RAPID COMMUNICATION

Glucocorticoid-induced glucose release is abolished in trout hepatocytes with elevated hsp70 content

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Abstract

The metabolic potential of cells with elevated heat shock protein 70 (hsp70) content was examined by measuring unstimulated and glucocorticoid-stimulated glucose release in trout hepatocytes maintained in primary culture. Exposure of hepatocytes to either heat shock (HS; +15 °C) or sodium arsenite (50 µM) did not affect cell viability, but resulted in significantly higher hsp70 levels over a 24 h recovery period. Hsp70 accumulation had no significant impact on unstimulated glucose release, but completely

abolished cortisol-induced glucose release in trout hepatocytes. This lack of glucocorticoid responsiveness corresponded with lower glucocorticoid receptor protein levels. Together, our results suggest that stressor-induced hsp70 accumulation, while important for maintaining cellular homeostasis, may impair metabolic adjustments to subsequent stressors in animals, especially those that are glucocorticoid-dependent.

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Introduction

Heat shock proteins (hsps) are a family of highly conserved proteins playing a major role in the cellular stress response process (Parsell & Lindquist 1993). The 70 kDa family of hsps (hsp70), the most widely studied of the hsps, is constitutively expressed in unstressed cells and is important in the normal functioning of cells. Upon exposure to stressors, particularly those affecting the protein machinery, the inducible form of hsp70 is synthesized in large amounts and is crucial for maintaining cellular homeostasis (Parsell & Lindquist 1993). Also, stressor-induced hsp70 synthesis represses the transcription and/or translation of other cellular proteins, whereas the removal of this inhibition correlates with decreased hsp70 synthesis during recovery from HS (DiDomenico *et al.* 1982). Thus hsp70 induction, while crucial for cellular stressor tolerance and survival, may impact the metabolic potential of cells to subsequent stressors. One of the well-characterized hormonal responses to stressor is the elevation of glucocorticoids in circulation and this stress-response is important for the homeostatic processes adapting animals to stress (Bamberger *et al.* 1996, Sapolsky *et al.* 2000). The tissue response to glucocorticoid is predominantly genomic and is mediated via intracellular glucocorticoid receptor (GR) that acts as transcription factors upon ligand binding to induce and/or repress transcription of glucocorticoid-responsive genes (Sapolsky *et al.*

2000). The glucocorticoid-mediated stimulation of gluconeogenesis, important for longer-term maintenance of glucose during stress in vertebrates, involves the synthesis of several enzymes, including phosphoenolpyruvate carboxykinase (PEPCK) (Hanson & Reshef 1997). Consequently, hsp70 synthesis and the resultant cellular re-programming, including repression of genes and proteins, may impact the gluconeogenic pathway. As cortisol is a key player in the glucose response to stress, we set out to test the hypothesis that stressor-induced hsp70 accumulation, while crucial for coping with proteotoxicity, lowers hepatocyte metabolic potential including glucocorticoid responsiveness. Rainbow trout hepatocytes in primary culture were used as a model to test this hypothesis because heat shock response (Iwama *et al.* 1998) and the glucocorticoid stimulation of gluconeogenesis (Mommensen *et al.* 1999) have been well characterized in this system. Our results suggest that glucocorticoid receptor (GR) down-regulation is a key factor in the lack of glucose response to cortisol stimulation in hsp70-accumulated trout hepatocytes.

Materials and methods

Primary culture of trout hepatocytes

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Rainbow Springs Trout farm (Thamesford, Ont). Fish

were maintained in running well-water (12 °C) at the University of Waterloo Aquatic facility under a 12 hL:12 hD photoperiod and fed to satiety once every two days using commercial trout feed (Martin Mills, Inc., Elmira, Ont). Trout were acclimated for at least a month prior to the isolation of hepatocytes. Trout hepatocytes were isolated using collagenase perfusion according to established protocols (Sathiyaa *et al.* 2001). Cells were plated in 6-well Primaria plates (Becton Dickinson Labware, NJ) at a density of 1.5×10^6 cells/well (0.75×10^6 cells/ml) in L15 media (Sigma, St Louis) and maintained at 13 °C. The experiment commenced after 16–24 h of plating as outlined below.

Heat shock and arsenite stimulation

Four experiments, each with cells isolated from a different fish, were conducted to examine the impact of HS or arsenite on hsp70 and GR accumulation in trout hepatocytes. The cells were stimulated with either 50 µM sodium arsenite (NaAsO₂; Sigma) or heat shock (HS; +15 °C) for 1 h, followed by recovery in fresh medium for 3 or 23 h at ambient temperature. Cells were sampled prior to, and at 3 or 23 h after stimulation with either HS or arsenite. Sampling of cells involved repeated pipetting with ice-cold medium, transferring the contents to microcentrifuge tubes followed by centrifugation (13 000 × g, 20 s), aspirating the supernatant and freezing the cell pellet on dry ice for immunoblotting (see below).

Cortisol stimulation

Hepatocytes from three independent fish were exposed to either HS or arsenite as described above for 1 h and allowed to recover for 3 h at ambient temperature. The cells were then exposed to fresh L-15 medium either in the absence (control) or presence of 100 ng/ml cortisol (hydrocortisone; Sigma) and the glucose release was determined 24 h after hormone addition. The cortisol dosage chosen was that typically seen in stressed rainbow trout (Wendelaar-Bonga 1997). The media and cells were collected and frozen on dry ice and the cell viability was determined by measuring the release of lactate dehydrogenase (see below).

SDS-PAGE and Western Blotting

The protein concentrations were determined by the BCA method (Pierce Chemical Co., IL) using bovine serum albumin (BSA) as the standard. Samples (30 µg protein loaded per lane) were separated on 8 or 10% polyacrylamide gels using the discontinuous buffer system of Laemmli (Laemmli 1970). The gels were transferred onto nitrocellulose membranes and immunodetection was carried out according to established protocols (Vijayan

et al. 1997). The primary antibodies consisted of either a polyclonal antibody to rainbow trout hsp70 (1:3000; Dr. Peter Candido, Biochemistry Department, UBC) or GR (1:1500; Tujague *et al.* 1998). The secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit (1:3000; Bio-Rad, CA) antibody. The bands were visualized with NBT (0.033% w/v) and BCIP (0.017% w/v) (Fisher Scientific, Ont). The protein band intensities were quantified with the Chemi imagerTM using the AlphaEase software (Alpha Innotech, CA).

Glucose and LDH analysis

Media glucose was determined colorimetrically using a commercially available kit (Trinder method; Sigma). LDH leakage was determined by measuring LDH activity in both the media and the cells and the leakage (medium) was expressed as percent of the total (medium+cells). LDH activity was measured over a 5 min period at 340 nm with NADH (0.12 mM) and pyruvate (1 mM) in imidazole buffer (50 mM, pH 7.5).

Statistical analysis

The changes seen with heat shock, arsenite and cortisol treatments were expressed as percent change from 100% control (no treatment), and the values were arcsine transformed and compared using paired student's *t*-test. The level of significance was set at 0.05.

Results and discussion

Heat shock protein 70 accumulation

The heat shock (+15 °C) treatment and arsenite exposure (50 µM) significantly induced hsp70 (Fig. 1), but did not affect cell viability as evidenced by the lack of change in LDH leakage (17, 18 and 13% for control, HS and arsenite exposed cells, respectively). These stressors induced hsp70 synthesis at 3 h of recovery and the protein accumulated over a 24 h period. While studies with transformed cells showed that HS- or arsenite-induced hsp70 synthesis is repressed upon recovery (DiDomenico *et al.* 1982, Kothary *et al.* 1984), we detected 35[S]methionine incorporation into hsp70 even at 24 h post-recovery from HS (A.N. Boone and M.M. Vijayan, Submitted). As the re-synthesis of other proteins correlates with repressed hsp70 synthesis, our results suggest that the 'normal' protein synthetic machinery in trout hepatocytes is perhaps inhibited for longer periods relative to mammalian or transformed fish cells (DiDomenico *et al.* 1982, Kothary *et al.* 1984). This inhibition may be due to either the intensity of the HS (+15 °C) and/or the lower ambient temperature (13 °C) in the present study.

Figure 1 Hsp70 accumulation after exposure to heat shock (A) or sodium arsenite (B). Top panel shows a representative western blot using anti-trout hsp70 and the lower panel shows the intensity of the bands expressed as percent change from 100% control. Values represent mean $\pm$ S.E.M. ($n=4$ fish). *significantly different from the control ($P<0.05$, paired student t -test); +significantly different from the 3 h ($P<0.05$, paired student t -test).

Glucose release

We show for the first time that the capacity for glucose release is not compromised in HS or arsenite exposed hepatocytes (Fig. 2). Gluconeogenesis is the major pathway responsible for glucose production in primary cultures of trout hepatocytes (Mommensen *et al.* 1999). As the gluconeogenic pathway is energy demanding, we hypothesized that preferential hsp70 synthesis, in order to cope with aberrations in protein machinery, will inhibit glucose production, but that does not appear to be the case. It is likely that in the present study HS or arsenite-induced hsp70 synthesis maintains the metabolic status of hepatocytes, including the functioning of proteins involved in gluconeogenesis. As glucocorticoids are key players in stress-induced gluconeogenesis, hepatocytes were exposed to physiological levels of cortisol (Wendelaar-Bonga 1997) to examine the impact of hsp70 on glucocorticoid responsiveness. The significantly higher glucose release seen in the control cells treated with cortisol (Fig. 2), further reiterates the gluconeogenic role of cortisol in fish

Figure 2 Effect of cortisol on glucose release after exposure to heat shock or sodium arsenite. Hepatocytes were exposed to either heat shock (HS; $+15^{\circ}\text{C}$) or sodium arsenite ($50\text{ }\mu\text{M}$) for 1 h and allowed to recover in ambient medium either in the presence (solid bar) or absence (open bar) of cortisol (100 ng/ml) for 24 h. Glucose in the medium is expressed as percent change from 100% control (no cortisol, HS or arsenite). Values represent mean $\pm$ S.E.M. ($n=3$ fish). *significantly different from the control ($P<0.05$, student t -test).

hepatocytes (Mommensen *et al.* 1999). This cortisol-induced glucose release, however, was abolished in cells exposed to HS or arsenite (Fig. 2) suggesting that hsp70 accumulation, while crucial for maintaining cellular metabolic capacity, alters cellular response to glucocorticoid stimulation. As glucocorticoids are crucial for re-establishing whole animal homeostasis to stressor insult (Bamberger *et al.* 1996, Sapolsky *et al.* 2000), our results argue for a decreased tolerance to subsequent stressors in animals with hsp70 induction. This attenuated response, however, may not be due to altered cellular energetics associated with hsp70 induction, but may perhaps be due to alterations in the cortisol signalling pathway(s), especially since both HS (Vedeckis *et al.* 1989) and arsenite (Kaltreider *et al.* 2001) affected the GR transcription machinery. The significantly lower glucose release with cortisol in HS- but not arsenite- exposed cells (Fig. 2) suggests a link between the HS response and the GR signalling pathway. As hsp90 and hsp70 are part of the GR heterocomplex, the HS-mediated alteration in the ratio of free to bound hsp/GR may be a mechanism modulating the activation and/or repression of GR-inducible genes (Kang *et al.* 1999; Sathiyaa *et al.* 2001).

GR expression

Our results using a physiologically relevant cell system show clearly that sublethal HS and arsenite exposure significantly decreased GR levels in trout hepatocytes (Fig. 3). This decrease was evident at 3 h and remained significantly lower even at 24 h post-exposure (Fig. 3). Previous studies have shown that HS *in vivo* resulted in GR translocation to the nucleus and activation of GRE even in the absence of ligand binding (Shen *et al.* 1993).

Figure 3 Glucocorticoid receptor (GR) protein accumulation after exposure to heat shock (A) or sodium arsenite (B). Top panel shows a representative western blot using anti-trout GR and the lower panel shows the intensity of the bands expressed as percent change from 100% control. Values represent mean $\pm$ S.E.M. ($n=4$ fish). *significantly different from the control ($P<0.05$, paired student t -test); +significantly different from the 3 h ($P<0.05$, paired student t -test).

Since we used whole cell homogenates for our immunodetection, the observed levels correspond to the total GR pool (cytosolic and nuclear) and the lower levels correspond to GR downregulation and not nuclear translocation. Studies using transformed cells showed that HS and arsenite potentiated glucocorticoid-mediated gene expression (Li *et al.* 2000, Wadekar *et al.* 2001). This potentiation was shown to require HSF activity (Li *et al.* 2000) suggesting a positive relationship between the cellular stress response and the glucocorticoid pathway at the transcriptional level. However, HS also decreased GR-binding activity and increased GR degradation in mouse AtT-20 cell lines (Ali & Vedeckis 1990–91) implying a decrease in cellular sensitivity to GR stimulation. Taken together, the use of transformed cell lines while important for the delineation of transcriptional and translational processes may not be physiologically relevant (Hanson & Reshef 1997).

Our results, using differentiated trout hepatocytes, demonstrate for the first time that the glucose

release capacity to cortisol stimulation is abolished in hsp70-accumulated cells. We show that this lack of responsiveness correlated with GR down-regulation (Figs. 2 and 3). Glucose release is an important aspect of the stress response process to fuel hepatic and extra-hepatic metabolic processes in order to cope with the increased energy demand. Consequently, our results suggest that hsp70 accumulation, while crucial for cellular stress tolerance, may hamper the metabolic potential to cope with subsequent stressors, especially those that are dependent on glucocorticoid stimulation.

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