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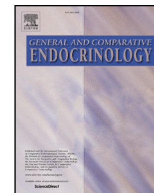

Research paper

Corticotropin-releasing factor regulates caspase-3 and may protect developing zebrafish from stress-induced apoptosis

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ABSTRACT

The corticotropin-releasing factor (CRF) system is expressed in the earliest stages of zebrafish development, long before its canonical function in the endocrine stress response is realized, and yet its function during embryogenesis is unknown. We tested the hypothesis that CRF protects embryos from stress-induced apoptosis. Here we confirm that a 1 h heat shock applied at either 6 h post-fertilization (hpf) or 30 hpf elicits an increase in caspase-3 activity, a key effector of apoptosis. Temporal changes in the expression of *crf* and its binding protein (*crf-bp*) during recovery from heat shock indicate that the CRF system is responsive to stressors experienced as early as gastrulation. Next, we heat shocked embryos that were microinjected with *crf* mRNA, and showed that caspase-3 induction is significantly reduced in embryos that overexpress CRF relative to control embryos. In addition, incubating embryos in the presence of the CRF receptor type 1 (CRF-R1) antagonist, antalarmin, during recovery from heat shock significantly increased caspase-3 activity, suggesting that CRF regulates caspase-3 via a CRF-R1-dependent pathway. Finally, we show that most heat shock-induced mortality occurred during the first hour of recovery, long before a significant increase in caspase-3 activity was detected. Indeed, the delayed caspase-3 induction coincided with a mortality plateau, and neither CRF overexpression nor antalarmin treatment altered heat shock induced mortality, supporting previous *in vitro* evidence that CRF-mediated cytoprotection occurs through the slow and tightly controlled apoptotic pathway. This study provides novel *in vivo* evidence that CRF regulates stress-induced apoptosis in a vertebrate model species, and demonstrates for the first time a function for the CRF system in early development that precedes its role in the endocrine stress response.

1. Introduction

Corticotropin-releasing factor (CRF) is best known for its role in regulating circulating glucocorticoid levels by initiating a hormone cascade known as the hypothalamus-pituitary-adrenal (HPA) or –interrenal (HPI) axis. CRF belongs to a family of related neuropeptides that includes urocortin (UCN; tetrapods), urotensin I (UI; fish), and sauvagine (amphibian). The CRF-like peptides signal through class II G-protein coupled transmembrane receptors (CRF-R1 and CRF-R2) and are further regulated by a specific binding protein (CRF-BP). Collectively, these components are called the CRF system, which is highly conserved in both form and function among vertebrates owing to its central role in the endocrine stress response (Denver, 2009). But the CRF system carries out numerous functions beyond HPA/I axis regulation. For example, the CRF system is broadly expressed in all major divisions of the central nervous systems of vertebrates, including teleost fish (Alderman et al., 2008; Alderman and Bernier, 2007; Pepels et al., 2002), and interacts with brainstem adrenergic and serotonergic neurotransmitter systems to affect context-specific behavioral and autonomic responses to stress (Lowry and Moore, 2006). There is significant potential, therefore, in using non-mammalian model species to define the broad functions of the CRF system in vertebrate physiology. To this end, the rapid external development of zebrafish offers an opportunity to study the endogenous role of the CRF system in vertebrate embryos that is independent of maternal factors.

Stress and/or elevated glucocorticoids experienced during early life stages can have lasting physiological consequences (Moisiadis and Matthews, 2014; Zannas and Chrousos, 2017). As the central regulator of circulating glucocorticoids, therefore, the CRF system is crucial to understanding the mechanisms that relate early life experiences to future phenotype. Interestingly, the CRF system is expressed at early embryonic stages of fish, prior to the onset of HPI axis maturation (Alderman and Bernier, 2009; Chandrasekar et al., 2007; Fuzzen et al., 2011). For example, in zebrafish, the CRF system is fully expressed by gastrulation or approximately 6 h post fertilization (hpf; Alderman and Bernier, 2009); whereas functional interaction between the pituitary

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and interrenal tissue occurs much later at 2 d post fertilization (dpf), around the time of hatching (To et al., 2007). A stressor-induced increase in cortisol, an indicator of HPI axis maturation, is further delayed to between 3 and 4 dpf (Alderman and Bernier, 2009; Alsop and Vijayan, 2008; Wilson et al., 2013). This suggests that the CRF system may have an early embryonic function that is independent of the HPI axis, but to date no such role has been described.

One intriguing function of the CRF system that may be relevant to embryonic development is its role in promoting cellular survival. The ability of CRF-related peptides to reduce *in vitro* stress-induced apoptosis is fairly well-established in mammalian neuronal (Bayatti and Behl, 2005; Koutmani et al., 2013) and cardiomyocyte (Davidson et al., 2009) cultures, but has also been demonstrated in other mammalian cell types (Blaabjerg et al., 2014; Radulovic et al., 2003). The molecular mechanisms responsible for these cytoprotective effects are likely mediated by CRF-R1 (Blaabjerg et al., 2014; Facci et al., 2003; Radulovic et al., 2003), and include transcriptional activation of survival factors (Bayatti et al., 2005; Brar et al., 2002) as well as suppression of caspase-3 (Madtes et al., 2004; Radulovic et al., 2003). Caspase-3 is a key effector enzyme in the apoptotic cascade, and its role in orchestrating programmed cell death is unequivocal (Nicholson and Thornberry, 1997; Strasser et al., 2000). There is limited evidence from non-mammalian taxa to support the evolutionary conservation of CRF-mediated cytoprotection (Boorse et al., 2006; Williams et al., 2017). Moreover, the mechanisms and outcomes of CRF-mediated cytoprotection have only been studied through *in vitro* and *ex vivo* experiments, and the present lack of direct *in vivo* evidence restricts our ability to understand this function at an organismal level. Therefore, we used a standardized acute heat shock stressor, previously shown to induce caspase-3 activity in zebrafish embryos as early as gastrulation (Yabu et al., 2001b), to test the hypothesis that early expression of the CRF system helps protect embryos from stress-induced apoptosis.

2. Materials and methods

2.1. Animals

Adult wild type zebrafish (*Danio rerio*) were obtained from AQUAlity Tropical Fish Wholesale (Mississauga, ON, Canada), and maintained as previously described (Alderman and Bernier, 2009) as per the principles of the Canadian Council for Animal Care. Embryos were collected from spawning groups of 6–8 fish and raised in egg water (6% w/v Instant Ocean; Spectrum Brands Inc, Atlanta, GA) at 28.5 °C, unless otherwise stated.

2.2. Experiment 1: Characterizing the endogenous response to heat shock

Embryos were raised either to the onset of gastrulation (~6 hpf; germ-ring stage) or to 30 hpf, and then subjected to a 1 h heat shock stressor at 40 °C or maintained at 28.5 °C (controls), as previously described (Yabu et al., 2001b). All embryos were then recovered at 28.5 °C for up to 10 h. Precise timing and temperature of the heat shock stress was ensured by directly transferring embryos between egg water at the desired temperatures, and control embryos were similarly handled. Embryos were sub-sampled at 4 h, 7 h and 10 h recovery for subsequent quantification of gene expression (n = 6 pools of about 20 embryos) or caspase-3 activity (n = 5 pools of 15 embryos) by euthanizing in tricaine and snap freezing on dry ice. For embryos heat-shocked at 30 hpf, samples for caspase-3 activity were only collected at the end of the 10 h recovery period.

2.3. Experiment 2: Effect of CRF overexpression on heat shock induced caspase-3

The open reading frame of *crf* (GenBank: BC085458) was generated by RT-PCR to include flanking BamH1 recognition sites using the primers: fwd 5′-gccggatccg ccaccatgaa gctcaatttt ctcg-3′ and rev 5′-ccccggatcc cttcccgaat atttccatc-3′. Following purification and sequence verification, the DNA fragment was ligated into either a pCS2p + or a pCS2p + eGFP vector linearized with BamH1 (kindly supplied by Dr. David Turner, University of Michigan). Messenger RNAs for native vectors and CRF-containing constructs were transcribed from 1 µg of purified plasmid DNA templates using the mMessage mMachine SP6 Kit (Ambion Inc, Austin, TX) according to manufacturer's instructions, and size-verified by gel electrophoresis. Synthesized mRNAs were stored in aliquots at −80 °C, then thawed on ice and diluted in sterile 1× Danieau (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.6) prior to microinjection of fertilized eggs (2 nl volume containing 0.5% phenol red). We used synthesized mRNAs from the pCS2p + eGFP vector constructs to validate the *in vivo* translation and in-phase insertion of *crf* (200 pg/embryo). Embryos were observed with fluorescent microscopy 4 h following microinjection, and a uniform distribution of GFP signal across blastomeres validated our constructs and microinjection technique. However, since this vector creates a large CRF-eGFP fusion protein with unknown CRF receptor affinity, all subsequent experiments were carried out with the pCS2p + vector constructs. To determine the effect of CRF overexpression on the caspase-3 response to heat shock, embryos were microinjected with 50, 100, or 200 pg/embryo mRNA from empty vector (injection control) or CRF constructs. At the onset of gastrulation, a subset of injected embryos was heat shocked and recovered for 10 h as above while the remaining embryos were maintained at 28.5 °C for the duration of the experiment. Total mortality was recorded at the end of the recovery period and viable embryos were collected as described above to quantify caspase-3 activity (n = 9 pools of 10 embryos).

2.4. Experiment 3: Effect of antalarmin on heat shock induced caspase-3

To determine if endogenous CRF is cytoprotective and if CRF cytoprotection in zebrafish embryos is mediated through CRF-R1, 30 hpf embryos were heat shocked as above, and then recovered for 10 h at 28.5 °C in egg-water alone or containing the CRF-R1 specific antagonist, antalarmin (10 nM; Tocris Bioscience, Bristol, UK). The dosage of antalarmin was selected based on its inhibitory constant (K_i = 1 nM; Webster et al., 1996). Survival was assessed every hour throughout the recovery period by visual inspection under a stereomicroscope (SMZ1500; Nikon Instruments Inc., Melville, NY, USA) to confirm heartbeat. Viable embryos were collected at the end of the recovery period as described above to quantify caspase-3 activity (20 embryos/n). This experiment was replicated 3 times, and final sample sizes varied by treatment (for survival, *N* represents one experimental replicate, therefore n = 3; for caspase-3 activity, n = 3–4 for control and n = 6 for heat shock treatments).

2.5. Gene expression analysis

Total RNA was extracted using Trizol (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's instructions, then 1 µg of total RNA was treated with DNase-I (Invitrogen) and reverse transcribed to cDNA using Superscript III following manufacturer's instructions (Invitrogen). Gene expression was measured in duplicate reactions by real-time PCR as described previously (Alderman and Bernier, 2009) using primers specific for *crf* (fwd: 5′-gccgcgcaaagttcaaaa-3′, rev: 5′-gcgaggagaatctgtgcgtaa-3′; GenBank accession no. NM_001007379) or *crf-bp* (fwd: 5′-cgagagttaccagaggagtttgtgta-3′, rev: 5′-accctctacggccaccatatc-3′; GenBank accession no. NM_001003459.1), as well as for two housekeeping genes (HKG), elongation factor-1α (*ef1α;* fwd: 5′-gggcaagggctccttcaa-3′; rev: 5′-cgctcggccttcagtttg-3′; GenBank accession no. NM_131263) and ribosomal protein L8 (*rpl8*; fwd: 5′-atagtctgctgtctggaggag-3′; rev: 5′-tcgggattgtgggaaataacg-3′; GenBank accession no. NM_200713). Average threshold cycle values for each sample were fitted to the antilog of standard curves generated from

serially diluted cDNA. To negate the modest increase in *ef1a* and *rpl8* expression induced by heat shock (< 2-fold; $p < 0.05$), HKG expression for each sample was first normalized to the average expression of the 4 h control treatment group, and then used to standardize the expression of each gene of interest. Primer-specific amplification efficiencies were: *crf* 81%, *crfbp* 88%, *ef1a* 92%, *rpl8* 98%. The absence of genomic DNA co-amplification was confirmed using non-reverse transcribed samples.

2.6. Caspase-3 assay

Caspase-3 activity in embryos heat shocked at 6 hpf (*Experiments 1* and *2*) was quantified using the Caspase-Glo® 3/7 Assay (Promega, Madison, WI) as per manufacturer's instructions. Briefly, samples were homogenized in 160 µl ice-cold homogenization buffer (20 mM HEPES-KOH pH 7.5, containing 250 mM saccharose, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT) and centrifuged for 15 min at 10,000 g. The resulting supernatant was mixed with Caspase-Glo® reagent and assayed. The luminescent signal was measured using a luminometer (LMax II; Molecular Devices, Sunnyvale, CA, USA) and is expressed as relative light units (RLU) standardized to the protein content of the supernatant (Bio-Rad Protein Assay with bovine serum albumin standards; Bio-Rad Laboratories, Hercules, CA). Caspase-3 activity in embryos heat shocked at 30 hpf (*Experiments 1* and *3*) was quantified based on the methods described by Stankiewicz et al. (2005). Briefly, the maximum rate of product formation from the fluorogenic caspase-3 substrate Ac-DEVD-AMC was quantified by fluorescence spectrophotometry. Samples were homogenized in lysis buffer (50 mM HEPES, 0.1% w/v CHAPS, 0.1 mM EDTA, 1 mM DTT, pH 7.4, 10 mg/ml aprotinin, leupeptin, and pepstatin A; Sigma Aldrich, Oakville, ON) and centrifuged at 12,000 g for 10 min. The supernatant was mixed with 3 volumes of assay buffer (50 mM HEPES, 100 mM NaCl, 0.1% w/v CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) containing 40 mM Ac-DEVD-AMC (Amresco, Solon, OH, USA). Fluorescence was measured at 460 nm (with excitation at 380 nm) for 1 h at 28 °C using a fluorescence plate reader (BioTek FLx800; BioTek, Winooski, VT, USA). The maximum reaction rate was standardized to the protein content as above (RLU/µg protein).

2.7. Data analysis

Data from *Experiment 1* were analyzed using a two-way ANOVA and Holm-Sidak test for the main variables of temperature (control, heat shock) and time (4h , 7 h , 10 h recovery), with the exception of caspase-3 activity in embryos heat shocked at 30 hpf which had a single time variable and was therefore analyzed using a Student's *t*-test. Gene expression data was independently analyzed using both *ef1a* and *rpl8* as HKG, and identical results were obtained. For *Experiment 2*, the percent change in caspase-3 activity between biological replicates of heat shock and control groups were determined for each injection type and concentration. These calculated values were used as the dependent variable to determine the effects of injection type (vector, CRF) and concentration (50, 100, 200 pg/embryo) on percent caspase-3 induction by heat shock (two-way ANOVA and Holm-Sidak test for multiple comparisons). One-way ANOVAs were used to detect concentration-specific effects on mortality for each injection type and temperature ($n = 9$; $p > 0.05$). Seeing none, mortality data was pooled across concentrations ($n = 27$) to determine the overall effect of mRNA microinjection and heat shock on embryo mortality (two-way ANOVA and Holm-Sidak test for multiple comparisons). Finally, for *Experiment 3*, differences in caspase-3 activity between control and antalarmin treated embryos were determined with a two-way ANOVA using the main variables temperature (control, heat shock) and concentration (0 nM, 10 nM antalarmin), followed by a Holm-Sidak test for multiple comparisons ($n = 3–6$ pools of 20 embryos). Cumulative induced mortality was calculated for experimental replicates ($n = 3$) as the difference between heat shock and control at each recovery time interval. These calculated values were used as the dependent variable to determine the effects of recovery time (hourly from 1 h to 10 h) and concentration (0 nM, 10 nM antalarmin) on heat shock-induced mortality (two-way repeated measures ANOVA and Holm-Sidak test for multiple comparisons). All analyses were performed in SigmaPlot 12.5 at $\alpha = 0.05$, with data normality and equal variance confirmed prior to ANOVA analyses. Data is expressed as mean ± SEM.

Table 1
The induction of caspase-3 activity following a 1 h heat shock stress. Embryos at 6 hpf were either transferred to egg water at 28.5 °C (control) or at 40 °C (heat shock) for 1 h, then returned to egg water at 28.5 °C. Caspase-3 activity (in relative light units, RLU/µg protein) was quantified after 4 h, 7 h, or 10 h recovery and differences were determined by two-way ANOVA and Holm-Sidak multiple comparison tests. Embryos at 30 hpf were treated to control or heat shock conditions as above and recovered for 10 h, and differences in caspase-3 activity were determined by a Student's *t*-test. An asterisk indicates differences between control and heat-shocked embryos at a given recovery time ($n = 5–10$; $p < 0.05$). Data is mean ± SEM.

Stage	Treatment	Recovery time (h)	Caspase-3 activity (RLU/µg protein)
6 hpf	control	4	0.36 ± 0.06
	heat shock		0.32 ± 0.06
6 hpf	control	7	0.31 ± 0.02
	heat shock		0.47 ± 0.06*
6 hpf	control	10	0.30 ± 0.03
	heat shock		0.64 ± 0.06*
30 hpf	control	10	11.71 ± 0.95
	heat shock		27.34 ± 2.08*

3. Results

3.1. Experiment 1

The endogenous response to heat shock was characterized by transient changes in caspase-3 activity and gene expression. For embryos heat shocked at 6 hpf, the induction of caspase-3 activity was dependent on recovery time (interaction of main variables $P = 0.007$), with a significant increase over controls at 7 h and 10 h recovery (Table 1). A similar heat shock-induced increase in caspase-3 activity occurred in 30 hpf embryos recovered for 10 h (Table 1). Changes in gene expression were analyzed independently with normalized *ef1a* and *rpl8* as HKG and yielded identical results; therefore only results for *ef1a* are presented. There was a significant interaction between the main effects of treatment and time on *crf* expression, but the specific changes were different at each developmental stage. In embryos heat shocked at 6 hpf, *crf* abundance increased relative to controls after 4 h recovery, but there were no differences at 7 h or 10 h recovery (Fig. 1A). Conversely, there was no change in *crf* expression during recovery from heat shock at 30 hpf; however, the non-heat shock treatment group experienced an ontogenic increase in *crf* expression between the 7 h and 10 h time points that was not observed in the heat shock treated group (Fig. 1B). The endogenous response to heat shock was also characterized by an increase in *crf-bp* mRNA levels independent of recovery time (main effect of heat shock $P < 0.05$), and this was true for embryos exposed to the stressor at 6 hpf (Fig. 1C) and at 30 hpf (Fig. 1D).

3.2. Experiment 2

CRF overexpression by mRNA microinjection reduced heat shock-induced caspase-3 activity by approximately 2-fold relative to control-injected embryos (main effect of injection $P < 0.05$, $n = 9$; Fig. 2A). Heat shock significantly increased embryo mortality relative to non-heat shocked embryos (main effect of temperature $P < 0.001$; $n = 27$; Fig. 2B). This occurred independently of injection type, with embryos overexpressing CRF experiencing similar mortality to control-injected

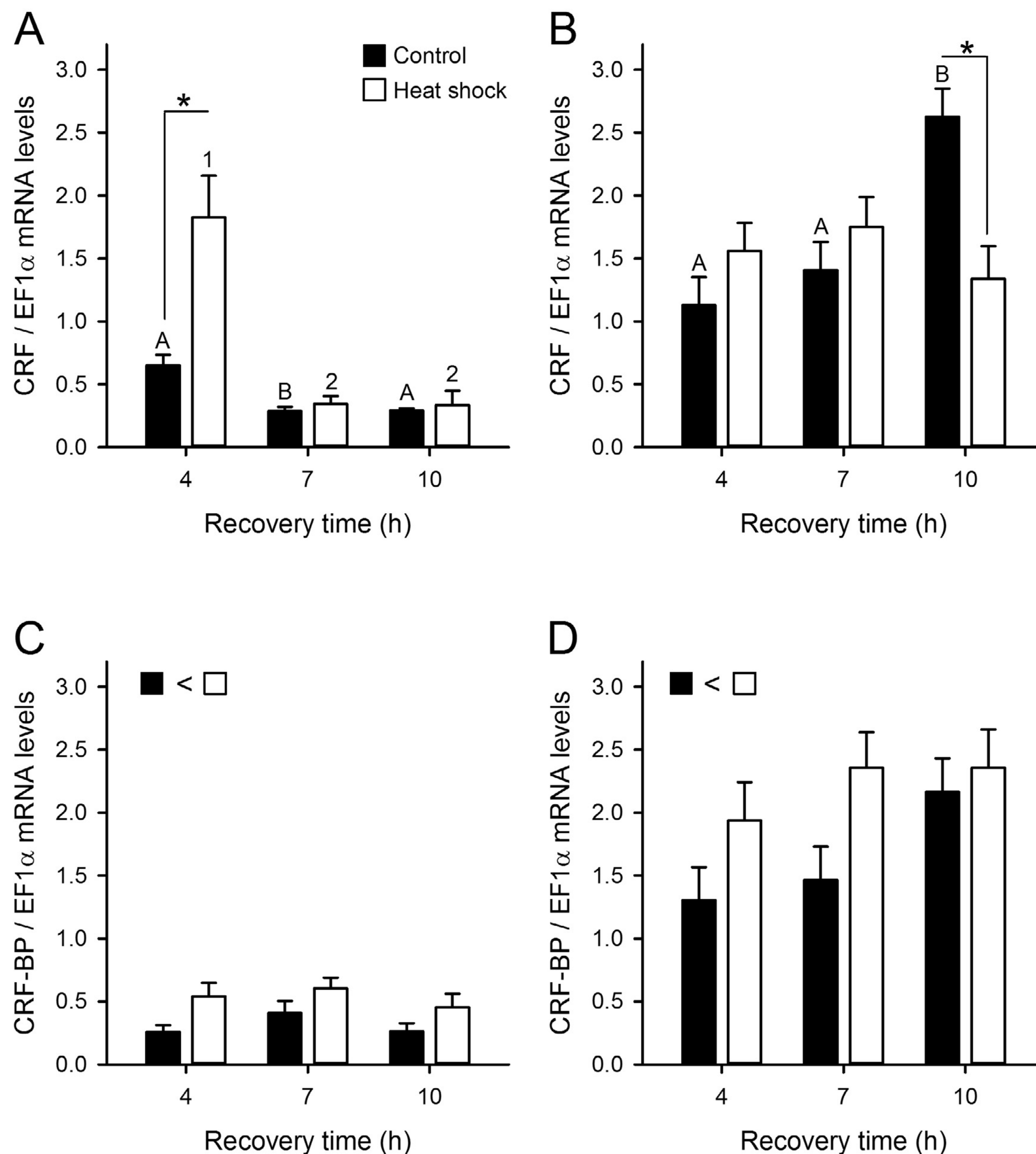

Fig. 1. Effect of heat shock exposure on *crf* (A, B) and *crf-bp* (C, D) gene expression. A heat shock stress was applied by transferring embryos to 40 °C egg water for 1 h and then returning the embryos to 28.5 °C egg water for 4 h, 7 h, or 10 h (white bars). Control embryos were similarly handled but maintained at 28.5 °C for the duration of the experiment (black bars). Heat shock was performed when embryos reached 6 hpf (A and C) or 30 hpf (B and D). Gene expression was quantified by qPCR and normalized to the expression of elongation factor 1α (*ef1α*). Data is presented as mean + SEM. Statistical differences were determined using a two-way ANOVA and a Holm-Sidak test for multiple comparisons (n = 6; $p < 0.05$). For *crf*, significant interactions between the main effects of temperature and time were present (*), and differences within control and heat shock over time are denoted with letters or numbers, respectively. For *crf-bp*, there was a significant difference in the main effect of temperature only.

embryos for both the non-heat shock and the heat shock treatments.

3.3. Experiment 3

As in previous experiments, all heat shock-treated embryos had elevated caspase-3 activity at 10 h recovery relative to non-heat shocked embryos. However, embryos that recovered from heat shock in the presence of the CRF-R1 antagonist, antalarmin, experienced a greater increase in caspase-3 activity than embryos not given the antagonist (interaction of main effects $P < 0.05$; n = 3–6; Fig. 3A). Final cumulative mortality (at 10 h recovery) in non-heat shocked embryos was low in both the 0 nM and 10 nM antalarmin groups (4.7% and 8.3%, respectively), and heat shock increased mortality in both groups (46.0% and 48.3%, respectively). The greatest heat shock-induced mortality occurred during the first 1 h of recovery and plateaued after 5 h recovery (main effect of time $P < 0.001$; n = 3; Fig. 3B), but the presence of antalarmin did not influence this response.

4. Discussion

Our data support a role for CRF, acting via CRF-R1, in regulating stress-induced increases in caspase-3 during zebrafish embryogenesis. We demonstrate this function using two independent experiments that either artificially increase CRF content in embryos using mRNA microinjection, or prevent endogenous CRF signaling using a specific CRF-R1 antagonist. As such, our results support the evolutionary

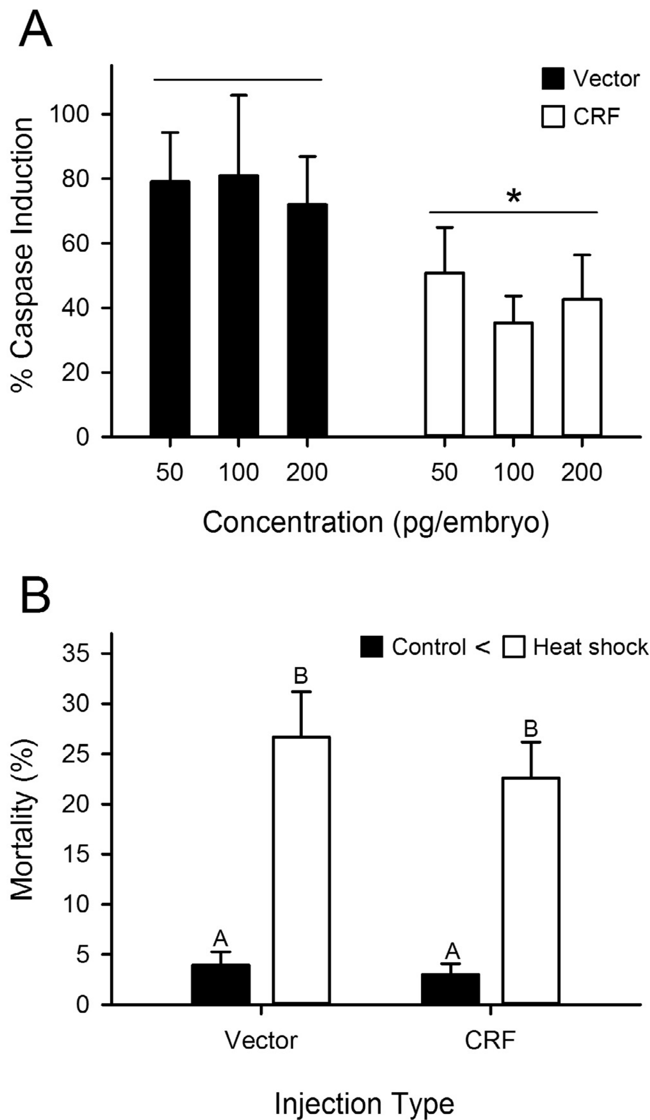

Fig. 2. Effect of CRF overexpression on heat shock-induced caspase-3 activity and mortality. Embryos were microinjected at the 1-cell stage with 50, 100, or 200 pg of synthesized mRNA from empty vector (Vector) or vector containing the coding sequence for *crf* (CRF). A 1 h heat shock stress was applied at 6 hpf and embryos were recovered for 10 h. (A) The change caspase-3 activity for heat shock relative to control treated embryos is expressed as percent caspase-3 induction for each injection concentration. Embryos that overexpressed CRF (white bars) experienced lower caspase-3 induction than embryos given the empty vector (black bars), but the response was not concentration-dependent (two-way ANOVA and Holm-Sidak multiple comparison test; n = 9; $p < 0.05$). (B) Mortality after 10 h recovery was significantly higher in heat shocked (white bars) relative to control (bars), irrespective of injection type (two-way ANOVA and Tukey test; n = 27).

conservation of a role for the CRF system in reducing stress-induced apoptosis, and invite further investigations that link CRF to cytoprotection using the zebrafish model. Equally important is that we present this as a novel function for CRF during a period of embryonic development when the HPI axis is not yet matured, and propose that it may help mediate the impacts of early life stress on offspring phenotype. Thus, we propose the developing zebrafish is a useful *in vivo* model for studying the cellular mechanisms and organismal outcomes of CRF-mediated cytoprotection.

In the past two decades, many have reported that CRF-related peptides act as potent cytoprotective agents for mammalian cells under conditions of stress-induced cell death. For example, Facci and

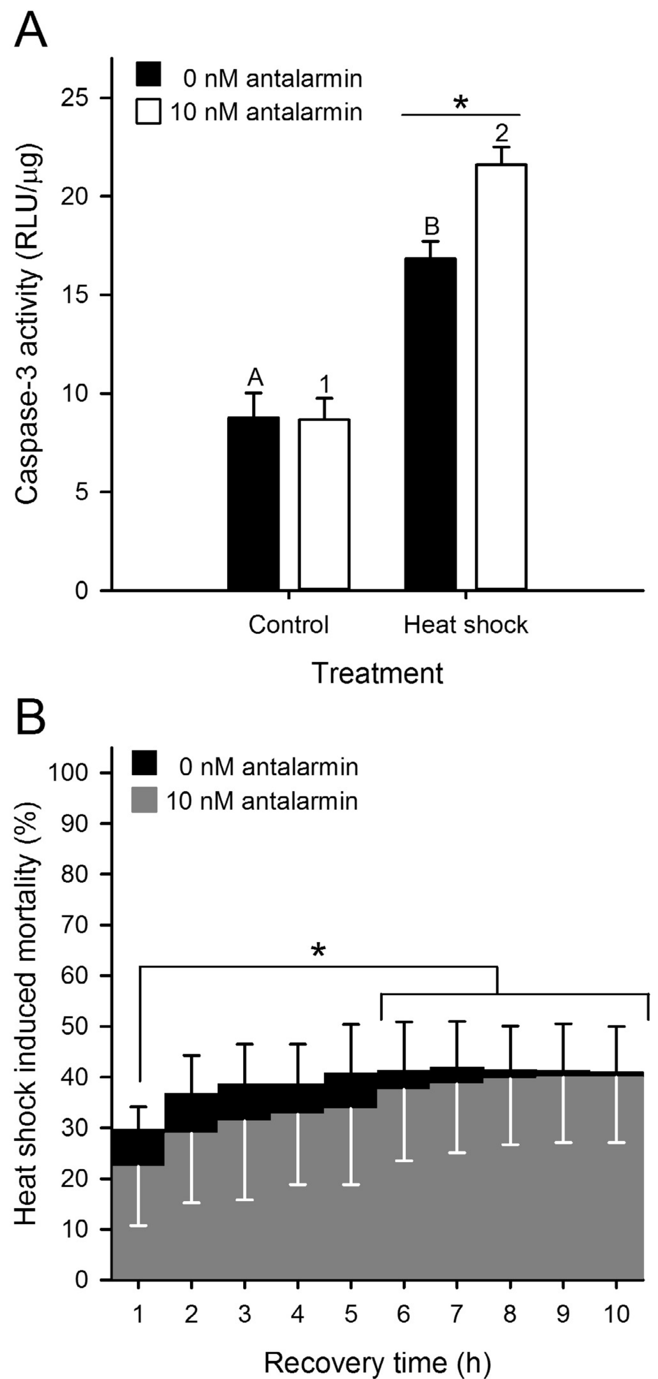

Fig. 3. Effect of the CRF-R1 antagonist, antalarmin, on heat shock-induced caspase-3 activity and mortality. A 1 h heat shock stress was applied at 30 hpf and embryos were recovered for 10 h in the presence or absence of antalarmin. (A) Caspase-3 activity (in relative light units, RLU/μg protein) in control (n = 3–4 pools of 20 embryos) and heat shocked (n = 6 pools of 20 embryos) embryos after 10 h recovery in the presence of 0 nM (black bars) or 10 nM (white bars) antalarmin, shown as mean ± SEM. Significant interactions between the main effects of temperature and concentration were present (*), and differences in the heat shock response for the 0 nM and 10 nM treatments are denoted with letters or numbers, respectively (two-way ANOVA and Holm-Sidak multiple comparisons test; $p < 0.05$). (B) The heat shock induced mortality for embryos recovered in the presence (gray) or absence (black) of 10 nM antalarmin was tallied hourly during the 10 h recovery period, and the experiment was repeated 3 times. Significant differences in the main effect of time (*) were detected using a two-way repeated measures ANOVA and Holm-Sidak multiple comparisons test ($p < 0.001$; n = 3), but no effect of antalarmin nor an interaction were detected. Data is shown as mean ± SEM (positive black error bars are for 0 nM; negative white error bars are for 10 nM).

colleagues showed that CRF and its related peptides UI, UCN, and sauvagine all reduced cell death induced by β-amyloid peptide (Facci et al., 2003). Remarkably, the cytoprotective effects of CRF-like peptides occur even at pico-molar concentrations and when application is delayed for hours after the lethal insult is applied (Elliott-Hunt et al., 2002; Facci et al., 2003; Pedersen et al., 2002). Like other ligands of class II GPCRs (Martin et al., 2005), CRF-related peptides activate cAMP-dependent pathways, including phosphorylation of PKA, that ultimately increase cell survival (Bayatti et al., 2003; Blaabjerg et al., 2014; Brar et al., 2002; Facci et al., 2003; Jonassen et al., 2012; Pedersen et al., 2002). Of relevance to the present study, both CRF and UCN can suppress apoptosis by coordinating a reduction in caspase-3 through PKA-mediated inhibition of the enzyme precursor, procaspase-3 (Madtes et al., 2004; Radulovic et al., 2003). To date, reduced caspase-3 and enhanced cell survival has only been demonstrated in two non-mammalian studies, using intact excised organs to demonstrate the cytoprotective effects of the CRF system *ex vivo*. Application of CRF to cultured tail explants from pro-metamorphic *Xenopus* tadpoles reduced caspase-3 activity and slowed the natural progression of tail resorption (Boorse et al., 2006). Similarly, both CRF and UCN3 reduced caspase-3 activity and the number of apoptotic cells in cultured zebrafish heart explants following hypoxia/re-oxygenation-induced cell injury (Williams et al., 2017). Our data add weight to the argument that cytoprotection offered by CRF-related peptides is indeed an ancient function of the CRF system (Boorse and Denver, 2006), and appears to function in diverse cell types and across a variety of contexts.

Intriguingly, the ontogeny of the CRF system bears some striking resemblance to that of caspase-3. Transcripts for components of the CRF system are maternally deposited into zebrafish oocytes, and endogenous transcription occurs as early as 6 hpf (Alderman and Bernier, 2009). This pattern is also observed for caspase-3, where mRNA of maternal origin is detected in zebrafish prior to endogenous transcription at the shield stage (Yabu et al., 2001a). While basal expression of the CRF system remains relatively low until after hatch (Alderman and Bernier, 2009; Alsop and Vijayan, 2009; Fuzzen et al., 2011), here we demonstrate a stress-induced increase in *crf* and *crf-bp* transcription as early as 11 hpf (4h after earliest heat shock). Although the endogenous response to heat shock is stage-specific for *crf* expression, our results indicate that the CRF system is responsive to environmental stressors from very early stages of development, and prior to a stress-induced cortisol response (Alderman and Bernier, 2009; Alsop and Vijayan, 2008; Wilson et al., 2013). Similarly, while basal caspase-3 activity remains low and unchanged until after 30 hpf (Yabu et al., 2001b), exposing zebrafish to cycloheximide, UV irradiation, or heat shock can induce caspase-3 activity as early as gastrulation (Negron and Lockshin, 2004; Yabu et al., 2001b). Importantly, transgenic embryos that overexpress procaspase-3 experience increased apoptosis during embryogenesis that leads to morphological changes in the eye, notochord, and heart (Yamashita et al., 2008), highlighting the importance of regulating pro-apoptotic pathways during early development. Thus the capacity for the CRF system to moderate stress-induced caspase-3 activity during embryogenesis may be critical for protecting sensitive embryos from disrupted development due to environmental fluctuations, and this may be especially relevant in organisms that develop externally.

The CRF-BP is a key regulator of CRF signaling, acting to coordinate the equilibrium of free versus bound ligand (Seasholtz et al., 2002). We observed a consistent and prolonged increase in *crf-bp* transcript abundance during recovery from heat shock, suggesting a role for this protein in regulating post-stress CRF signaling. Indeed, an increase in CRF-BP may allow the selective regulation and termination of CRF signaling to fine-tune the cellular response to stress. Such a role may be important given that CRF can also promote apoptosis (Androulidaki et al., 2009; Chen et al., 2014; Dermitzaki et al., 2002). For example, over-activation of CRF-R1 in response to persistently uncontrolled responses or prolonged CRF treatment can induce apoptosis and activate apoptotic genes (Chen et al., 2014). Future studies should consider the role of CRF-BP in mediating the biphasic effects of CRF on apoptosis.

Despite clear evidence that the CRF system regulates heat shock-induced caspase-3 activity, organismal survival was not enhanced through this pathway during the time course of our study. Mortality was substantially increased by heat shock, but neither CRF overexpression nor antalarmin treatment influenced this result. By regularly monitoring mortality during heat shock recovery, we show that most deaths occurred during the first hour of recovery, and nearly zero deaths occurred between 6 h and 10 h recovery. Importantly, a heat shock-induced increase in caspase-3 does not occur until at least 6 h recovery (present study; Yabu et al., 2001b), indicating that the mortality observed in our study likely occurred via non-apoptotic and CRF-independent pathways, such as necrosis. Therefore, our novel *in vivo* results are consistent with previous *in vitro* evidence from established models (i.e. cardiomyocyte and primary neuronal cultures) showing that CRF-mediated cytoprotection manifests through the regulated and slower apoptotic process and independent of the rapid and uncontrolled necrosis-induced cell death (Bayatti and Behl, 2005; Davidson et al., 2009; Koutmani et al., 2013).

5. Conclusion

This study presents the first *in vivo* evidence that supports CRF-mediated cytoprotection via regulation of caspase-3, thereby establishing the developing zebrafish as an important model for understanding the molecular regulation of stress-induced apoptosis. Studies during the first 2 dpf of zebrafish development can investigate the regulation and mechanisms of CRF-mediated cytoprotection in intact organisms without the potentially confounding effects of the endocrine functions of the CRF system. In addition, the long-term organismal outcomes inferred by CRF-mediated cytoprotection can be readily quantified by taking advantage of embryo transparency (morphological endpoints) as well as high-throughput technologies for assessing movement (behavioral endpoints). We predict, therefore, that zebrafish will be instrumental in understanding how the CRF system engages with and modulates pro-apoptotic signaling. Ultimately, this will generate an appreciation for the CRF system in connecting shifts in the external environment with internal physiology via the HPI axis after hatching, as well as via reducing stress-induced apoptosis during embryogenesis.

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