine receptor blockade, provided by treatment with aminophylline, was present prior to the final anoxic episode, under these circumstances, the level of Hsp70 induced was significantly higher than in the pair-matched saline treated controls. An understanding of the molecular and metabolic basis for neuroprotective switches, which result in an up-regulation of neuroprotective Hsp70 expression in the brain, is needed so that intervention strategies can be devised to manage CNS pathologies and minimise damage caused by ischemia and trauma. In addition, the current findings indicate that measurements of HSP expression *per se* may provide a useful correlate of the level of neuroprotection achieved in the switch to an autoprotected phenotype.

2. INTRODUCTION

Constitutive levels of the Heat shock protein super-family have been found in all eukaryotic cells (1) and while they have diverse functions, the precise cascade and interaction of signals, which trigger increased HSP

expression are not completely understood. Members of the HSP super-family act as molecular chaperones to inhibit abnormal protein associations (2), facilitate the repair of damaged proteins (1,3), maintain the tertiary structure of proteins during metabolic stress (4) and have a pivotal role in protein transport (2). Increased levels of inducible HSP's have been used as biomarkers of previous exposure to physiological stressors, some of which underlie a number of CNS pathologies (5). The presence of inducible HSP's not only indicate that a substantial stressor has been encountered but also that some degree of neuroprotection to subsequent stressors has been initiated (6). The ability of trauma victims to respond by expressing elevated levels of the HSP family is correlated with an increased chance of survival (7).

Alteration of gene expression in the brain resulting in the induction of specific HSPs appears to be initiated in response to a diverse array of physiological stressors. These include temperature change, hypoxia, ischemia, environmental insult, (8, 9); psychoactive drugs (10, 11); neurodegenerative disease (12); or cellular injury (13). It is not known whether a number of triggers converge on a common regulator or whether sensors can act as synergists to up-regulate the production of protective HSPs. Lutz and Prentice (14) suggest that there are two categories of detectors that respond to hypoxia. The molecular sensors, which signal through promoter elements to alter gene expression and the metabolic sensors, which respond to a cellular energy crisis.

Examining the conditions under which inducible HSPs are expressed may further clarify the nature of the signals that activate the molecular sensors responsible for maintaining cellular homeostasis during cellular stress. During ischemia and subsequent reperfusion, ROS levels increase and initiate the neuronal apoptotic pathway (6). However the physiological stress of ischemia also triggers the protective up-regulation of HSPs as part of a suite of genes turned on by the Hypoxia response element (HRE) (15, 16, 17), which is activated via Hypoxia inducible factor (HIF) and non HIF transcription factor pathways (18). Alteration in gene expression confers neuroprotection and counteracts progression along the apoptotic pathway via caspase 3 activation (6). The induction of HSP may be of importance in such times of crisis because they chaperone the ATP-dependent refolding of damaged proteins (1, 3) and confer an autoprotected phenotype, which can be blocked by administering an antibody to Hsp70 (19).

It seems likely that induction of Hsp70 is linked not only to an oxygen sensor but also to an energy sensor because Hsp70 promoter activation responds to a negative cellular energy balance (20) as well as to oxidative stress (21). We examined the induction of Hsp70 protein in a recently described hypoxia and anoxia tolerant tropical vertebrate, the epaulette shark (22, 23, 24) to determine whether oxygen sensors and energy sensors are involved as synergists in altering gene expression in the brain. Most vertebrates are highly vulnerable to decreased oxygen levels; as a result of even a brief exposure to hypoxia or anoxia they suffer irreparable damage to the brain and heart tissue. Consequently, examining the induction of HSP in hypoxia and anoxia intolerant species would make the interpretation of sensor activation difficult because any change in inducible HSP may have been caused by the presence of damaged protein. Hypoxia tolerant animals have evolved diverse autoprotective physiological mechanisms, which act to delay or avert the energy crisis that normally accompanies exposure to reduced oxygen levels (25). Consequently, examination of the up-regulation of HSP in a hypoxia-tolerant animal could reveal whether oxygen and energy sensors can be activated separately and whether there is a cumulative activation of gene expression in the brain resulting in elevated levels of Hsp70 protein.

The Epaulette shark is one of the few vertebrates that can tolerate extended periods of hypoxia at 25^0C without suffering neuronal damage (23). Most hypoxia and anoxia tolerant vertebrates (18) evolved their tolerance to these stressors at temperatures close to freezing so the autoprotective mechanisms they use may be influenced by their response to over-wintering at low temperature. The epaulette shark can live on reef platforms that are subjected to intermittent hypoxia. During nocturnal low tides when the water on the reef platform and the ocean water are separated by a fringing reef crest, the nocturnal respiration of the reef community dramatically lowers dissolved oxygen levels (26). This shark is unique in its hypoxia and anoxia tolerance at tropical temperatures making it a suitable candidate in which to examine the effect of stimulating oxygen and/or energy sensors on the level of Hsp70 expression. Repeated sub-lethal hypoxia (hypoxic-preconditioning) further increases the natural level of hypoxia tolerance in the epaulette shark by lowering its resting metabolic rate and decreasing oxygen consumption (27). We examined the level of Hsp70, in the brain stem and the more hypoxia sensitive cerebellum, after hypoxic-preconditioning to determine whether hypoxia tolerance is achieved by elevated levels of Hsp70, as has been reported for non-hypoxia tolerant animals (19).

The epaulette shark enters a state of metabolic depression in response to anoxic-preconditioning with the result that brain energy charge is maintained. In the epaulette shark, such metabolic depression can be blocked by aminophylline, a non-specific adenosine receptor blocker, with a subsequent loss of brain energy charge (24). We were interested in whether Hsp70 levels increased when brain energy charge fell in aminophylline treated animals exposed to anoxia because this would indicate the involvement of an energy sensor in the up-regulation of HSP.

In other species, the time course and intensity of Hsp70 induction in response to stressors closely parallels the vulnerability of regions of the CNS to insult (4). After ischaemic insult Hsp70 is increased in the hypoxia-sensitive hippocampus and cerebellum prior to other regions of the CNS (28, 29). Increased Hsp70 may serve to indicate not only that exposure to a stressor has occurred but also that at least one autoprotective mechanism has begun to control cellular damage. The close involvement of

Hsp70 in neuroprotection raises two questions. First, whether the protective effect of hypoxic-preconditioning and/or anoxic-preconditioning in this hypoxia and anoxia tolerant species is associated with an induction of Hsp70. Secondly, whether an oxygen sensor and/or an energy sensor is involved in up-regulating Hsp70. A comparison was made of Hsp70 levels after anoxic-preconditioning with and without metabolic stress caused by an aminophylline-induced reduction in brain energy charge. In addition, the Hsp70 levels were examined after hypoxic-preconditioning to determine whether Hsp70 is part of the first group of autoprotective strategies switched on to achieve the increased hypoxia tolerance observed in response to hypoxic-preconditioning (27) or anoxic-preconditioning (24) in this species.

3. MATERIALS AND METHODS

3.1. Hypoxic and anoxic-preconditioning.

Epaulette sharks, mean wt 473.625g and length 55.45cm, were caught from the reef platform of Heron Island (23°26'S, 151°55'E). Five sharks were exposed to a hypoxic-preconditioning regimen to elicit the shark's neuroprotective responses to hypoxia using the protocol previously described (30, 27). Briefly, this consisted of two hours of hypoxia (5% of normoxia) every twelve hours for four consecutive days. Five sharks were exposed to normoxia as controls under identical conditions. Twelve hours after the last exposure, sharks were anaesthetised (benzocaine, 0.0075% in seawater). Brains were rapidly removed, frozen and stored at -70^0C.

The method used in the anoxic-preconditioning regimen has been described previously (24). Briefly, eight epaulette sharks were exposed to anoxia on day 1 until they lost of their righting reflex, sharks were pair matched for the time to loss of righting reflex in this first exposure. Twenty-four hours later, one member of each pair of matched sharks was injected with either aminophylline (n=4), an adenosine antagonist that reduces hypoxia tolerance, or saline (n=4) and re-exposed to a final 50 minutes of anoxia. Four sharks were exposed to normoxia, at the same times, to act as untreated normoxic controls. At the conclusion of the experiment, sharks were anaesthetised (benzocaine, 0.0075% in seawater) and the brains were rapidly removed, hemisected, frozen and stored at -70^0C.

3.2. One Dimensional SDS Page Electrophoresis

The cerebellum was removed from the frozen brains and homogenised, using a glass homogeniser, in cold (4^o C) lysis buffer prepared according to Seubert, *et al* (31) in a ratio of 10:1. After centrifugation for 2 minutes at 400g, protein concentration was determined using the Nano-Orange Protein quantification kit (Molecular probes, N-6666) according to the manufacturer's directions, to enable an equal amount of protein to be was calculated for each sample. Then a standard amount of protein (20µg) with 5µl of protein tracking dye (Comassie blue) was added to an eppindorf tube and heated in a water bath at 100^oC for three minutes to denature the proteins. The preparation was centrifuged and the supernatant retained. Each stained denatured protein sample was loaded into a well of a 12% acrylamide resolving gel layered with a 5% acrylamide stacking gel using a 0.5ml syringe attached to a 30 gauge needle, under Sodium dodecyl (laurel) sulfate (SDS) running buffer (25mM Tris pH7.6, 0.1% SDS and 0.2glycine). Three additional wells were loaded, one contained an aliquot of molecular weight markers (Sigma), and another contained Hela cells as a positive control and served as a common calibration point and the last well was a negative control, in which no protein was loaded. Electrophoresis at a constant voltage of 60-70V was applied to fractionate the proteins until the tracking dye reached the end of the gel. Gels were removed, equilibrated in Polyvinylidene fluoride (PDVF) transfer buffer for 15 minutes, immersed in methanol for twenty seconds, followed by distilled water to equilibrate the gel. Finally, gels were soaked for fifteen minutes in PVDF transfer buffer prior to staining one gel with the Coomassie blue to stain protein bands and the other gel was transferred to PVDF membrane over 10- 14 hours at 10 volts in PVDF transfer buffer then transferred to 10% methanol and stained with Ponceau red for 1 min to confirm protein transfer. The Ponceau red was washed out prior to immunodetection.

3.3. Immuno-detection

PVDF membranes were immersed in a blocking buffer consisting of 5% skim milk in 1M Tris buffered saline (TBS: 10 mM Tris, 25 M NaCl, 0.05% Tween, pH 8.0) for 30 minutes. Rinsed in TBS then incubated in Mouse anti-Hsp70 (Signal Transduction laboratories) diluted 1:1000 and placed on an orbital shaker for one hour at room temperature. Membranes were rinsed then washed 3 times for fifteen minutes with blocking buffer. Followed by incubation in HRP labelled Rabbit anti-mouse (Amersham) diluted 1:2000 for 30 minutes. Gels were run four times with separate samples to ensure reproducibility. The membranes were rinsed three times in TBS prior to incubation with the substrate Lumigen PS-3 (Amersham Pharmacia Biotech) for five minutes. The membranes were then scanned using a Fluroimager (Fluroimager 595 – Molecular dynamics). Fluorescence intensities for each band were recorded and analysed using Image Quant 5.0. Semi quantitative analysis was based on the intensity of Hsp70 staining relative to that of the same calibration sample included on each gel, namely the Hela cells.

3.4. HPLC analysis

The brain energy charge of animals, which showed elevated Hsp70 was measured using HPLC as described previously (24). Briefly, frozen hemi-sections were homogenised in 5 ml of 4 % perchloric acid, centrifuged at 13000rpm for 5 min and after neutralising the supernatant with 1M KOH the concentration of ATP, ADP and AMP was determined using HPLC as described by Van Der Boon et al, (32) and the adenosine concentration was measured using HPLC as described by (33). Then the adenylate energy charge (EC) was calculated using the standard formula: EC = ([ATP] + ½ [ADP]) / ([ATP] + [ADP] +[AMP])

3.5. Statistical analysis

Means and standard deviations were calculated the results analysed using a two tailed paired students t test for pair matched animals for samples run on the same gels

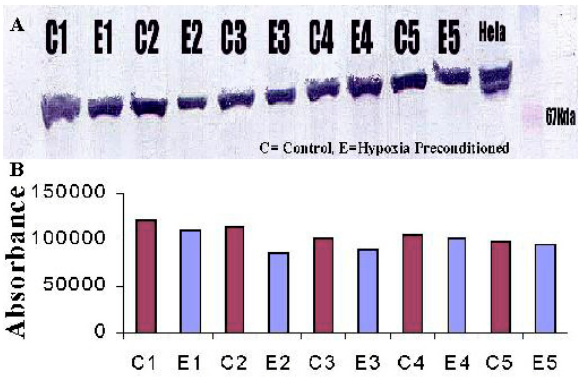

Figure 1. (A) Shows the western blot of Hsp70 protein bands for the brainstem from hypoxia-preconditioned animals and their pair matched controls. Hela cells were used as a positive control for Hsp70. The 67Kda molecular weight marker appears in the last column. (B) Shows the absorbance measure for each of the brainstems. Abbreviations: the series denoted by C refers to brainstems from control animals exposed to normoxia and the series denoted by E refers to brainstems from hypoxia-preconditioned animals.

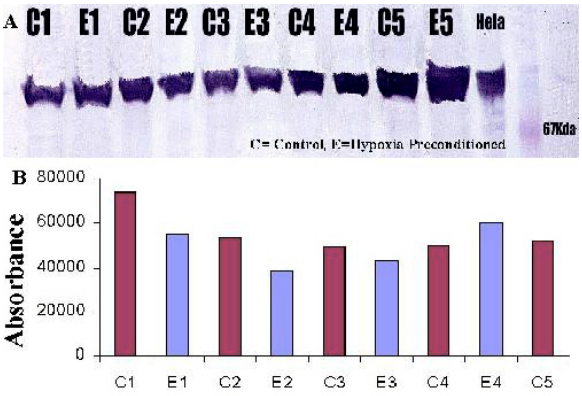

Figure 2. (A) Shows the western blot of Hsp70 protein bands for the cerebella from hypoxia-preconditioned animals and their pair matched controls. Hela cells were used as a positive control for Hsp70. The 67Kda molecular weight marker appears in the last column. (B) Shows the absorbance measure for each of the cerebella. Abbreviations: the series denoted by C refers to the cerebella from control animals exposed to normoxia and the series denoted by E refers to the cerebella from anoxia-preconditioned animals.

or an unpaired students t test, when comparison was made to the non-pair matched untreated normoxic controls such as those included in the anoxic-preconditioning experiment.

4. RESULTS

4.1. Oxidative stress without metabolic stress

Interestingly, the constitutive level of Hsp70 was 1.94 fold lower in the cerebellum (55,673.68 ± 10,349.46) than in the brainstem (107,869.33 ± 8,901.4). Western analysis revealed that exposure to hypoxic-preconditioning caused no significant difference in the level of Hsp70 in the brainstem (Figure 1) or the cerebella (Figure 2) to that in the pair matched control animals. Conversely, exposure to the more severe regimen of anoxic-preconditioning resulted in 2.79 fold elevation of mean Hsp70 expression in the cerebella of saline treated anoxia-preconditioned animals (155,550.17 ± 41,279) compared to their pair matched normoxic controls run on the same gel (55,675.47 ± 26,294.04, shown in Figure 3). Indicating that Hsp70 expression was significantly elevated in response to anoxic-preconditioning ($p < 0.005$) but not by hypoxic-preconditioning.

4.2. Oxidative stress coupled with metabolic stress

The Hsp70 levels in cerebella from untreated normoxic controls and pair matched anoxia-preconditioned animals with and without aminophylline prior to episode 2 are shown in Figure 3. Comparison of the total brain energy charge in these three groups confirmed that the aminophylline treated animals had experienced a decreased brain energy charge at the conclusion of the experiment. After anoxic-preconditioning, the mean brain energy charge of saline treated animals (0.739 ± 0.02) did not significantly differ from the non-preconditioned normoxic controls (0.806 ± 0.017). The mean brain energy charge of aminophylline treated animals (0.694 ± 0.011) was significantly lower ($p < 0.001$) than that of their pair matched saline treated controls (anoxia-preconditioned) and the untreated normoxic controls. Thus confirming that metabolic stress had been induced only in the aminophylline treated anoxia-preconditioned animals

There was a 7.4 fold increase in Hsp70 expression, above the mean constitutive level in normoxic controls, when exposure to anoxic-preconditioning was preceded by the blockade of the adenosine receptor by the administration aminophylline (412,466.07 ± 171,818.97, $p < 0.005$). This represents a significant increase ($p<0.01$) in Hsp70 expression over the increase in Hsp70 expression generated by anoxic-preconditioning alone. Taken together these data revealed that exposure to anoxia in the presence of metabolic stress, namely the significantly reduced brain energy charge in the aminophylline treated animals, resulted in enhanced Hsp70 expression.

5. DISCUSSION

Hsp70, a potent neuroprotective agent is synthesised in tissues, including the brain, in response to physiological stressors. Its pre-emptive role ranges from refolding damaged proteins to subverting the progression of the apoptotic pathway (6). The data presented here support the proposal by Lutz and Prentice (14) that both metabolic sensors and molecular, in this case oxygen, sensors trigger gene regulation in physiological crisis and reveal that these sensors can act as synergists to produce a graded neuroprotective response. In the hypoxia and anoxia tolerant epaulette shark anoxic-preconditioning resulted in a significant elevation of Hsp70. Since brain energy charge did not change after exposure to repeated anoxia, these data suggest that the activation of an oxygen sensor alone was sufficient to trigger the increased level of Hsp70. However, the finding that exposure to hypoxia was not associated

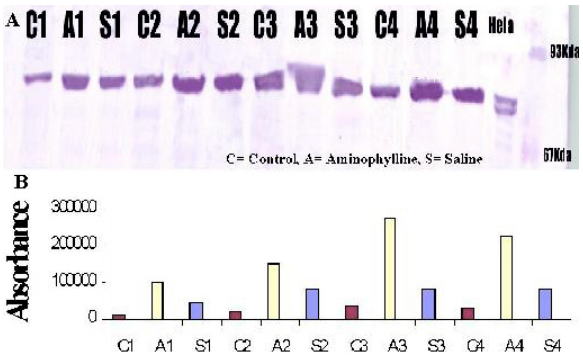

Figure 3. (A) Shows the western blot of Hsp70 protein bands for the cerebella from normoxic untreated animals and pair matched anoxia-preconditioned animals treated either with aminophylline or saline prior to their final anoxic episode. Hela cells were used as a positive control for Hsp70. The 67Kda molecular weight marker appears in the last column. (B) Shows the absorbance measure for each of the cerebella. Abbreviations: the series denoted by C refers to the cerebella from control animals exposed to normoxia, the series denoted by A refers to the cerebella from anoxia-preconditioned, aminophylline treated animals and the series denoted by S refers to the cerebella from anoxia-preconditioned, saline treated animals.

with elevated Hsp70 levels may indicate that there is an O_2 sensor threshold that has to be exceeded. In other species, hypoxic-preconditioning confers protection, in part, via up-regulation of the molecular chaperone Hsp70. While repeated exposure to severe hypoxia does not cause delayed neuronal death (23) the mode of neuroprotection does not appear to be dependent on the up-regulation of Hsp70. In contrast, a few apoptotic profiles have been observed after exposure to an identical regimen of repeated anoxia (Renshaw and Kerrisk, unpublished data) and the data reveal that there was a significant up regulation of Hsp70 in the brain. An alternative view is that exposure to hypoxia did not achieve a "damage threshold" required to initiate up-regulation of Hsp70 in this species.

When cells from hypoxia-sensitive teleosts, such as rainbow trout are exposed to hypoxia, the level of Hsp70 mRNA is significantly elevated (34). However, it is not known whether the decreased level of oxygen *per se* or an associated metabolic stress elevates Hsp70 mRNA. To address the nexus between energy level and HSP gene transcription in the rainbow trout, Currie et al., (35) reduced ATP levels by inhibiting glycolysis and oxidative phosphorylation and detected no increase in HSP gene transcription. We used the epaulette to investigate diminished O_2 with and without metabolic stress. The data showed that metabolic stress, reflected by decreased brain energy charge in aminophylline treated sharks exposed to anoxia, caused a further significant increase in the level of Hsp70 revealing that metabolic sensors are involved in the up-regulation of Hsp70.

While the nature of the metabolic signal required to up regulate Hsp70 is unknown, these data suggest some promising lines of research. Since Hsp70 maintains the tertiary structure of proteins during metabolic stress (4), the combination of anoxia with decreased brain energy charge may have provided a sufficient stimulus to damage proteins and thereby trigger transcription of Hsp70 mRNA. Alternatively, it is possible that adenosine receptors may play a direct role in the up-regulation of Hsp70 in the epaulette shark brain since Hsp70 protein levels were significantly elevated when the adenosine receptor was blocked with aminophylline. While the precise cascade of signals triggering hypoxic-preconditioning remain obscure there is considerable support for the purine nucleotide adenosine as an O_2 / metabolic sensor and mediator of hypoxic tolerance. In hypoxia- and anoxia-tolerant teleosts that over-winter at 0°C in hypoxic or anoxic conditions brain adenosine levels rise as ATP utilisation exceeds its generation. This rise in adenosine triggers the onset of metabolic depression, which in turn conserves ATP (18). In the epaulette shark exposure to anoxia with or without aminophylline resulted in a 3.5-fold increase in brain adenosine levels (24). It is likely that in this species, the significant increase in adenosine acts not only as a trigger to initiate metabolic depression (27) but also may act as a signal of metabolic stress to increased Hsp70. A negative cellular energy balance activates the Hsp70 promoter (20) and activation of the adenosine receptor A_1 also increases Hsp70 via a Protein Kinase C dependent pathway (36). While anoxic-preconditioning before an anoxic insult confers neuroprotection, this can be blocked by administering an anti-body to Hsp70 (19) revealing a pivotal role for Hsp70 in switching to an autoprotective phenotype.

In summary, the induction of Hsp70 above constitutive levels appears to be dependent on the severity of the stressor, since anoxia but not hypoxia triggered the autoprotective response. The data revealed that a further significant elevation in the level of Hsp70 in response to anoxia was associated with the blockade of the adenosine receptor and diminished brain energy charge. Hsp70 may be a retaliatory neuroprotective agent, which is elevated in response to injury above a certain threshold. If this is so, then measurements of Hsp70 levels may be a useful clinical tool to quantify not only the level of prior insult but also to gauge the extent of the autoprotective response in victims of stroke, birth hypoxia and brain ischemia.

6. ACKNOWLEDGEMENTS

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Abbreviations: HSP: Heat shock protein; Hsp70: Heat shock protein 70; SDS: Sodium dodecyl (laurel) sulfate; PVDF: Polyvinylidene Fluoride; TBS: Tris buffered saline.

Key Words: Neuroprotection, Gene Expression, Hypoxic – preconditioning, Anoxic-preconditioning, Hypoxia Tolerance, Anoxia Tolerance, Review

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