Rapid and Nongenomic Glucocorticoid Signaling in Rainbow Trout

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Corticosteroids are key regulatory hormones involved in many aspects of physiology and have long been known to exert rapid and delayed effects. The delayed corticosteroid effects are mediated by transcriptional events downstream of glucocorticoid receptor activation. Conversely, the rapid effects are mediated independently of transcriptional regulation and are thought to involve non-classical steroid receptors and signaling pathways. Lately, research has begun to focus on delineating the rapid and nongenomic actions of glucocorticoids but most of these studies have been on mammalian models. Cortisol, the primary corticosteroid in teleosts, is an established genomic regulator of the physiological response to stress, but very little is known about either the rapid effects or their mechanisms of action in non-mammalian vertebrates. Additionally, nongenomic glucocorticoid action in the liver is poorly characterized in all animal species despite the importance of this organ in regulating glucocorticoid-mediated physiological adjustments during stress adaptation. The primary objective of this thesis is to investigate the rapid glucocorticoid effects, and their mode of action, associated with stressor-induced corticosteroid elevation in fish liver. The overriding hypothesis is that rapid effects of cortisol on acute stress adaptation involve changes to liver membrane order and rapid modulation of stress signaling pathways in rainbow trout (*Oncorhynchus mykiss*), a well studied teleost model. This hypothesis was tested by examining rapid plasma membrane and intracellular responses following stressor-induced cortisol elevations *in vivo*, as well as to cortisol treatment *in vitro* using liver plasma membrane, tissue slices, hepatocytes in suspension, or primary culture of hepatocytes.

Steroid hormones are lipophilic molecules and freely incorporate into the plasma membrane. Through noncovalent interactions (hydrogen bonds and Van der Waal forces), glucocorticoids can potentially alter physical properties of the plasma membrane, thus leading to intracellular responses. The effect of stressor-induced cortisol elevations on physical changes to the hepatic plasma membrane was investigated by measuring the microviscosity of the plasma membrane. Plasma membrane fluidity (inverse of microviscosity) is an important determinant of transmembrane protein function, and changes to lipid order can transmit extracellular signals by activating membrane-associated signaling pathways. Fluidity of purified liver plasma membranes was monitored using steady-state fluorescence polarization of 1,6-diphenyl-1,3,5 hexatriene, a well characterized membrane probe. In addition to measuring lipid dynamics, the effect of cortisol on plasma membrane structure and surface properties were also investigated using atomic force microscopy. The effect of

cortisol on the activation of key stress signaling pathways, including protein kinase (PKA), protein kinase (PKC), Akt, and mitogen-activated protein kinase (MAPK), was tested in fish liver. Also, as acute stress adaptation is regulated by an integrative hormonal response involving catecholamines (primarily epinephrine), the rapid effect of cortisol action on adrenergic signaling in the liver was evaluated *in vitro*. Finally, an attempt was made to identify cortisol-binding plasma membrane protein, as glucocorticoids are also thought to mediate rapid effects through a novel membrane glucocorticoid receptor.

The results demonstrate for the first time that stressor exposure significantly increases liver plasma membrane fluidity (decreased microviscosity). A role for cortisol in mediating stressor-induced fluidization was confirmed *in vitro*, as physiological stress levels of this steroid (≥100 ng/ml) significantly increased liver plasma membrane fluidity. In addition to increasing lipid fluidity, acute stress and cortisol treatment altered membrane topography, including changes to membrane microdomains. The stressor-induced cortisol elevation also rapidly modulated major signaling cascades in rainbow trout liver, including PKA, PKC, and ERK1/2 MAPKs. A role for cortisol in the activation of these kinase pathways was confirmed *in vitro*. Specifically, cortisol rapidly and transiently increases cyclic AMP (cAMP) accumulation and induces the phosphorylation of PKA substrate proteins, including cAMP response element-binding (CREB) protein. In addition to activating PKA signaling, cortisol rapidly induced phosphorylation of PKC and Akt substrate proteins, while stimulating p38 MAPK dephosphorylation *in vitro*. Moreover, rapid cortisol signaling may stimulate metabolite oxidation in order to maintain the energy balance within liver tissue as cortisol acutely depleted key liver metabolites (including liver glucose), suggesting enhanced turnover without impacting the steady-state adenylate energy charge ratio (measure of the energy status of the cell). Also, rapid effects of cortisol alter the hormonal responsiveness of hepatocytes to adrenergic stimulation, including suppression of epinephrine-stimulated cAMP-CREB activation and glucose production. Preliminary results point to a plasma membrane protein that specifically binds cortisol in trout liver, but this remains to be characterized. Also, in addition the membrane-mediated response, mifepristone, a glucocorticoid receptor (GR)-antagonist, blocked some rapid cortisol effects suggesting the possible involvement of GR signaling pathway.

Until now, cortisol has been primarily thought to play a role in the long-term recovery process to acute stress by enhancing plasma glucose levels through the upregulation of liver gluconeogenic capacity. The results presented in this thesis provide evidence for a novel role for rapid

cortisol action on the acute metabolic adjustments that support liver function immediately following acute stressor exposure. Particularly, the results lead to the proposal that acute cortisol action stabilizes the energy status of the cell by maintaining ATP levels through increased metabolite turnover, suggesting enhanced metabolic activity of the liver immediately following acute stressor exposure. While the mechanism is unclear, plasma membrane alterations in response to cortisol intercalation may facilitate rapid steroid signaling either through mechanotransduction or by altering activity of plasma membrane proteins. The structural changes to the plasma membrane in response to acute stressor exposure and/or cortisol treatment highlight a novel membrane-mediated mechanism of rapid stress adaptation in hepatic tissue.

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List of Abbreviations

1,2-dipalmitoylphosphatidylcholine (DPPC)
1,6-diphenyl-1,3,5-hexatriene (DPH)
11-deoxycorticosterone (DOC)
2-aminobutyrate (2-AB)
3,3'-dioctadecyloxacarbocyanine perchlorate (DiO)
Adenosine diphosphate (ADP)
Adenosine monophosphate (AMP)
Adenosine triphosphate (ATP)
Adenylate cyclase (AC)
Adrenocorticotropic hormone (ACTH)
Alanine (Ala)
AMP-activated protein kinase (AMPK)
Asparagines (Asn)
Aspartate (Asp)
Atomic force microscopy (AFM)
cyclic AMP (cAMP)
cAMP-response element binding protein (CREB)
Cholesterol (C)
Corticotrophin releasing factor (CRF)
Cycloheximide (cyclo)
Dexamethasone (DEX)
Free fatty acids (FFA)
Glucocorticoid receptor (GR)
Glucocorticoid response elements (GREs)
Glucocorticoids (GC)
Glucose-6-phosphate dehydrogenase (G6PDH)
Glutamate (Glu)
Glutamine (Gln)
Glycerophosphocholine (GPC)
Glycine (Gly)
Glycosylphosphatidylinositol (GPI)
G-protein coupled receptors (GPCR
Hypothalamic-pituitary-adrenal (HPA)
Hypothalamic-pituitary-interrenal (HPI)
Isoleucine (Ile)
Isocitrate dehydrogenase (ICDH)
Lactate dehydrogenase (LDH)
Leucine (Leu),
Lipoprotein lipase (LPL)

Malic enzyme (ME)
MAPK phosphatase-1 (MKP-1)
Melanocortin 2 receptor (MC2R)
Membrane glucocorticoid receptors (mGR)
Methionine (Met)
Mifepristone (RU486)
Mineralocorticoid receptor (MR)
Mitogen-activated protein kinase (MAPK)
Nuclear magnetic resonance (NMR)
Paraventricular nucleus (PVN)
Phenylalanine (Phe)
Phosphatidylcholine (PC)
Phosphatidylethanolamine (PE)
Phosphatidylinositides (PI)
Phosphatidylserine (PS)
Phosphocholine (PC)
Phosphoenolpyruvate carboxykinase (PEPCK)
Phosphoinositide 3-kinase (PI3K)
Protein kinase A (PKA)
Protein kinase C (PKC)
Sarcosine (Sar)
Sphingomyelin (SM)
Synthetic peptide (PEP)
T cell receptor (TCR)
Taurine (Taur)
Threonine (Thr)
Trimethylamine N-oxide (TMAO)
Tyrosine (Tyr)
Valine (Val)
Zeta-chain-associated protein kinase of 70 kDa (ZAP-70)

Chapter 1
General Introduction

1.1 Introduction

In response to stressful stimuli (physical, chemical or psychological) the neuroendocrine stress-axis is activated, which co-ordinates physiological adjustments, ultimately resulting in altered digestive, respiratory, metabolic, osmoregulatory, immunologic and reproductive status (Sapolsky et al., 2000). The neuroendocrine stress system is a well conserved and integrated physiological response which involves the rapid secretion of stress hormones, principally catecholamines (norephinephrine and epinephrine) and glucocorticoids (GC; cortisol in mammals and teleost or corticosterone in rats, mice, birds, amphibians and reptiles; Sapolsky et al., 2000). The mechanism of action and cellular effects of catecholamines and GC are of primary concern as dysregulation of the stress axis has several harmful consequences, including impaired growth, metabolism, immune function and reproduction (Romero et al., 2009; Vegiopoulos and Herzig, 2007). Consequently, sustained activation or suppression of the stress-axis can result in several stress-related diseases, which not only wreak havoc on the individual but can potentially damage population dynamics (Romero et al., 2009).

Whereas catecholamines are generally associated with rapid activation of the fight-or-flight response, GC is generally thought to elicit delayed effects (within hours or days) that are essential for stress adaptation (Mommsen et al., 1999). The delayed effects of GC are thought to be mediated by genomic action and include the activation of the ligand-bound transcription factor, the glucocorticoid receptor (GR) (Bury and Sturm, 2007). In addition to genomic regulation, steroid hormones also stimulate rapid effects, which are independent of gene transcription (Borski, 2000). Unlike the classical steroid signaling pathway, the nongenomic role of GC in mediating the acute stress response is underrepresented in the scientific literature (Borski, 2000). Although the mechanisms are still under investigation, it is well established that GC elicit rapid inhibitory effects on the mammalian stress-axis (Groeneweg et al., 2011), while the contribution of rapid GC action in peripheral tissues, including liver, following acute stress is poorly characterized across vertebrates (Borski, 2000). The liver is a primary target tissue for GC as it is an essential contributor to the metabolic readjustments in response to stressor exposure. To date, most studies have examined the delayed cellular responses to cortisol, whereas few studies have examined whether this steroid hormone also elicits rapid effects in

the liver. Considering plasma cortisol levels increase several minutes after an acute stressor it is likely that cortisol acutely regulates liver function and plays an important physiological role in supporting homeostatic recovery from the stressor. Also, research conducted to date has primarily examined rapid GC action in mammalian models, whereas the function of non-classical GC signaling in teleost is largely unknown. Therefore, the purpose of this thesis was to investigate whether cortisol mediates rapid effects in rainbow trout and to elucidate the possible mode of action leading to the overall contribution of rapid cortisol action on liver function.

1.2 Stress Response

The generalized stress response is activated in response to homeostatic disruption by either internal (e.g. infection) or external (e.g. predation, environmental changes) stressors (Sapolsky et al., 2000). Although less studied, the stress response in fish appears to be very similar to the response seen in mammals (Mommsen et al., 1999). The physiological response to acute stress (Fig.1-1) is often characterized as two phases, a primary and secondary response (Mommsen et al., 1999). The primary response involves neuroendocrine activation, resulting in an initial induction of the sympathetic nervous system, stimulating the release of catecholamines from chromaffin cells within the head kidney (analogous to adrenal medulla). Simultaneously, the hypothalamic-pituitary-interrenal (HPI) axis (hypothalamic-pituitary-adrenal (HPA) axis in mammals) activation coordinates the synthesis and release of cortisol into the circulation (Bonga, 1997). Briefly, activation of the HPI axis is initiated by central input into the hypothalamus, which in turn stimulates the release of corticotrophin releasing factor (CRF). CRF then stimulates pituitary secretion of adrenocorticotropic hormone (ACTH) into the general circulation, where it induces *de novo* synthesis and secretion of cortisol by activating melanocortin 2 receptor (MC2R) receptor on steroidogenic cells located in the head kidney (analogous to adrenal cortex; Aluru and Vijayan, 2008; Bonga, 1997). Once catecholamines and cortisol are secreted into the blood, they mediate physiological adjustments critical for overcoming and coping with the stressor (Mommsen et al., 1999). Particularly, stress hormones are essential to meet the enhanced energy and oxygen demands, by augmenting cardiovascular function and enhancing metabolic capacity (Mommsen et al., 1999).

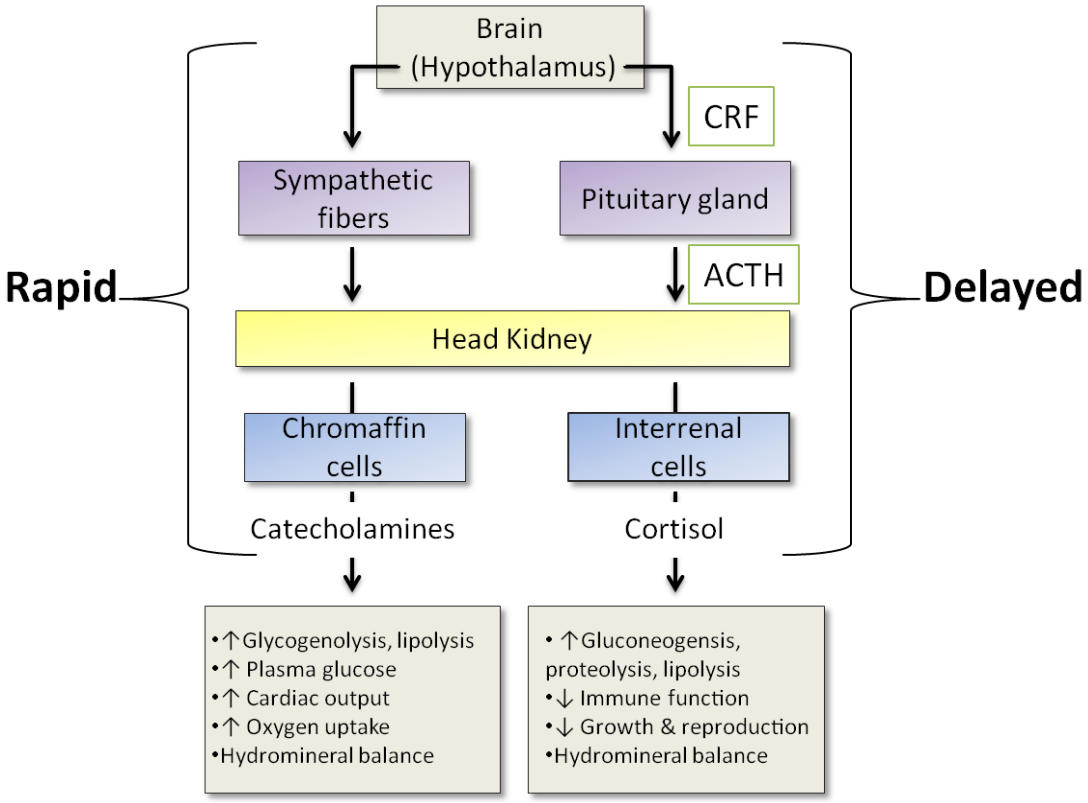


Figure 1-1. The teleost stress-axis.

In response to an acute stressor (predation, environmental changes, trauma, etc.) a neuroendocrine response is immediately mounted to provide major tissues with adequate oxygen and nutrients to meet increased metabolic demand associated with acute stress. Activation of the stress-axis involves rapid release of catecholamines through sympathetic nerve fibers, whereas activation of hypothalamus-pituitary-interrenal axis results in increased plasma cortisol levels. Elevation of these stress hormones mediate important physiological adjustment required for stress adaptation, such as increased oxygen and energy substrate availability. CRF; corticotrophin-releasing factor (hormone), ACTH; adrenocorticotropic hormone.

Due to the catabolic nature and suppressive effects of stress hormones on growth and reproduction, plasma GC levels are strictly regulated through a negative feedback mechanism, whereby elevated levels of GC inhibit the activation of the HPA axis (Miller and O'Callaghan, 2002). In mammalian species, GC has been shown to exhibit regulation at each region of the HPA axis, including the hippocampus and pituitary (Herman and Cullinan, 1997). While not yet investigated in teleost, mammalian negative feedback regulation of GC secretion involves rapid and delayed effects and, therefore, thought to involve both nongenomic and genomic signaling (de Kloet et al., 2008). In mammals, GC rapidly inhibits ACTH release within minutes of glucocorticoid treatment, accounting for about half of the total inhibition of ACTH release (Hinz and Hirschelmann, 2000). Also, studies in mammals indicate GC regulates ACTH secretion via transcription-dependent mechanisms by regulating CRH (Malkoski and Dorin, 1999) and proopiomelanocortin mRNA expression (Birnberg et al., 1983).

The sensitivity of the stress-axis is dependent on genetic and environmental factors (Federenko et al., 2004) and recently it has become evident that developmental conditions have life-long consequences on stress reactivity (Welberg and Seckl, 2001), but relatively less is known in teleosts (Schreck et al., 2010). On a shorter time-scale recent stressful events also influence the response to subsequent stressors (Sapolsky et al., 2000), and therefore the magnitude of stress-axis activation and the subsequent physiological response will be dependent on a variety of parameters, including past environmental (both recent and developmental) conditions.

1.3 Glucocorticoid Signaling

As stated above, GC, like other steroid hormones, initiates both rapid and delayed effects on cellular physiology (Borski, 2000). The delayed effects are mediated by the classical steroid hormone signaling pathway (Fig. 1-2), which involves gene regulation mediated by the activation of the ligand-dependent transcription factor, GR (Scoltock and Cidlowski, 2011). Until recently, research has focused on the genomic consequences of GC action; however attention is now shifting towards delineating the rapid nongenomic action (Fig. 1-2) of this regulatory hormone (Lösel and Wehling, 2003). The rapid GC effects cannot be explained by changes in mRNA levels and protein synthesis as these effects occur within seconds to minutes after steroid treatment (Lösel and Wehling, 2003). Instead the rapid GC effects involve activation of second messenger-protein kinase signaling

pathways, which in some cases also leads to genomic alteration (Lösel and Wehling, 2003). Although our understanding of GC action lags behind that of other steroids, current research is revealing diverse nongenomic GC-activated signaling pathways as well as pleiotropic genomic effects (Revollo and Cidlowski, 2009). The following two sections will provide detailed examples of genomic and nongenomic GC effects and the proposed mechanisms of action.

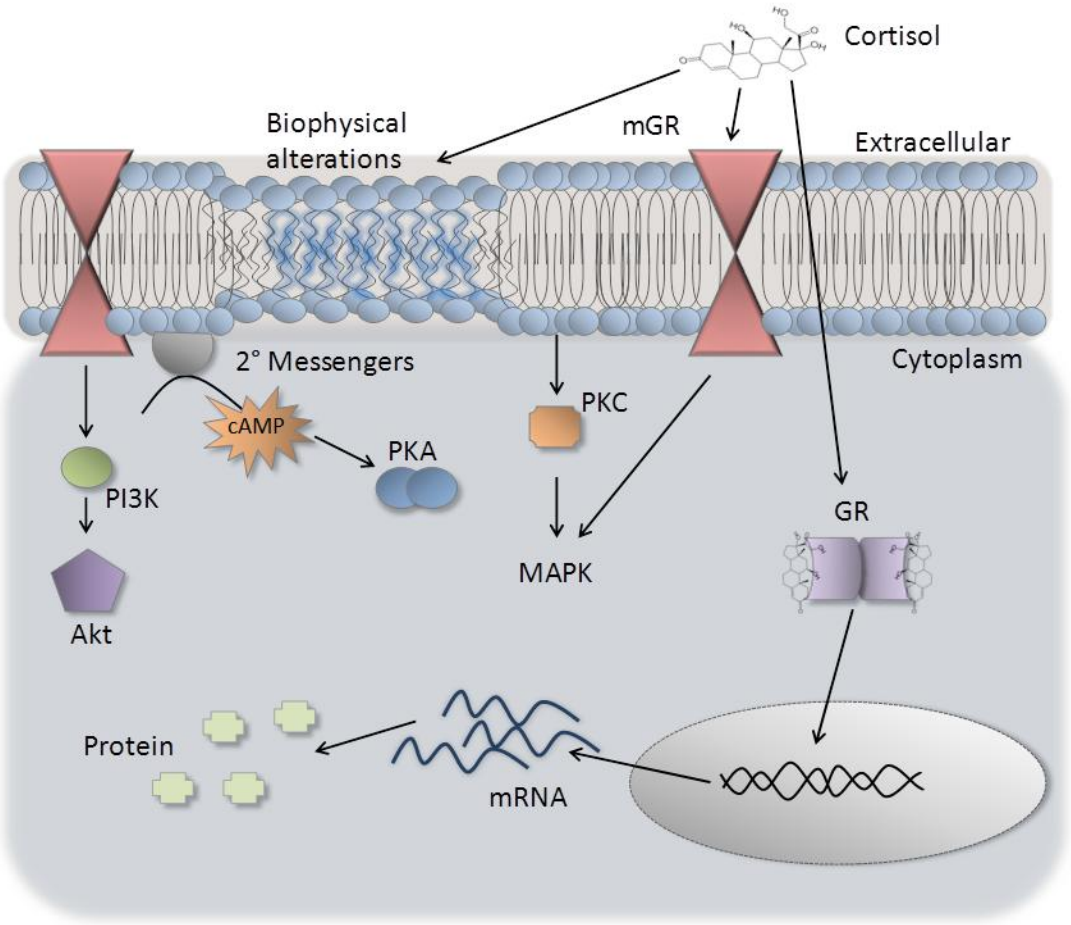


Figure 1-2. Schematic summary of glucocorticoid (GC) signaling

The most well defined mechanism of GC action involves steroid diffusion through the plasma membrane leading to activation of the intracellular glucocorticoid receptor (GR), which regulates protein levels through transcriptional regulation. GC also activates diverse signaling pathways leading to nongenomic cellular adjustments. Although still under intense investigation, these signaling events are initiated at the plasma membrane due to biophysical modulations to the plasma membrane and/or specific membrane glucocorticoid receptors (mGR). Alternatively, GR has also been implicated in nongenomic GC action. PI3K; phosphoinositide 3-kinase, PKA; protein kinase A, PKC; protein kinase C, MAPK; mitogen-activated protein kinase.

1.3.1 Genomic Pathway

Although plasma GC elevations occur within minutes after the onset of stress many GC induced effects are observed several hours later (Lösel and Wehling, 2003). The delayed effects of GC are elicited through the classical mechanism of steroid action, which involves gene regulation, and hence is referred to as the genomic pathway (Bury and Sturm, 2007). The classical steroid signaling pathway is relatively well defined and involves ligand-activated transcription factors belonging to the steroid nuclear receptor family. Cortisol binds both the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), but the role of MR in mediating cortisol effects is not well understood (Colombe et al., 2000; Prunet et al., 2006). Genomic regulation by corticosteroids involves ligand-induced GR activation followed by receptor dimerization and translocation to the nucleus. Once in the nucleus, GR binds to glucocorticoid response elements (GREs) within the promoter region of glucocorticoid-receptive genes leading to enhanced or reduced transcription (Prunet et al., 2006). In the absence of corticosteroid binding, GR forms a multi-protein complex with heat shock proteins and immunophilins (Nicolaides et al., 2010), although this hasn't been confirmed in teleost.

The first GR was cloned in rainbow trout in the mid-90s and was initially believed to be the primary corticoid receptor in teleost (Ducouret et al., 1995). It is now known that most teleost possess two genes encoding GR (GR1 and GR2) (Bury et al., 2003), the only exception being zebrafish (*Danio rerio*) with one GR in the genome (Alsop and Vijayan, 2009). In addition to expressing two GRs, teleost also express an analogous MR receptor (Colombe et al., 2000). Currently it is controversial whether fish possess a distinct endogenous MR ligand other than cortisol. Over the last few years there is increasing evidence that 11-deoxycorticosterone (DOC) might be a physiological agonist for the teleost MR as it is a potent MR-binding compound (Milla et al., 2008; Stolte et al., 2008; Sturm et al., 2005). GR1 and GR2 have distinct activation properties, such that GR2 is more sensitive than GR1 and is subsequently activated at lower cortisol concentrations, whereas GR1 is thought to be activated at high, stress-induced levels of cortisol. This is due to a higher binding affinity and a greater transactivation rate of the GR2 compared to GR1(; Li et al., 2012; Sturm et al., 2011; Prunet et al.,2006; Bury et al., 2003). In rainbow trout, GR mRNA and protein are detected in all major tissue systems including the liver and brain (Aluru and Vijayan, 2004; Mommsen et al., 1999; Alderman et al., 2012), and a recent study revealed that the central and peripheral GR is essential for the negative feedback regulation of plasma cortisol levels (Alderman et al., 2012).

Circulating levels of cortisol stimulate wide-spread genomic regulation in central and peripheral tissues. In the central nervous system cortisol reduces CRF mRNA levels in the preoptic brain area, which has been demonstrated in rainbow trout (Doyon et al., 2006) and goldfish (*Carassius auratus*; Bernier et al., 1999). In trout cortisol also reduces neuropeptide Y (NPY) mRNA levels in the preoptic area, suggesting these peptides are co-regulated in response to GC treatment (Doyon et al., 2006). In addition to regulating CRF levels during negative feedback, GR signaling also appears to maintain basal levels of this peptide in rainbow trout (Alderman et al., 2012).

In the periphery, glucocorticoids are well known for their induction of gluconeogenesis through the up-regulation of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme in the gluconeogenic pathway (Aluru and Vijayan, 2007). In teleost, the effects of GR signaling on transcript levels have been well documented in the liver, and includes regulation of genes involved in energy metabolism, cell stress response, reproduction and xenobiotic metabolism (Aluru and Vijayan, 2007; Aluru and Vijayan, 2009; Wiseman et al., 2007). In addition, piscine GR signaling appears to be involved in liver and muscle proteolysis, by regulating cathepsin D mRNA levels (Aluru and Vijayan, 2009; Dardevet et al., 1995). In teleost, cortisol genomically affects steroidogenic capacity of the interrenal tissue by modulating the steroidogenic acute regulatory protein (StAR) expression, a key rate-limiting step in steroidogenesis involved in transport of cholesterol into the inner mitochondrial membranes (Alderman et al., 2012).

Lastly, in addition to regulating adult physiology, GR signaling is also critical for regulating proper development in teleost (Nesan and Vijayan, 2012). For example, recent studies in zebrafish show that intact GR signaling is required for proper muscle development, as GR morpholino injection disrupts myogenic mRNA levels (Nesan et al., 2012). In addition to muscle development, GR signaling also appears to be important for mesodermal development as GR promoter activity, as well as GR mRNA and protein levels are observed at high levels at somite boundaries (Nesan et al., 2012).

1.3.2 Non-genomic Glucocorticoid Signaling

Nongenomic steroid effects, occur independently of gene expression or *de novo* protein synthesis, and are persistent in the presence of transcriptional and translational inhibitors (e.g. actinomycin D and cycloheximide, respectively) (Lösel and Wehling, 2003). In addition to occurring independently of

genomic activation, the effects are rapid (seconds to minutes) compared to the delayed effects (hours) of transcriptional regulation (Lösel and Wehling, 2003). The ability of membrane-impermeable steroid analogs (e.g. cortisol covalently coupled to BSA or synthetic peptide) to mimic the rapid nongenomic effects has often been used to differentiate between genomic and nongenomic effects. This approach has several drawbacks, such as the possibility of internalization or decoupling of the steroid from the impermeable substrate. Moreover, there is increasing support that rapid nongenomic effects, particularly for GC, might be mediated by the intracellular GR (Löwenberg et al., 2007). Therefore, the insensitivity to transcription and translation inhibitors and time of onset are considered to be the most important criterion for establishing nongenomic steroid effects (Lösel and Wehling, 2003). The mechanisms mediating nongenomic corticosteroid effects are diverse and largely uncharacterized. Experimental evidence thus far suggests three possible mechanisms including 1) biophysical transduction upon cortisol incorporation into the plasma membrane, 2) novel membrane receptor 3) the classical glucocorticoid receptor (Fig. 1-2). Once initiated, nongenomic glucocorticoid effects may be mediated by several different signaling cascades (Fig. 1-2).

1.4 Rapid Glucocorticoid Effects

The following section highlights what is currently known regarding nongenomic cortisol action and the proposed pathways mediating these effects. It is important to note that currently, research investigating nongenomic cortisol action has been primarily conducted in mammalian models, whereas research on rapid GC action in non-mammalian vertebrates, particularly teleosts, is limited. Moreover, research conducted to date, has focused heavily on the central nervous system, cardiovascular system and immune function, with few reports on other peripheral organ systems, including liver (Haller et al., 2008). As described below, GC have diverse rapid effects and may be mediated by equally diverse mechanisms. Thus, nongenomic GC action is highly tissue and context-specific, and possibly species-specific as well.

1.4.1 Nervous system

The physiological relevance of nongenomic GC action was first realized with regards to negative feedback control of the mammalian HPA axis, as GC elevation rapidly inhibits HPA hormone secretion (Jones et al., 1972). Although GC also genomically regulate HPA activity, they also have rapid effects occurring within minutes, which are independent of mRNA and protein synthesis (Abou-samra et al., 1986). Since the initial characterization of rapid GC-mediated deactivation of the

mammalian HPA axis, progress has been made at delineating the mechanisms involved in this nongenomic feedback circuit (Evanson et al., 2010). Earlier studies using mouse pituitary cells, reported that dexamethasone, through a G-protein pathway, inhibits stimulated-cAMP production and subsequent ACTH secretion (Iwasaki et al., 1997). However, in a human pituitary cell line, GC reduce ACTH secretion through a rapid GR-mediated pathway (Solito et al., 2003). Together this suggests that within mammalian models there may be divergent rapid signaling pathways, by which GC inhibit ACTH secretion.

In addition to inhibiting CRH-stimulated ACTH release, GC inhibit HPA axis activity in part by nongenomic actions on cannabinoid signaling within the paraventricular nucleus (PVN) of the hypothalamus (Evanson et al., 2010). This is achieved by activation of G-protein signaling, which results in the release of endocannabinoids from post-synaptic neurons (Di et al., 2003). Through retrograde transmission, the endocannabinoids activate pre-synaptic cannabinoid CB1 receptors leading to the inhibition of glutamate release (Di et al., 2003). Until recently, GC-mediated G-protein activation has mainly been attributed to a transmembrane GPCR (Tasker et al., 2006); however, there is recent evidence that PVN endocannabinoid signaling is mediated by GR (Haam et al., 2010). Thus, future studies will be needed to establish whether GC activate multiple pathways (i.e. GR-mediated versus GPCR-mediated) leading to altered endocannabinoid signaling and physiological effects. Moreover, the rapid GC effects on the hypothalamus and pituitary has been restricted to mammals, with no studies examining rapid GC effects on central function in any teleost species.

In mammalian species, GC are known to have important nongenomic effects in the limbic systems (hippocampus, amygdala and prefrontal cortex) which influences HPA axis sensitivity and plays a role in memory formation (Groeneweg et al., 2011). Interestingly, throughout the limbic systems initiation of corticosteroid effects are extremely diverse, involving both classical receptors, MR and GR, as well as non-classical membrane receptors (Groeneweg et al., 2011). For instance in the rat hippocampus, corticosterone rapidly modulate neuron excitability, which appears to be mediated by membrane localized MR (Karst et al., 2005). In the rat amygdala, MR mediates rapid effects of corticosterone on glutamate activity (Karst et al., 2010) and MR resides at the plasma membrane in amygdala synapses (Prager et al., 2010). Studies using electron microscopy also found evidence that GR localizes in rat lateral amygdala plasma membranes, which likely plays a role in the nongenomic GC alteration of stress-related memory consolidation (Johnson et al., 2005). In contrast, GC nongenomically interact with noradrenergic signaling within the rat amygdala ultimately affecting

memory consolidation through a GR-independent modification of the β-adrenorecptor-cAMP pathway (Roozendaal et al., 2002). Lastly, in the cortex of rats, corticosterone via a membrane receptor, nongenomically modulates memory acquisition through PKA- cAMP-response element binding protein (CREB) activation of histone acetylation (Roozendaal et al., 2010). Together, research on the limbic system in mammals highlights that rapid action of GC is substantially diverse as they can be mediated by the intracellular GR and MR as well as a putative membrane GR. The rapid central GC effect described above have only been examined in mammalian models thus far, and the rapid effects exerted by GC on HPI axis activity is less clear in non-mammalian models. In teleost, nongenomic cortisol action has been characterized in the pituitary (*Oreochromis mossambicus*) with regards to prolactin release (Borski et al., 2002). Specifically, cortisol perfusion rapidly inhibits prolactin release from the pituitary, which is dependent on inhibition of cAMP-PKA signaling and Ca^{2+} accumulation (Borski et al., 1991; Hyde et al., 2004). Further studies indicated that this rapid cortisol effect is membrane-mediated and is independent of protein synthesis (Borski et al., 2001). Nongenomic GC action (Lewis and Rose, 2003) and partial purification of a membrane glucocorticoid receptor (Evans et al., 2000) has also been characterized in the brain of the rough-skinned newt (*Taricha granulosa*). In this amphibian species, corticosterone rapidly suppresses sexual behaviours by activating endocannabinoid signaling in the hindbrain region (Coddington et al., 2007), likely through a GPCR kappa opioid-like receptor (Evans et al., 2000). Clearly, central nongenomic GC action in non-mammalian species demands greater research focus as our understanding of the rapid GC action is greatly lacking, especially compared to what is known in mammals.

1.4.2 Peripheral Nervous System

Chromaffin cells, which secrete catecholamines in response to neuronal stimulation, are a key target for nongenomic GC action and play a role in regulating the HPA axis activity (Park et al., 2008; Wagner et al., 1999). Overall, GC treatment rapidly inhibits stimulated-chromaffin secretion of catecholamines, while this has only been demonstrated in mammals (Park et al., 2008; Wagner et al., 1999). In guinea-pigs (*Cavia porcellus*), cortisol rapidly suppresses stimulated-catecholamine secretion by nicotinic receptor inhibition or suppression of PKC activity (Yonekubo et al., 2003). In bovine and rat chromaffin cells, GC rapidly modulate potassium channel gating *via* a GR-independent regulation of catecholamine secretion (Lovell et al., 2004). Studies from rat adrenal medulla cell line (PC12 cells) underscore how multiple signaling cascades may be involved in regulating nongenomic

GC action. For instance, in this cell line GC rapidly inhibit stimulated-cytosolic Ca^{2+} spikes by activating a G-protein-PKC pathway (Lou and Chen, 1998; Qiu et al., 1998; Qiu et al., 2003), which leads the activation of MAPKs including, Erk1/2 MAPK (Qiu et al., 2001), p38 and c-Jun NH_2-terminal protein kinase (JNK) (Li et al., 2001). Although the above studies underscore a novel GR-independent pathway in mammalian chromaffin tissue, whether GC mediate activate similar pathways in teleost chromaffin cells has not been investigated.

Cardiovascular System

In the mammals, GC rapidly modulate vascular contractility, inflammatory responses and regulate blood pressure (Lee et al., 2012). Furthermore, the activation of PI3K-Akt pathway has been implicated in mediating many of the rapid nongenomic GC responses in the cardiovascular system (Lee et al., 2012). For instance, in cardiac tissue and endothelial cells dexamethasone rapidly induces vascular protective effects through the stimulation of endothelial nitric oxide synthase (eNOS) *via* PI3K-Akt-dependent mechanisms, leading to reduced inflammation (Hafezi-Moghadam et al., 2002). In addition to the vascular-protective effects, GC also nongenomically alter cardiac energy metabolism, including rapid phosphorylation of AMP-activated protein kinase (AMPK) and p38 MAPK ultimately leading to increased low lipoprotein lipase (LPL) transport (Kewalramani et al., 2008).

Reports of rapid GC effects in non-mammalian vascular systems are almost non-existent. A study conducted in rainbow trout reported rapid vasoconstriction of coronary vessels within 10 min of cortisol treatment (Agnisola et al., 2004), while the mechanism involved is unknown. In teleost branchial tissue, cortisol rapidly (5-30 min) induces ATPase activity both *in vitro* and *in vivo* in the Mozambique tilapia (*Oreochromis mossambicus*), and this response was independent of protein synthesis (Sunny and Oommen, 2001). Rapid cardiovascular adjustments are critical during stress adaptation and based on the studies above nongenomic cortisol action appears to play a critical role in cardiac and vascular functions. Together the rapid cardiovascular action of cortisol highlights the potential importance of non-classical GC signaling on overall homeostasis.

1.4.3 Immune System

Glucocorticoids are well known for having rapid and delayed anti-inflammatory and immune suppressive properties. Due to its clinical significance, there has been intense research focus into the mechanisms leading to rapid T cell suppression by GC in mammals (Löwenberg et al., 2007).

Although the mechanism of action is still being unraveled, there is mounting evidence for a role of the classical intracellular GR in T cell inhibition (Löwenberg et al., 2007). Upon GC binding, GR causes rapid dissociation of the T cell receptor (TCR) complex, inhibiting proper TCR signaling (Löwenberg et al., 2007). In addition to directly disrupting TCR complex dynamics, GC *via* a GR-dependent mechanism also rapidly inhibit Lck and Fyn kinase activity, both of which positively regulate TCR downstream signaling (Löwenberg et al., 2005). Subsequently, GC-induced suppression of pathways downstream of TCR activation was also observed, including decreased Akt, PKC, and MAPK (Erk, p38 and JNK) activity (Löwenberg et al., 2005). In contrast, GC enhance zeta-chain-associated protein kinase of 70 kDa (ZAP-70), which is a TCR inhibitory signaling kinase (Boldizsar et al., 2013). Again, the above studies highlight the intricate and diverse signaling pathways involved in rapid GC action and future studies will be needed to determine whether nongenomic T cell effects are conserved across vertebrates.

Nongenomic GC signaling regulates macrophage function in mammals (Long et al., 2005), and this was also reported in a teleost model notably by altering phagocytosis activity (Roy and Rai, 2009). For instance, in mice macrophage corticosterone treatment rapidly reduces phagocytosis through a membrane-mediated mechanism that is independent of the classical GR (Long et al., 2005). Similarly, in a piscine model, cortisol rapidly reduces phagocytosis, both *in vitro* and *in vivo*, by an adenylate cyclase-cAMP-PKA pathway (Roy and Rai, 2009). The cortisol-mediated inhibition of phagocytosis was also initiated at the plasma membrane, as BSA-cortisol mimicked the response (Roy and Rai, 2009). However, unlike in mice, nongenomic cortisol-mediated phagocytosis in fish was prevented by the GR antagonist, mifepristone (RU486) suggesting a GR mediated response. This suggests that mechanisms mediating nongenomic action GC action are not necessarily conserved across vertebrates. Moreover, future studies investigating the interaction of cortisol on teleost immune function should consider the rapid cortisol action as a potential mediator of immune function.

1.4.4 Liver

From genomic studies it is evident that GC play a critical role in regulating liver metabolism, but a role for nongenomic signaling in the metabolic responses is unclear (Aluru and Vijayan, 2009). Despite reports of high-affinity liver plasma membrane GC binding sites (Alléra and Wildt, 1992; Grote et al., 1993; Ibarrola et al., 1996), a hepatic membrane glucocorticoid (mGR) has not been cloned and only a handful of studies have examined the nongenomic GC signaling in this key target tissue (Borski, 2000). Considering that glucose levels rise within 30 min after stressor exposure

(Bracewell et al. 2004), non-genomic GC signaling, in addition to catecholamine response, may be playing a role in this rapid response. Evidence for this was provided by Gomez-Munoz et al. (1989), who reported that corticosterone rapidly stimulates glycogenolysis by inducing the rapid phosphorylation and subsequent activation of glycogen phosphorylase through a Ca^{2+} signaling in rat hepatocytes (Gomez-Munoz et al., 1989).

Nongenomic GC signaling in teleosts liver is relatively unknown. A recent study suggested the possible rapid effect of cortisol on liver metabolic capacity, including alterations to malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PDH) and isocitrate dehydrogenase (ICDH) activities, and this response was not blocked by transcription inhibitors (Sunny et al., 2002). In contrast, testosterone enhanced intermediary enzyme activity suggesting that nongenomic effects on liver metabolism are steroid-specific (Sunny et al., 2002). Most recently in rainbow trout it was shown that cortisol rapidly alters liver plasma membrane fluidity, microdomain reorganization and rapid induction of kinase signaling pathways (Dindia et al., 2012). To date, the research conducted in our laboratory along with the studies mentioned above are the only reports investigating the physiological effects and mechanisms underlying nongenomic GC signaling in liver. Considering that liver is a key target for genomic GC signaling, future research should be focused on determining the physiological relevance of rapid GC effects, the possible mechanisms of action and the potential genomic consequence.

1.5 Nonspecific Membrane-mediated Steroid Effects

As lipophilic compounds, steroid hormones can freely intercalate into the lipid bilayer by aligning their planar rings parallel to the phospholipids (Massey and Pownall, 2006). Upon plasma membrane incorporation, steroids have the ability to modulate lipid-lipid, lipid-protein and protein-protein interactions, thus resulting in functional changes to membrane protein activity (Whiting et al., 2000). The steroid-induced biophysical alteration of membrane function represents an additional mechanism by which steroid hormones can influence cell physiology (Whiting et al., 2000). The next section will briefly describe the plasma membrane structure along with their biophysical properties essential for cellular function. The following sections will describe the effect of GC on plasma membrane and how these effects might facilitate nonspecific activation of the cellular response.

1.6 Plasma Membrane Characteristics

The plasma membrane acts as a specialized permeability barrier that protects intracellular processes from the external environment and, control cellular homeostasis (McElhaney, 1975). In addition to acting as a semi-permeable barrier the plasma membrane is also critical for structural stability, cellular transport (Grefen et al., 2011), cell motility (Fackler and Grosse, 2008) and signal transduction (Groves and Kuriyan, 2010). In addition to proteins, the plasma membrane is primarily composed of phospholipids, which are amphipathic molecules containing both hydrophobic (fatty acid chains; "tails") and hydrophilic (phosphate group; "head") regions. Phospholipids spontaneously form lipid bilayers when placed in a polar environments as the non-polar fatty acid chains will aggregate towards each other, whereas the polar head will face outwards, interacting with the aqueous environment (Van Meer et al., 2008).

Since the proposal of the fluid mosaic model (Singer and Nicolson, 1972), research has revealed that the biophysical structure of the plasma membrane is quite complex with asymmetrical distribution of lipid classes between the two lipid layers, as well as uneven lateral distribution of membrane proteins (Simons and Toomre, 2000). The lipid species of eukaryotic plasma membrane is very diverse; the extracellular lipid layer is enriched in phosphatidylcholine (PC), sphingomyelin (SM), and glycosylsphingolipids, while the cytoplasmic leaflet is enriched in phosphatidylethanolamine (PE) and phosphatidylserine (PS) along with phosphatidylinositides (PI) (Daleke, 2003). Along with preferential transbilayer lipid synthesis, membrane asymmetry is achieved through several transporter groups, referred to as flippases, floppases, and scramblases (Daleke, 2003).

Lateral compartmentalization of plasma membrane proteins is achieved by natural phase separations of liquid-disordered (liquid crystalline state) and liquid-ordered (gel state) regions (Fig. 1-3; Edidin, 2003). The liquid –ordered regions form small (10-200 nm) microdomains, termed lipid rafts that are rich in cholesterol, glycosphingolipids and sphingomyelin (Edidin, 2003). Due to cholesterol enrichment, these domains are more ordered compared to liquid-disorder regions, which form the bulk of the plasma membrane. Whether raft-formation is cholesterol driven or primarily due to protein-protein interactions remains controversial, nonetheless lipid rafts are accepted as critical regulators of signal transduction. For instance, G-protein coupled receptors (GPCR; Chini and Parenti, 2004), tyrosine kinase receptors (Coskun et al., 2011) and glycosylphosphatidylinositol

(GPI)-linked proteins (Moran and Miceli, 1998) preferentially partition into raft regions, supporting a functional role of lipid rafts in regulating intracellular signaling activation.

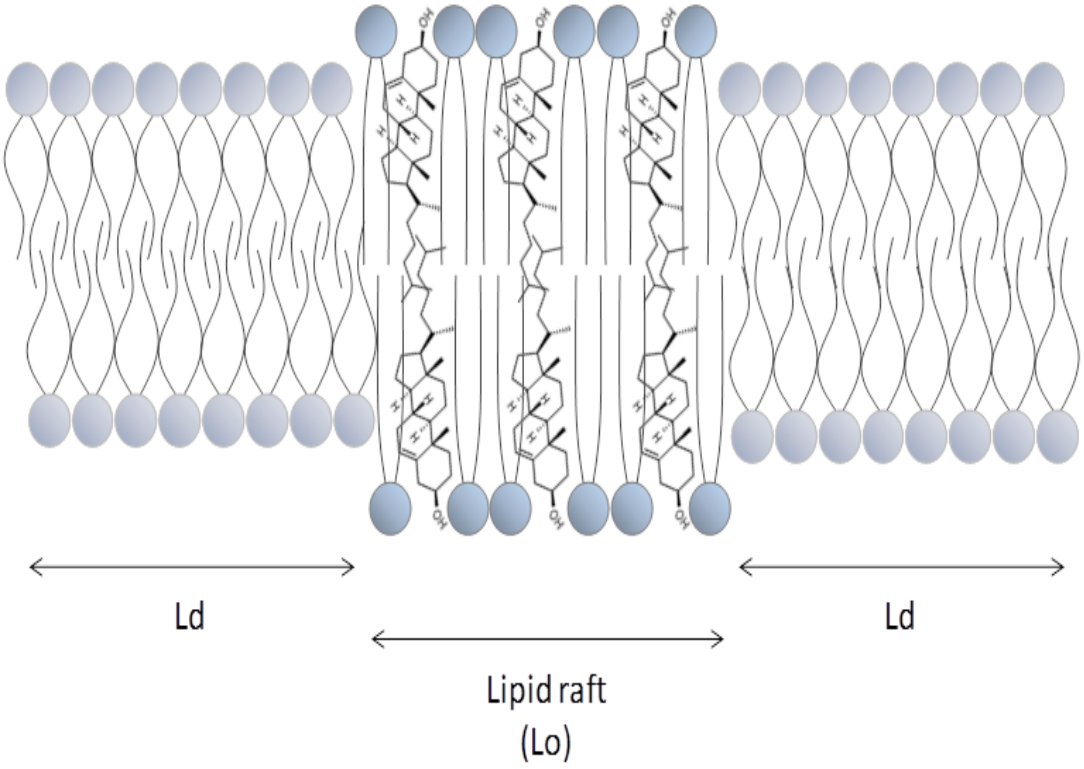


Figure 1-3. Diagram depicting the plasma membrane lipid phases

Distinct membrane regions occur within the plasma membrane due to the different lipid and protein components. The majority of the plasma membrane is in the liquid-disordered state (Ld; liquid-crystalline), while microdomains are laterally dispersed throughout the lipid layer. These microdomains are referred to as lipid rafts and are enriched in cholesterol, glycosphingolipids and sphingomyelin, causing them to be more ordered (liquid-ordered (Lo)) compared to the rest of the plasma membrane.

In addition to protein compartmentalization, plasma membrane physical properties, such as microviscosity (inverse of membrane fluidity; Fig. 4), greatly influence cellular functions through lipid-protein interactions (Van Meer et al., 2008). The protein-lipid interaction is often very specific and is required for proper protein conformation and, therefore, directly impacts transmembrane protein function (Dowhan and Bogdanov, 2011). In addition to impacting protein function, the viscosity of the lipid environment also affects lipid raft formation and stability, thus affecting protein segregation into these lipid microdomains (García-Sáez and Schwille, 2010). The lipid-protein composition of the plasma membrane is the primary determinant of basal plasma membrane molecular dynamics (Van Meer et al., 2008). For instance, proportion of different phospholipid classes along with the species of fatty acid tails (saturation level, acyl chain length, lipid peroxidation) will largely influence bulk membrane properties (Van Meer et al., 2008). Although rarely directly investigated, tissue differences in plasma membrane composition are thought to be responsible for tissue-specific fluidity effects. For instance, raft-dependent signaling cascades, including insulin signaling are reportedly more sensitive to membrane disturbance in a hepatic cell lines that lack caveolae, a subtype of lipid rafts (Vainio et al., 2002). The intrinsic dynamics of the plasma membrane are readily altered by external stimuli (Fig. 1-4), including temperature (Williams and Hazel, 1994), shear stress (Haidekker et al., 2000) and pressure (Mastrangelo et al., 1979). Chemical agents, including endogenous ligands can also impact lipid dynamics (Whiting et al., 2000).

Cholesterol, a major sterol component of eukaryotic plasma membranes, controls numerous membrane properties, including membrane thickness, bulk fluidity and phase separations (Ipsen et al., 1990) and as a result cholesterol is a major determinant of membrane protein activity (Andersen and Koeppe, 2007). Cholesterol can also impact protein activity through direct protein-cholesterol interactions, as several proteins containing cholesterol binding sites have been identified (Gimpl, 2010). Together, cell membranes are composed of a complex repertoire of lipid and protein components, which interact in specific and nonspecific ways to control cellular function.

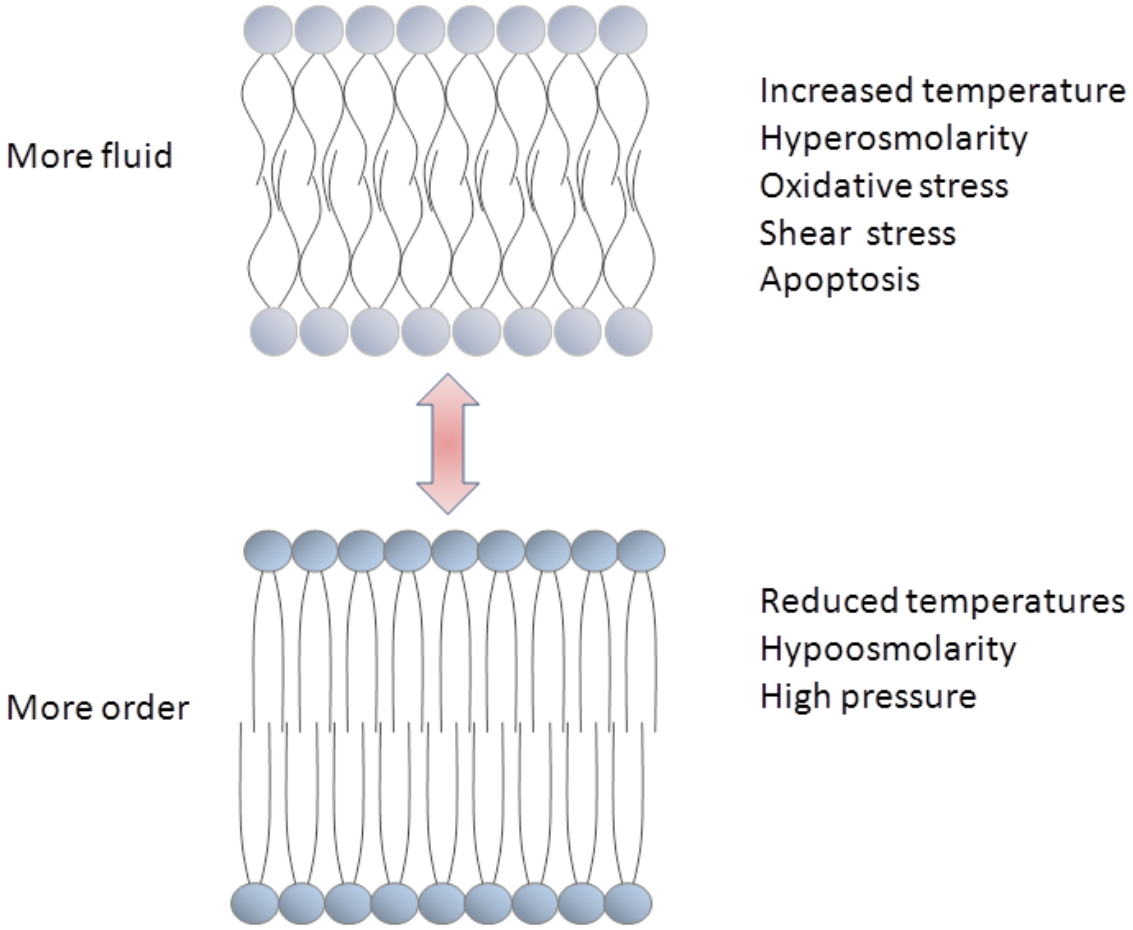


Figure 1-4. Diagram illustrating the phospholipid response to environmental changes

The phase of the lipid bilayer is highly sensitive to external stimuli, including temperature, osmolarity, mechanical stress and homeostatic state of the cell. Lipid transition into the liquid-disordered state (become more fluid) occurs in response to high temperatures, hyperosmolarity, oxidative stress, mechanical stress and during apoptosis, whereas membrane lipids become more ordered in response to low temperatures, hypoosmolarity and high pressures. Aquatic species, including rainbow trout are potentially exposed to all of the above stressors and, therefore, regulation of plasma membrane properties is critical in these species.

1.7 Techniques for Studying Membrane Biophysical Properties

1.7.1 Fluorescence polarization

Measurement of 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence polarization is a valuable and commonly used technique to study membrane fluidity (Fig. 1-5). DPH is a long, linear, hydrophobic fluorescent molecule, which freely incorporates into lipid bilayers preferentially aligning parallel to the fatty acid chains (Lentz, 1989). The rationale for using DPH to monitor fluidity dynamics is based on the fact that the emitted polarized fluorescence is dependent on the rotational freedom within the lipid environment, since rotations orthogonal to the molecular axis cause fluorescence depolarization (Lentz, 1989). Therefore, the degree of polarized light emitted from DPH is related to the microviscosity of the surround lipids. This technique has been widely used in synthetic and biological membranes, including rainbow trout hepatocytes (Hazel et al., 1998).

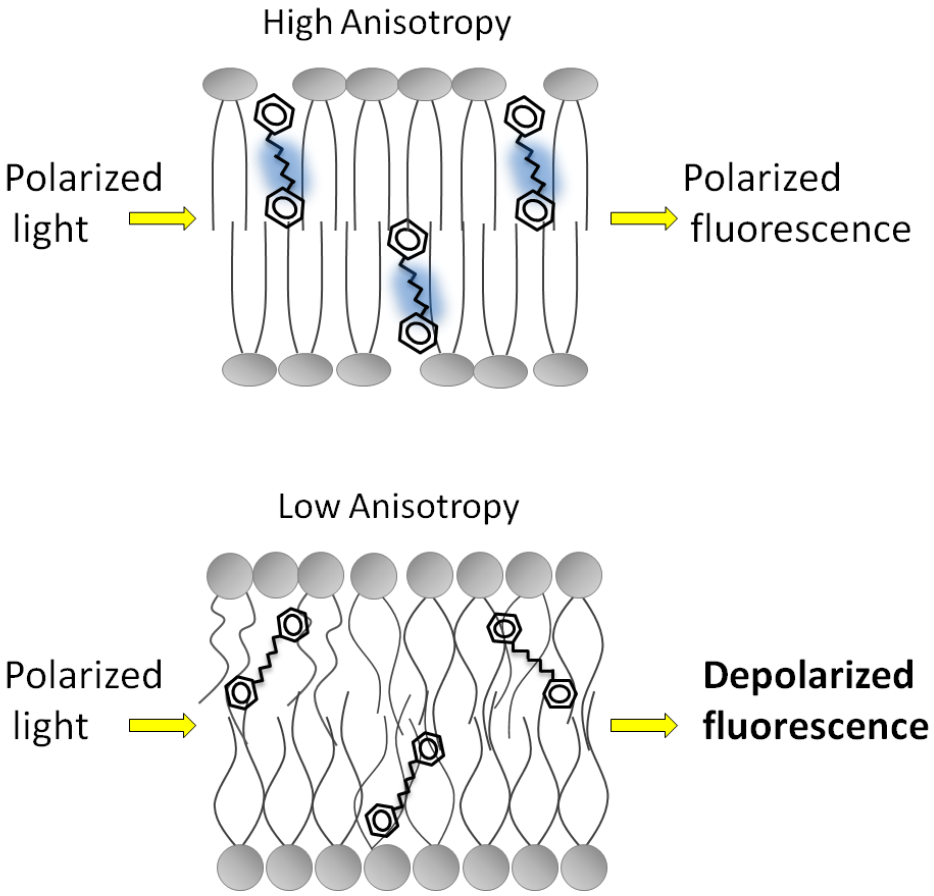


Figure 1-5. Theoretical explanation of fluorescence polarization measurements

Schematic diagram explaining fluorescence polarization measurements of 1,6-diphenyl-1,3,5-hexatriene (DPH) as an indicator of lipid bilayer fluidity. Rotations orthogonal to the long axis of DPH results in rapid fluorescence polarization. Therefore, fluorescence polarization is directly related to the rotational freedom of DPH within the lipid environment. Lipid bilayers which are more ordered and restrict the movement of DPH will have higher fluorescence polarization values compared to membranes which are more fluid.

1.7.2 Atomic Force Microscopy

Atomic force microscopy (AFM; Fig. 1-6) is a scanning microscope, which is gaining tremendous popularity in biological membrane research due to the powerful nano-scale resolution of surface properties, such as height and surface adhesion measurements (Frederix et al., 2009). Previously, biological application of AFM were primarily limited to studying synthetic lipid monolayers, however due to the ability to measure biological properties under physiological conditions, AFM imaging is beginning to provide critical information on cellular functions. For example, imaging biological membranes with AFM has provided valuable information regarding lipid raft properties, such as their dimensions (Cai et al., 2012; Orsini et al., 2012). At the cellular level, several studies have successfully used AFM to characterize structural and surface properties of living cells (Hu et al., 2009). In addition to measuring surface properties, AFM can also be used to characterize single molecule properties, such as protein conformations and interactions (McAllister et al., 2005). Lastly, AFM is powerful for characterizing membrane proteins, as they can be analyzed in their native lipid environment without detergent extractions (Muller and Engel, 2008). Therefore, AFM is an excellent high-resolution tool for characterizing physical properties of biological molecules under physiological conditions.

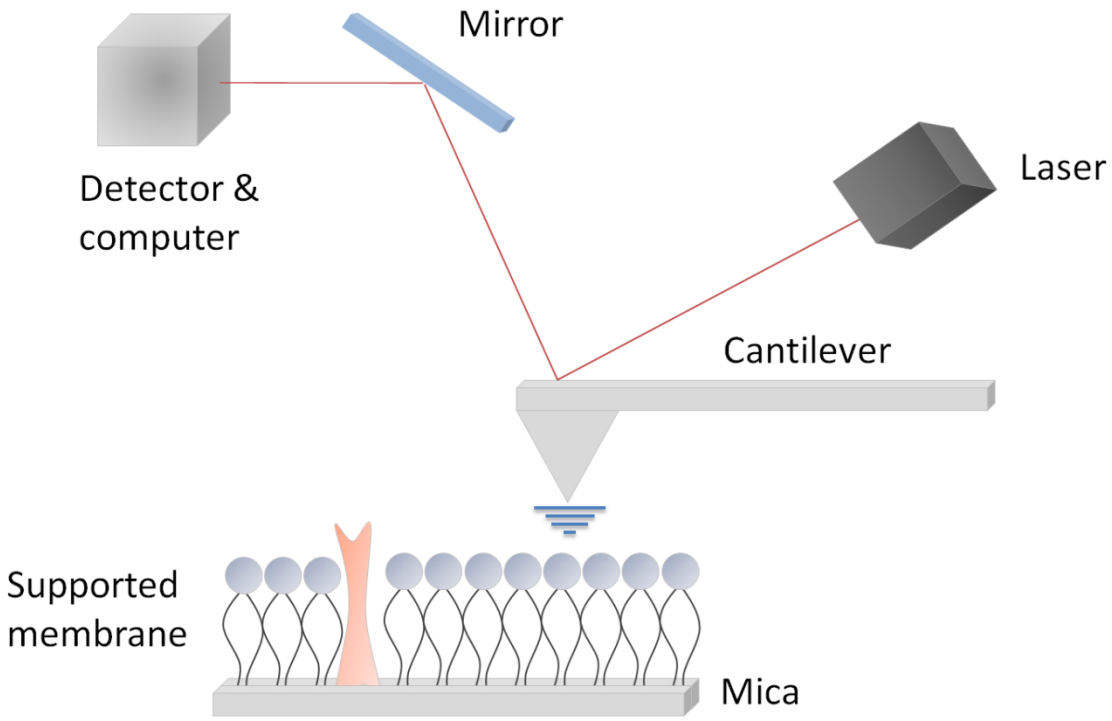


Figure 1-6. Schematic diagram of an atomic force microscope (AFM)

AFM is a high-resolution scanning microscopy technique that uses a cantilever which moves along (or scans) the surface of the sample. The Van der Waals force interactions between the cantilever tip and the molecules of the sample are monitored by the deflection of the laser which is emitted off the cantilever. Depending on the instrument settings, the degree of deflection is detected and used to construct sample properties such as topography and elasticity.

1.8 Membrane-activated Signaling Pathways

The biophysical lipid molecular dynamics affect several plasma membrane properties, including lateral protein and lipid diffusion, membrane component interactions (lipid-lipid, protein-protein and lipid-protein interactions), and regulates protein conformation (Van Meer et al., 2008). As a result, changes in membrane fluidity induced by steroid hormones can directly alter signal transduction pathways (Whiting et al., 2000). Along with steroid hormones several other agents can influence lipid fluidity, including peptide hormones (Massa et al. 1975), trace elements (Garcia et al., 2005), and proteins, including albumin (Beck et al., 1998). Due to the research into mechanotransduction (Huang et al., 2004), there is increasing recognition of the essential role played by the biophysical environment of the plasma membrane in regulating stress-activated signaling pathways (Vigh et al., 2007). In particular, it is well documented that G-protein signaling pathways are sensitive to sheer stress, which is accompanied by increased fluidity (Gudi et al., 1998). For instance, in model liposome, devoid of other cellular constituents, G proteins are activated in response to fluidizing agents, indicating that lipid conformation, can directly impact G protein activity (Gudi et al., 1998). In human endothelial cells, B_2 bradykinin GPCR is activated in response to fluidizing stimuli including fluid shear stress, hypotonic shock and benzyl alcohol (Chachisvilis et al., 2006). As heteromeric G-proteins are linked to several signaling cascades, including phospholipase C-PKC and cAMP-PKA pathways, fluidity-dependent activation of G protein signaling has the ability to influence several intracellular processes (Papadaki and Eskin, 2008). In addition to GPCR signaling, endothelial MAPKs pathways are also sensitive to membrane lipid dynamics, such that lipid fluidizers (benzyl alcohol) increase whereas excess cholesterol (increased membrane order) suppresses MAPK activity (Butler et al., 2002). In addition to chemical fluidization, MAPK is also activated in response to mechanical fluidization in endothelial cells, which might be due to G protein–dependent PKC activation as well as altered tyrosine kinase activity (Papadaki and Eskin, 2008).

In addition to affecting cellular signaling pathways, changes to membrane fluidity also impact cellular transport. For instance, several mammalian studies have shown that Ca^{2+} signaling is impacted by membrane microviscosity, as membrane cholesterol content is typically inversely related to intracellular Ca^{2+} levels, suggesting that Ca^{2+} influx is enhanced with increased membrane order (Bastiaanse et al., 1997). In support, Squier et al., (1988) reported that the rotational mobility, which determines the rate of calcium transport, is directly related to the membrane fluidity (Squier et al., 1988). In rat liver, benzyl alcohol (< 30 mM), increases enzyme activity of several membrane

proteins, including adenylate cyclase, 5'-nucleotidase, cAMP phosphodiesterase and Na^+/K^+-ATPase (Gordon et al., 1980). In contrast, activity of the nicotinic acetylcholine receptor does not appear to be dependent on overall viscosity, but instead depends on lipid composition as similar receptor activities were achieved at both ordered and disordered phases in the electric organ of the *Torpedo californica* (Sunshine and McNamee, 1994).

1.9 Effect of Glucocorticoids on Cell Membranes

There is the potential that nongenomic steroid action is initiated, at least in part by direct interaction between steroid hormones and the lipid bilayer, as sterols are known to alter lipid bilayer fluidity (Whiting et al., 2000). The interaction and subsequent physiochemical effect of compounds on cellular membranes is currently an important field of research, especially with regards to therapeutically relevant agents, such as GC. The membrane effects of GC are therapeutically relevant due to their potential modulation of cellular processes along with their ability to directly alter uptake and effectiveness of vector base chemotherapies, such as Lipiodol (Becker et al., 2010). The reported membrane effects of glucocorticoids are outlined in greater detail below and are summarized in Table 1-1.

Table 1-1. Selected effects of glucocorticoids on model and biological lipid membranes

Glucocorticoid	Membrane Effect	Meth-od	Organism/tissue	Dose	Exposure Duration	Reference
Cortisol, DEX	↑Fluidity	FP	DPPC liposomes	1 mM	Incorporated in lipid film	(Ghosh et al., 1996)
Methyl-prednisolone	↓ Fluidity	FP	Human granulocytes	0.5-2 mg/ml	2-5 min	(Lamche et al., 1990)
DEX	↓ Fluidity	FP	Rabbit cardiac muscle and endothelial	10^{-7} M	16 hours	(Gerritsen et al., 1991a)
DEX	↑Fluidity	FP	Rat small intestine	1 µg/ day g body wt	4 days	(Brasitus et al., 1987)
DEX	↑Fluidity	FP	Rat small intestine Golgi membranes	1 µg/ day g body wt	4 days	(Dudeja et al., 1988)
TA	↑Fluidity; diffusion	FP	Murine B lymphocytes	10^{-7} M	6 hours	(Keating et al., 1988)
DEX	↑Fluidity; diffusion	FP	HeLa cells	10^{-6} M	24 hours	(Boullier et al., 1982)
DEX	↑Fluidity	FP	Rat distal colon	1 µg/ day g body wt	4 days	(Dudeja et al., 1987)
DEX	↓ Fluidity	FP	Human leukemia cells	2-200 nM	15-60 min	(Kiss et al., 1990)

Table 1-1. Continued

Glucocorticoid	Membrane Effect	Meth-od	Organism/tissue	Dose	Exposure Duration	Reference
TA	Structural changes in NE	AFM	*Xenopus laevis* oocytes	10^{-6} M	5 min	(Shahin et al., 2005)
DEX	↑Fluidity of rafts	FP	Murine T cells	10^{-6} M	18 hours	(Laethem et al., 2003)
Cortisol	Conformation change in the AChr receptor;	-	*Torpedo californica* electric tissue	600 µM	30 min	(Nievas et al., 2008)
DEX	↑Fluidity; enhance vector uptake	FP	Hepatocellular carcinoma	10-20 µM	1 hour	(Becker et al., 2010)
Cortisol	Altered topography; ↑ elasticity	*AFM*	*Porcine cerebral endothelium*	500 nM	7 days	(Schrot et al., 2005)

DEX; dexamethasone, FR; fluorescence polarization, DPPC; Dipalmitoylphosphatidylcholine, TA; triamcinolone acetonide, NE; nuclear envelope

Emergent research indicates that sterol effects on plasma membrane properties can be highly structure-specific (Massey and Pownall, 2006; Rog et al., 2008;Olsen et al., 2009; Olsen et al., 2010) and are likely to be dependent on the lipid composition, as this will affect sterol orientation (Kucerka et al., 2009). Although biophysical membrane actions of GC has been widely described, these studies are limited to a select mammalian membranes and artificial membrane systems (Table 1). For example, in synthetic 1,2-dipalmitoylphosphatidylcholine (DPPC) lipid bilayers, corticosteroids increased membrane fluidity (Ghosh et al., 1996). In animal systems, GC have fluidizing and ordering effects. For instance, in rat physiological cortisol levels (10^{-7} M) significantly fluidize erythrocyte membranes (Massa et al., 1975), and fetal liver microsomes (Kapitulnik et al., 1986). In the rat, GC associated fluidization is thought to facilitate postnatal maturation of small intestine brush border membranes (Neu et al., 1986). Physiological cortisol levels also fluidize dog brain synaptosomal plasma membranes, which are associated with increased Na^+/K^+-ATPase activity (Deliconstantinos, 1985). This effect was attributed to incorporation of cortisol into the membrane rather than specific binding of cortisol to Na^+/K^+-ATPase as the effect was easily reversed by washing the membrane fraction.

Numerous studies have also reported membrane ordering effects of GC. For example corticosteroids reduced membrane fluidity of granulocytes (Lamche et al., 1990). An ordering glucocorticoid effect also occurs in porcine myocardial tissue which is accompanied by inhibition of phospholipase A2, reduction of arachidonic acid and free fatty acid levels (Engelman et al., 1989). In rabbit cardiac muscle endothelial cells, inhibition of eicosanoid production is attributed to ordering effect of dexamethasone at physiological concentrations (Gerritsen et al., 1991).

At pharmacological levels, glucocorticoids alter the physicochemical properties of cell membranes, which lead to inhibition of ion cycling across the cell membrane (Buttgeriet et al. 2000). Maier et al. (2005) reported that dexamethasone (DEX) concentrations of 60 μM induced competition with the lipophilic membrane marker 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) for membrane incorporation, but membrane fluidity was not affected. Lastly, pharmacological GC fluidization of T cell membranes is hypothesized to play a role in glucocorticoid-mediated dissociation of TCR signaling complex, leading to rapid immunosuppressive effects (Van Laethem et al., 2003).

The effect of sex steroids on membrane fluidity has also been widely documented and appears to be tissue specific. For instance, in female rat chondrocytes, 17β-estradiol enhances

membrane order, which was dose- and stereo-specific (Schwartz et al., 1996), whereas 17β-estradiol increases membrane fluidity in rat vaginal cells (Reddy et al., 1989). In non-mammalian studies, progesterone induces a transient decrease in oocyte fluidity (Morrill et al., 1989), which is essential for resumption of meiotic divisions (Morrill et al., 1993). More importantly, studies on steroid membrane effects are revealing that most anti-tumor properties are imposed by the accompanying plasma membrane effects (Baritaki et al., 2007). For instance, the ordering effect of 17β-estradiol in human breast cancer cells is thought to reduce DNA synthesis and cell proliferation in this carcinoma cell line (Clarke et al., 1990). Similarly, progesterone is known for its anti-ovarian epithelial tumor properties, which are thought to be partly mediated by the ordering properties of this steroid on adenocarcinoma cells (McDonnel et al., 2003). Other lipophilic anti-cancer agents are also associated with reduced membrane fluidity, and although the causative relationship is still under investigation, the effect on lipid dynamics is thought to be a key anti-tumorigenic property (Sade et al., 2012).

Taken together, steroid hormones of all classes impact biophysical properties of the plasma membrane which likely have functional cellular consequences. Given that steroid hormones are established modulators of plasma membrane fluidity, characterizing their effects on the plasma membrane is a critical step to understanding their mechanism of action and realizing their full physiological and clinical relevance.

1.10 Plasma Membrane Perturbation and the Stress Response

As mentioned above, the physicochemical properties of the plasma membrane are acutely responsive to extracellular stimuli, including temperature, salinity, sheer stress and endogenous ligands (Fig. 1-3). Consequently, the sensitivity of the plasma membrane to various stimuli has led to the proposal of the membrane-regulated cellular stress response, in which the plasma membrane acts as the primary sensor and initiator of cellular responses to stressful stimuli (Vigh et al., 2007). This model has been finely developed with regards to thermal stress, as it is well recognized that lipid phases are highly temperature sensitive. Moreover, subtle changes in mammalian membrane dynamics in response to temperature have been shown to activate the heat shock response, including synthesis of heat shock proteins accompanied by increases in cytosolic Ca^{2+} (Balogh et al., 2005).

It is important to note that the heat shock response is not uniformly activated by all fluidizing agents, suggesting that stress-activated pathways are sensitive to specific nanomolecular alterations

induced by fluidizing agents, such as microdomain reorganization (Nagy et al., 2007). Lastly, the heat shock response was activated in the absence of protein denaturation (Török et al., 2003) suggesting the plasma membrane is more acutely sensitive to thermal changes compared to intracellular protein conformation. Although membrane-regulated stress response model has mainly been examined in the context of physical stressors (Vigh et al., 2007), chemical agents such as endogenous ligands, including GC, also induce physiochemical membrane changes. Consequently, this leads to the possibility that in addition to receptor-mediated cortisol signaling, the plasma membrane might play a role in regulating the generalized stress response following GC elevation (Fig. 1-7) (Dindia et al., 2012).

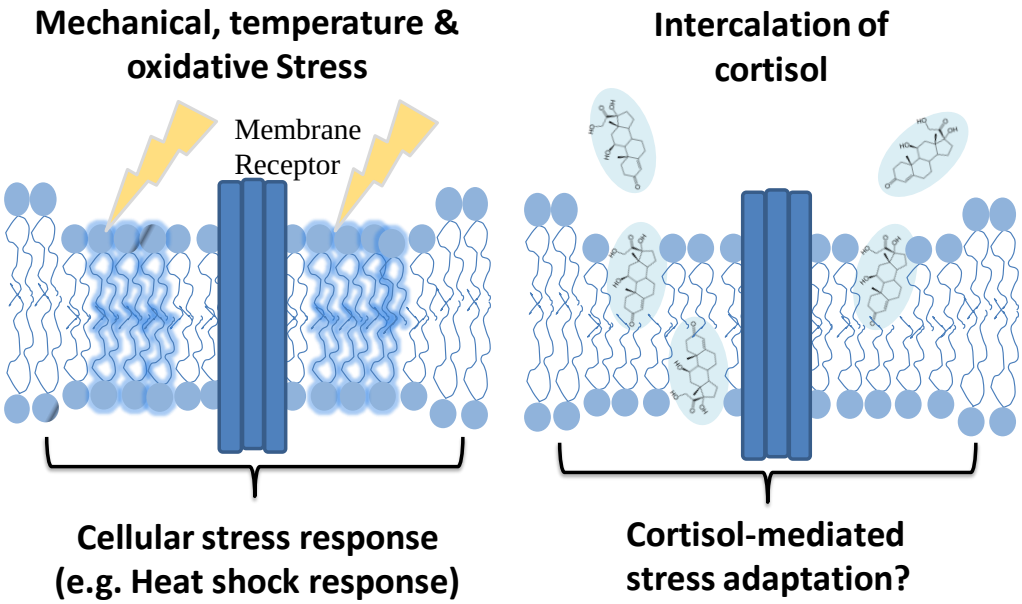


Figure 1-7. Plasma membrane and the ability to sense cellular stress

The plasma membrane is thought to be the primary cellular sensor to extracellular stressors. Due to the sensitivity of phospholipids to external stimuli, changes in biophysical properties of the plasma membrane are thought to be the primary activator of cellular response to stressors, including temperature. While the role of the plasma membrane in initiating the thermal cellular stress response has been well documented, how the biochemical changes in response to GC intercalation and the associate rapid cellular stress effects remain to be determined.

1.11 Thesis Objectives

The primary objective was to test the general hypothesis that rapid, nongenomic cortisol signaling plays an important role in liver function during the stress response in rainbow trout (*Oncorhynchus mykiss*). The rapid, nongenomic effects of cortisol on liver function are poorly understood despite the importance of this organ in maintaining homeostasis. Therefore, the hypothesis that nongenomic cortisol signaling is important for acute metabolic re-programming to cope with stress, and regain homeostasis, was tested *in vivo*, while the characterization of the nongenomic signaling pathways and downstream effects were carried out *in vitro* using either purified plasma membrane, liver slices, hepatocytes in suspension or primary culture of hepatocytes. The specific objectives are to determine whether:

1. Cortisol affects physicochemical properties of liver plasma membrane and rapidly modulates stress signaling pathways (Chapter 2)
2. Stressor-evoked plasma cortisol elevation in trout alters liver membrane physicochemical properties and signaling pathways (Chapter 3)
3. Cortisol rapidly regulates key stress signaling pathways in the liver (Chapter 4)
4. Cortisol rapidly alters liver metabolite levels (Chapter 5)
5. Cortisol rapidly modulates epinephrine signaling in trout liver (Chapter 6)

In addition to the above objectives, an attempt to identify a plasma membrane protein that specifically binds cortisol was also undertaken (Appendix A).

Chapter 2: **Novel nongenomic signaling by glucocorticoid may involve changes to liver membrane order in rainbow trout**

2.1 Introduction

The neuroendocrine response to stress is highly conserved among vertebrates and essential to reestablish homeostasis (Sapolsky et al., 2000). The principal stress hormones, epinephrine and glucocorticoids, have critical functions in the stress adaptation process (Charmandari et al., 2005). The fight-or-flight response involves the activation of the sympathetic nervous system leading to the rapid release of epinephrine from chromaffin cells (Charmandari et al., 2005). Glucocorticoid hormone release occurs in response to activation of the hypothalamus-pituitary-adrenal (HPA) axis (Charmandari et al., 2005), and reaches peak levels only after the catecholamine response (Sapolsky et al., 2000). Most glucocorticoid effects are mediated by the glucocorticoid receptor (GR), a ligand-bound transcription factor, which regulates protein synthesis (Charmandari et al., 2005). In addition to the slower genomic actions, glucocorticoid also elicits rapid effects that are independent of gene transcription and are broadly categorized as nongenomic signaling (Borski et al., 2002), but a role for this in stress adaptation is unclear (Tasker and Herman, 2011).

Nongenomic steroid signaling involves activation of membrane-bound receptors, but a glucocorticoid-specific membrane receptor is yet to be identified (Borski et al., 2002; Groeneweg et al., 2011). In addition to receptor-mediated effects, biophysical changes in lipid bilayers due to steroid intercalation may activate signaling pathways (Rog et al., 2008), (Lösel and Wehling, 2003). Despite studies showing that changes in plasma membrane properties can rapidly affect the cellular stress response (Vigh et al., 2007), very little is known about the effect of stress steroids on membrane properties and the associated signaling events (Borski, 2000). We tested the hypothesis that acute stress levels of cortisol, the principal glucocorticoid in teleost fishes, rapidly modulate cell stress signaling pathways by altering the biophysical state of the plasma membrane. This was tested using rainbow trout (*Oncorhynchus mykiss*) liver, an ideal model as lipid dynamics and plasma membrane properties have been well characterized (Zehmer and Hazel, 2004). We utilized steady-state fluorescence polarization and atomic force microscopy (AFM) to investigate the effect of cortisol on trout liver plasma membrane properties. Rapid changes in phosphorylation status of putative protein kinase A (PKA), protein kinase C (PKC) and Akt substrate proteins in trout hepatocytes were used to confirm modulation of cell signaling pathways in response to cortisol treatment. Benzyl alcohol, a known membrane fluidizer and initiator of the cellular stress response, was also used as a positive control to assess whether changes in plasma membrane fluidity by cortisol may be involved in the cell signaling events. Our results demonstrate for the first time that stressed

levels of cortisol rapidly activate stress-related signaling pathways in rainbow trout hepatocytes. We propose alteration in membrane fluidity as a novel nonspecific glucocorticoid-mediated stress response leading to the rapid modulation of stress signaling pathways.

2.2 Materials and Methods

2.2.1 Animals & Sampling

Juvenile rainbow trout (*Oncorhynchus mykiss*; 100–300 g) purchased from Alma Aquaculture Research Station (Alma, ON) were maintained at the University of Waterloo aquatic facility exactly as described before (Sandhu and Vijayan, 2011). The tanks were supplied with a constant flow of aerated well water (12±2°C) and were maintained under a 12 hL:12 hD photoperiod. Trout were acclimated for at least two weeks prior to experiments and were fed commercial trout feed (Martin Mills, Elmira, ON) to satiety once daily, 5 days a week. Experiments were approved by the University of Waterloo Animal Care Protocol Review Committee and adhere to guidelines established by the Canadian Council on Animal Care for the use of animals in teaching and research.

2.2.2 Liver Plasma Membrane

Liver plasma membranes were isolated using sucrose gradient as described previously (Sulakhe, 1987). The membrane pellet was resuspended in TCD buffer (300 mM sucrose, 10 mM Tris-HCl, 1 mM dithiothreitol (DDT), 0.5 mM $CaCl_2$, 1X protease inhibitor cocktail, pH 7.5; Sigma) and frozen at −70°C. All steps, including centrifugation, were carried out at 4°C. The enrichment of the membrane fraction was determined as described previously by measuring the activities of Na^+/K^+-ATPase (McGuire et al., 2010), 5′-nucleotidase (Solyom and Trams, 1972) and lactate dehydrogenase (Gravel et al., 2009). The six-fold higher Na^+/K^+-ATPase (H: 1.2 ± 0.1 vs. M: 6.9 ± 1.1; n = 7–8) and thirteen-fold higher 5′- nucleotidase (H: 16 ± 1.5 vs. M: 178 ± 48; n = 7–8) activities (U/g protein) in the membrane (M) fraction compared to the initial tissue homogenate (H), confirm membrane enrichment. The ~90% drop in LDH activity (H: 1055 ± 40 vs. M: 124 ± 14; n = 7–8) in the membrane fraction further confirms enriched plasma membranes with negligible cytosolic contamination.

2.2.3 Membrane Cortisol and Cholesterol Analysis

Membrane cortisol (after diethyl ether extraction) concentrations were determined by radioimmunoassay exactly as described previously (Alsop et al., 2009). Diethyl ether extracted

plasma membrane cholesterol levels were measured using a cholesterol oxidase enzymatic kit (Wako Chemicals, Richmond, VA).

2.2.4 DPH Anisotropy

The hepatic plasma membrane fluidity was analyzed by measuring the steady-state fluorescence polarization using the membrane fluorescent probe, 1,6-diphenyl-1,3,5 hexatriene (DPH; Life Technologies Inc., Burlington, ON) exactly as described previously (Katynski et al., 2004). The degree of fluorescence polarization (anisotropy) is directly related to the mobility of DPH within the lipid environment, therefore, DPH anisotropy is inversely related to membrane fluidity. Membrane samples were added to 96-well opaque plates (Corning Incorporated, New York, USA; approximately 300 ng protein/µl; 100 µl total) and incubated with DPH (1:100 of 4.7 mM stock) for 30 min in the dark. To assess membrane order, isolated plasma membranes (0.5 mg/ml protein) were incubated with control, cortisol (10–1000 ng/ml (0.0275-2.75 µM), n = 6), peptide moiety (PEP; 2.75 µM, n = 5), cortisol-PEP (2.75 µM, n = 5), 17β-estradiol (1 µM, n = 6), testosterone (1 µM, n = 6) or benzyl alcohol (25 mM, n = 7) for 30 min while shaking at room temperature. This peptide conjugate has previously been used to differentiate between intracellular (genomic) versus membrane-mediated (nongenomic) steroid effect (Nagler et al., 2010). Readings were taken at various temperatures starting at 2°C thirty min post-treatment, followed by 12, 24 and 37±1°C. The required temperature (reached within approximately 5–10 min) was maintained and this was confirmed by temperature monitoring within each well, using a digital thermometer, immediately prior to and after the anisotropy measurements. Experiments were conducted on a minimum of five independent trout plasma membrane samples. Mifepristone (a GR antagonist) had its own effect on membrane order (data not shown) and, therefore, was not used as a tool for blocking GR effects in the present study.

2.2.5 Preparation of Cortisol-peptide (Cortisol-PEP) Conjugate

Conjugation of cortisol to form a derivative was carried out as reported by (Erlanger et al., 1957). Cortisol-carboxymethyl oxime (Cortisol-CMO (4-pregnen-11b,17,21-triol-3,20-dione3-O-carboxymethyloxime,catalog number Q3888-000) was purchased from Steraloids Inc. (Newport, RI). The peptide conjugated to the CMO is a 15 amino acid sequence of the steroidogenic acute regulatory protein (N-terminus-SGGEVVVDQPMERLY-C-terminus; Proteomics Core Facility, Washington State University, Pullman, WA). The PEP is conjugated via the serine to the CMO using a mixed anhydride technique (Erlanger et al., 1957) using *N*,*N*-dimethylformamide (DMF) as solvent, tri-*N*-

butylamine, and isobutyl chloroformate. This conjugation procedure produces a product of 1:1 stoichiometry of a cortisol molecule to a single PEP sequence. The reaction is added to LH-20 Sephadex column to separate the cortisol-PEP, free cortisol, and free PEP. Based on the absorbance at 280 nm, three peaks are derived from the separation on the column with the first peak as cortisol-PEP. This method of obtaining just the hormone conjugate has been confirmed for E2-PEP (Davis et al., 2011) using Waters QTOF-micro electrospray mass 89 spectometer with the sample introduced by direct infusion (Macromolecular Resources, Colorado State University, Fort Collins, CO).

2.2.6 Atomic Force Microscopy (AFM)

Plasma membrane surface topography (height changes) and phase (viscoelastic changes) were measured simultaneously using atomic force microscopy (AFM). AFM measurements were carried out in a fluid cell (Molecular Imaging) using the Agilent Technologies 5500 Scanning Probe Microscope in intermittent contact mode (MAC mode) at 0.7 ln/s as described before (Moores et al., 2011). Precise force regulation was obtained in MAC mode by using a magnetically coated cantilever (MacLevers Type II from Agilent Technologies; force constant: 2.8 N/m, tip radius: 7 nm, and height: 10–15 μm) Membrane samples were transferred onto a freshly cleaved piece of mica placed within the liquid cell and equilibrated for 10 minutes followed by a quick rinse with nanopure water. Plasma membranes were scanned immediately after rinsing (control, 0 min) or 30 min after incubation with or without cortisol (100 ng/mL). The scan immediately after the rinse was used to ensure that there were no time-dependent effects on plasma membrane topography and phase. Control membranes scanned 30 min after the rinse were compared to cortisol-treated membranes to determine cortisol treatment effects. Scanning at 0.7 ln/s takes approximately 15 min, therefore, the initial scan (referred to as 0 min) was completed within 15 min and the post-treatment scans (referred to as 30 min) within 45 min.

Imaging was repeated with membranes from four independent fish. Images were analyzed using Agilent Image Processing software. Quantitative analysis of surface coverage of higher domains was carried as described previously (Hane et al., 2010) using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA; http://rsbweb.nih.gov/ij/). Briefly, surface coverage was calculated by taking the sum of pixels associated with regions above a certain height (to select for higher domains) divided by the sum of the total number of pixels in the image. Membrane roughness for topography and differences in

viscoelasticity was calculated by taking the average difference in height or phase between lower and higher membrane regions, respectively.

2.2.7 Hepatocyte Experiment

Rainbow trout hepatocytes were isolated using *in situ* collagenase perfusion and maintained exactly as described previously (Sathiyaa et al., 2001). Hepatocyte viability was >95% and the cells were suspended in L-15 (Sigma, St. Louis, MO) medium and plated in six-well tissue culture plates (Sarstedt, Inc., Newton, NC) at a density of 1.5 million cells/well (0.75 million cells/ml). Cells were maintained at 13°C for 24 h at which time the L-15 media was replaced and the cells were allowed to recover for an additional 2 h before the start of experiments. Cells were treated for 10 min either in the absence (0.01% ethanol) or presence of cortisol (100 or 1000 ng/mL) or benzyl alcohol (25 mM). We were unable to use PEP as a tool to separate membrane receptor-mediated effects of cortisol from changes due to membrane order because PEP by itself affected acute signaling pathways in trout hepatocytes (data not shown). The reaction was stopped by replacing L-15 media with 100 µl ice cold lysis buffer (50 mM Tris, 0.25 M sucrose, 1% SDS, 10 mM NaF, 5 mM EDTA, 5 mM NEM, 0.1% Nonidet-P40). Lysed cells were quickly heated at 95°C for 5 min followed by brief sonication (sonic dismembrator, Fisher Scientific). Experiments were repeated with hepatocytes isolated from three independent fish.

2.2.8 Immunoblotting

Protein concentration was measured using the bicinchoninic acid (BCA) method using bovine serum albumin as the standard. All samples were diluted in Laemmli's sample buffer (1 M tris-HCl, pH 6.8, 60 mM, glycerol 25%, SDS 2%, β-mercaptoethanol 14.4 mM, bromophenol blue 0.1%). Total protein (40 µg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane and blocked with 5% solution of non-fat dry milk in 1 X TTBS (2 mM Tris, 30 mM NaCl, 0.01% Tween, pH 7.5) for 1 h at room temperature. This was followed with an overnight incubation (1:1000 dilution) with either phospho-(Ser) PKC substrate, phospho-Akt substrate (RXXS/T) or phospho-PKA Substrate (RRXS/T) polyclonal rabbit antibodies (Cell Signaling Technology, Beverly, MA). Blots were incubated for 1 h at room temperature with anti-rabbit horseradish peroxidase (HRP)-labeled secondary antibody (Bio-rad; 1:3000 dilutions in 5% skim milk). Protein bands were detected with ECL Plus™ chemiluminescence (GE Health Care, Baie d'Urfe, QC) and imaged using either the Typhoon 9400 (Amersham Biosciences) or the Pharos FX Molecular Imager (Bio-rad). Total lane or

protein band intensity was quantified using AlphaImager HP™ (Alpha Innotech, CA). Equal loading was confirmed by incubation of membranes with Cy3™ conjugated monoclonal mouse β-actin antibody (Sigma, 1:1000) for 1 h at room temperature.

2.2.9 Statistical Analysis

A one-way or two-way analysis of variance was used for multiple comparisons and a least significant differences (LSD) *post hoc* test was used to compare within factor effects. Statistics were performed either on raw or log transformed data (when necessary to meet normality and equal variance assumptions). A probability level of $p<0.05$ was considered significant. All statistical analyses were performed using SigmaPlot 11 software (Systat Software Inc., San Jose, CA, USA).

2.3 Results

2.3.1 Membrane Properties – Cortisol Exposure in vitro

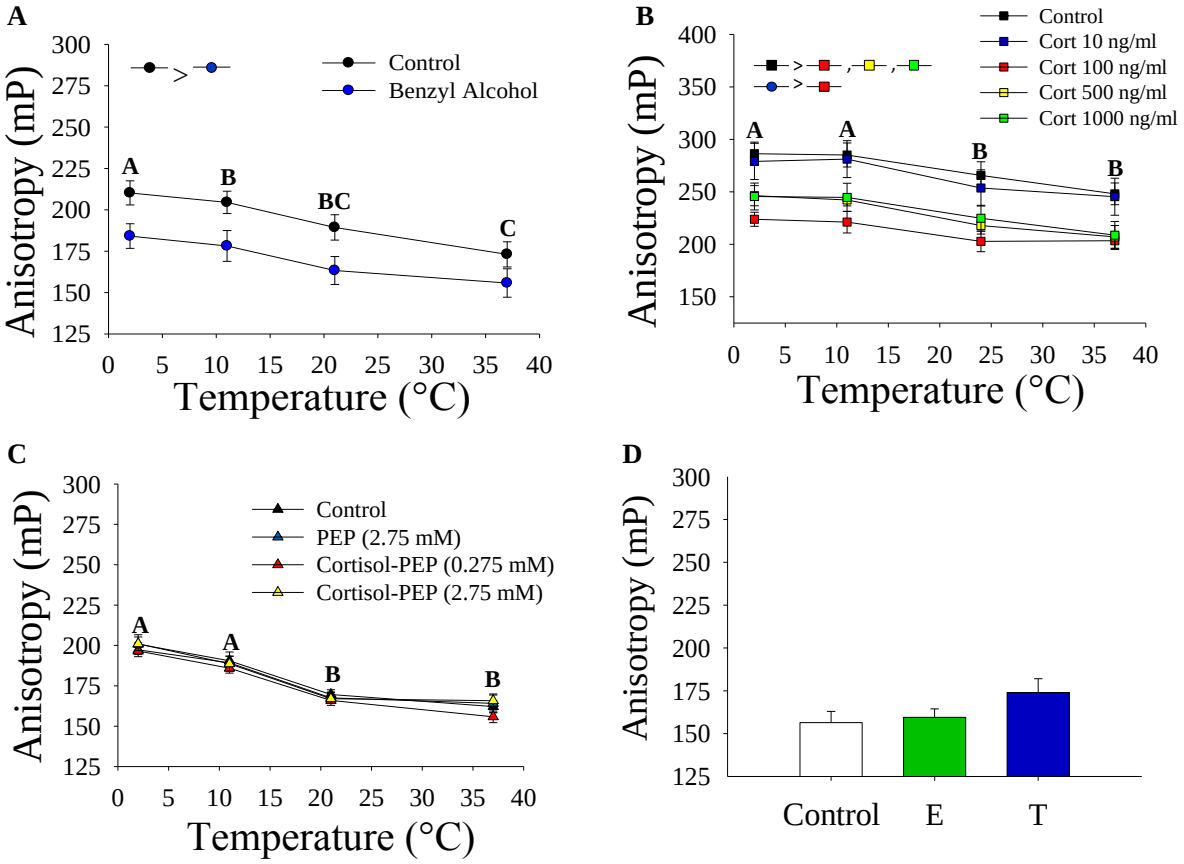
To determine whether cortisol accumulates within plasma membranes, enriched liver plasma membranes from unstressed trout were exposed to stress levels of cortisol (100 ng/mL) for 30 min *in vitro*. Cortisol treatment caused a significant five-fold increase in membrane cortisol content (0.50±0.26 ng/mg protein) compared to untreated membranes (0.11±0.06 ng/mg protein, n = 3, Paired Student's t-test), while membrane cholesterol levels were not significantly different between the two groups (60.9±10.9 µg/mg protein in control versus 55.2±9.4 µg/mg protein in cortisol-treated membranes).

2.3.2 Steady-state fluorescence polarization.

As expected, DPH anisotropy decreased with increasing temperatures (Fig.2-1). Benzyl alcohol significantly increased hepatic plasma membrane fluidity compared to the control membrane (Fig. 2-1A). Exposure to stressed levels of cortisol (100–1000 ng/mL) significantly increased hepatic plasma membrane fluidity, whereas resting level of cortisol (10 ng/mL) reported in trout had no significant effect on fluidity compared to the control group (Fig. 2-1B). When cortisol was coupled to a peptide moiety (PEP) to make it membrane impermeable (cortisol-PEP), there was no significant effect on membrane fluidity (Fig. 2-1C). Also, neither pharmacological levels of 17β-estradiol (10 µM) nor testosterone (10 µM) significantly affected trout liver plasma membrane order (Fig. 2-1D).

Figure 2-1. Cortisol effect on plasma membrane order *in vitro*

A) 1,6-Diphenyl-1,3,5-hexatriene (DPH) fluorescence anisotropy of enriched hepatic plasma membranes isolated from rainbow trout treated with or without benzyl alcohol for 30 min prior to anisotropy measurement at various temperatures. Data represents mean ± S.E.M (n = 7 independent fish). Different upper case letters indicate significant temperature effects and inset indicates significant treatment effects (two-way repeated measures ANOVA, $p<0.05$). B) DPH fluorescence anisotropy of enriched hepatic plasma membrane fractions treated with cortisol (0, 10, 100, 500, and 1000 ng/ml) for 30 min at various temperatures. Data represents mean ± S.E.M (n = 6 independent fish). Different upper case letters indicate significant temperature effects and inset indicates significant treatment effects (two-way repeated measures ANOVA, $p<0.05$). C) DPH fluorescence anisotropy of isolated hepatic plasma membrane fractions treated with the peptide conjugate (PEP, equivalent to 1000 ng/ml, 2.75 µM), or cortisol-conjugated peptide (cortisol-PEP, 0.275 µM and 2.75 µM) for 30 min prior to anisotropy measurement at various temperatures. Data represents mean ± S.E.M (n = 5 independent fish). Different upper case letters indicate significant temperature effects (one-way repeated measures ANOVA, $p<0.05$). D) DPH fluorescence anisotropy of enriched trout hepatic plasma membrane fractions treated with 17β-estradiol (E; 1 µM) or testosterone (T; 1 µM) for 30 min. Data shown as mean ± S.E.M (n = 6 independent fish).


A
Anisotropy (mP)
Temperature (°C)
Control
Benzyl Alcohol
A
B
BC
C
B
Anisotropy (mP)
Temperature (°C)
Control
Cort 10 ng/ml
Cort 100 ng/ml
Cort 500 ng/ml
Cort 1000 ng/ml
A
A
B
B
C
Anisotropy (mP)
Temperature (°C)
Control
PEP (2.75 mM)
Cortisol-PEP (0.275 mM)
Cortisol-PEP (2.75 mM)
A
A
B
B
D
Anisotropy (mP)
Control
E
T

2.3.3 Atomic force microscopy (AFM).

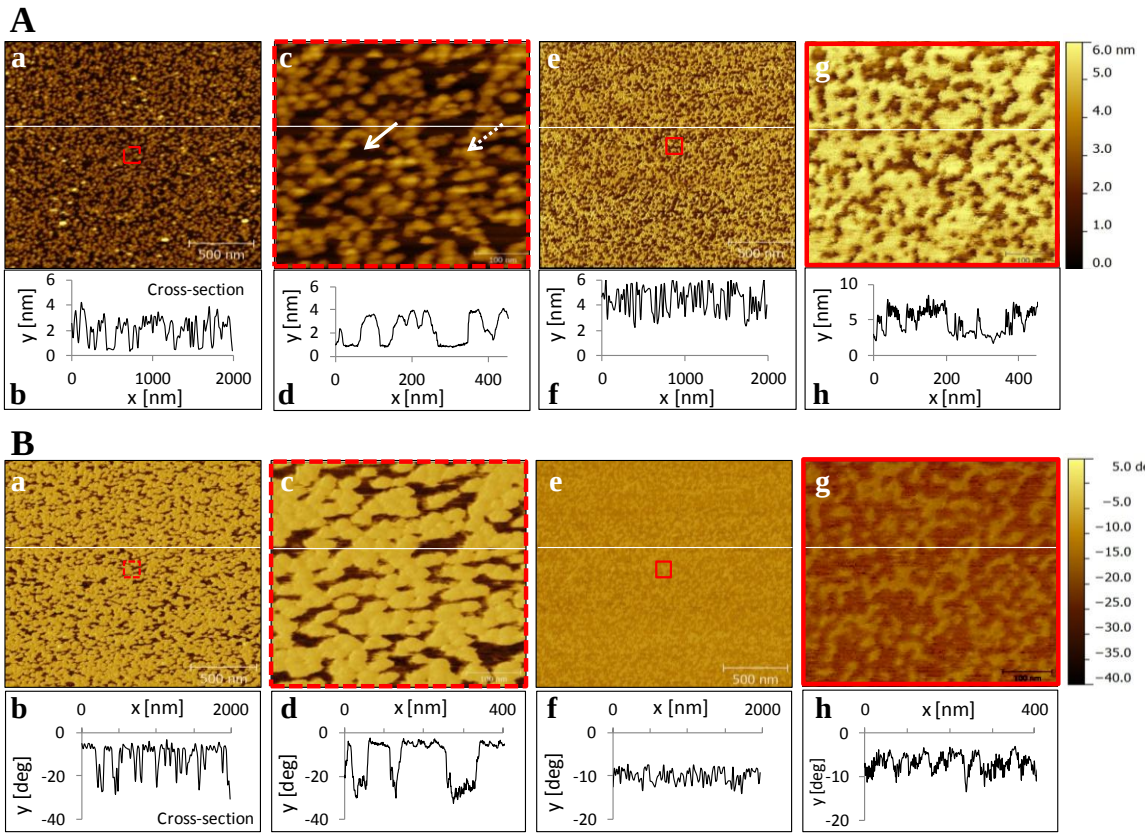
The surface topography of control (Figs. 2-2A, a,c) membranes and their corresponding cross-section plots (Figs. 2-2A, b,d) reveal membrane domains within the plasma membrane that differ in height. The solid arrow points to a lower membrane domain (darker regions), while the dotted arrow denotes a higher domain (lighter regions, Fig. 2-2A, c). The difference in height between the low and high domains (average membrane roughness) of control plasma membranes did not vary over the 30 min incubation (0 min: 2.60 nm±0.073 nm versus 30 min: 2.49 nm±0.11 nm). However, surface topography differed considerably after cortisol treatment (Figs. 2-2A, e,g, cross-sections Figs. 2-2A, f,h) compared to control membranes at 30 min (Figs.2- 2A, a,c, cross-sections Figs. 2-2A, b,d). In particular, by comparing the cross-sections, maximum roughness was higher for membranes treated with cortisol (3.98 nm±0.13) compared to control membranes (2.49 nm±0.11 nm).

In addition to domain height, the phase image (Fig. 2-2B), which maps the degree of surface adhesion of the cantilever as it interacts with the surface (Magonov et al., 1997), also indicates that the different domains differ in their relative hardness (viscoelastic properties). As with topography, the control phase images did not change over the 30 min period. Unlike topography, cortisol treatment decreased the degree to which the phase differed between the higher and lower regions (Figs. 2-2B, e,g) compared to control membranes (Figs.2- 2B, a,c). Specifically, in control membranes there was a nine-fold difference in the phase image (Fig.2- 2B, b) between the soft versus the most rigid points, whereas there was only a twofold difference after cortisol treatment (calculated from corresponding cross sections; Fig. 2-2B, d). As seen in the cross-sectional plots of control (Figs.2- 2B, b,d) and cortisol (Figs. 2-2B, f,h) treated membranes, this is due to an increase in the surface adhesion of the lower (fluid) domain, whereas the surface adhesion of the upper domain remained unchanged following cortisol treatment (i.e. phase of lower domains increases, whereas phase of upper domains is unchanged in response to cortisol treatment; Fig. 2-2C).

Lastly, as seen in both the topography and phase images following cortisol treatment (Figs. 2-2A and 2B [e,f,g,h]), the micro-domains (lipid rafts) increased in frequency and size and, therefore, cover a greater surface area compared to control (Figs. 2-2A and 2B [a,b,c,d]) membranes. Specifically, surface coverage of higher domains for control membranes was 51%, whereas the surface coverage of higher domains increased to 66% following cortisol treatment.

Figure 2-2. Cortisol effect on liver plasma membrane topography and surface adhesion

A) Representative atomic force microscopy (AFM) images of supported hepatic plasma membrane topography (membrane height). Images were taken prior to (a) and 30 min after cortisol (100 ng/mL) treatment (e) in liquid cell at room temperature. A zoomed in scan is also shown for the control (c) and cortisol-treated (g) membranes that was scanned for 60 min. The approximate scan region of the zoomed in image is indicated by the dashed red box in the control image (a) and solid red box in the cortisol-treated image (e). Two distinct domains, which differ in height, are visible in both control and cortisol-treated membranes. A representative higher domain is indicated by the dotted arrow, while the lower domain is indicated by the solid arrow (c). Short-term cortisol treatment altered the topography of the plasma membrane. The cross-section graph featured below each image was calculated from points along the white horizontal line. The y-axis represents vertical height (nm), whereas the x-axis represents the horizontal distance (nm). B) Representative AFM images of supported hepatic plasma membrane phase (surface adhesion properties). Images were taken prior to (a) and 30 min after cortisol (100 ng/mL) treatment (e) in liquid cell at room temperature. A zoomed in scan is also shown of the control (c) and cortisol treated (g) membranes that was scanned for 60 min. The approximate scan region of the zoomed in image is indicated by the dashed red box in the control image (a) and solid red box in the cortisol-treated image (e). Two distinct domains, which differ in their viscoelastic (surface adhesion) are visible in both control and cortisol-treated membranes. Acute cortisol treatment altered the viscoelastic properties of the plasma membrane within 30 min of treatment. The cross-section graph featured below each image was calculated from points along the white horizontal line. The y-axis represents degree of deflection (degrees), whereas the x-axis represents the horizontal distance (nm). C) A schematic representation of cortisol's effect on plasma membrane properties. Short-term incubation with cortisol (b) increased surface roughness (height difference between higher and lower domains) compared to control membrane (a).

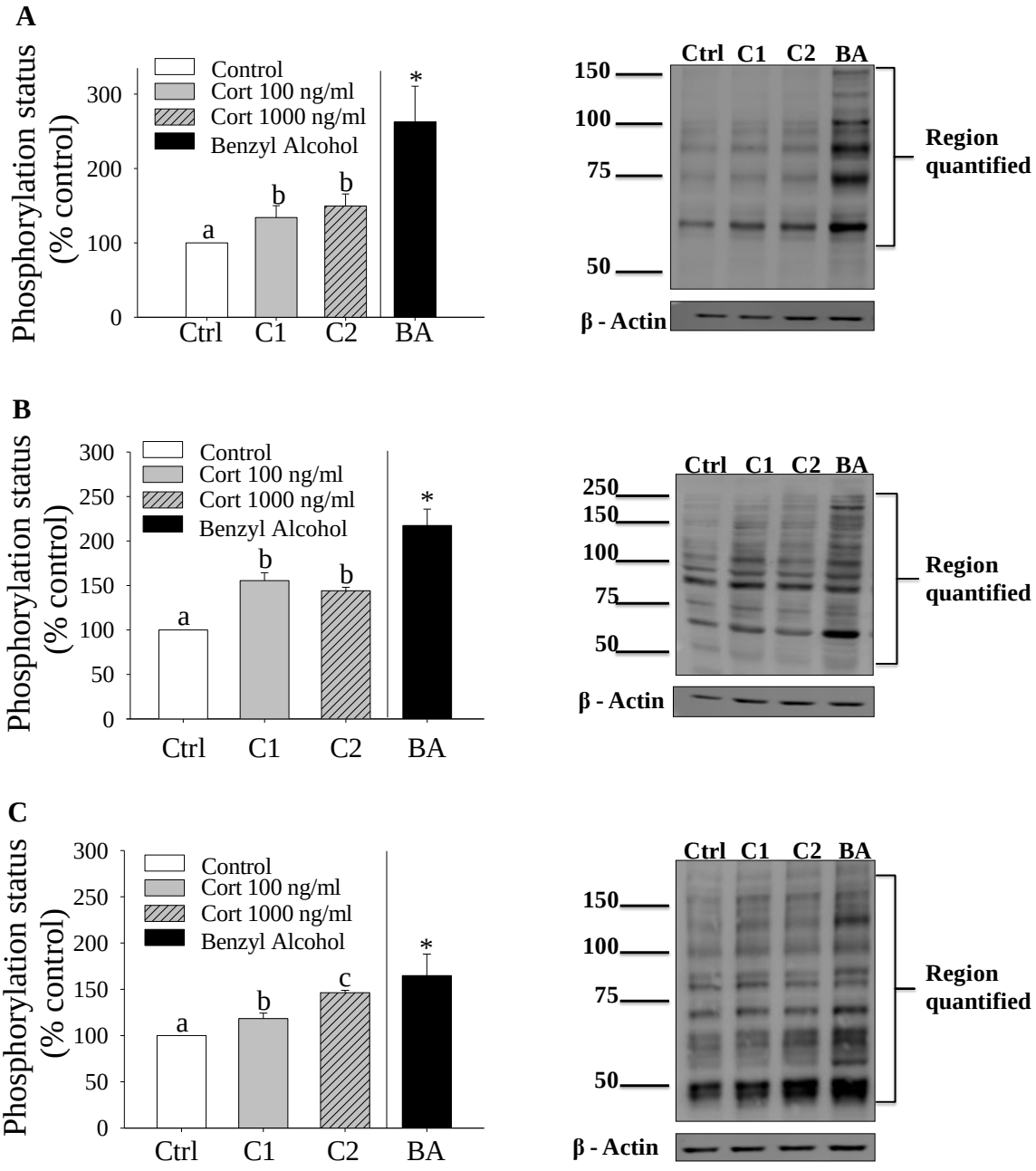

A
a
c
e
g
6.0 nm
5.0
4.0
3.0
2.0
1.0
0.0
500 nm
Cross-section
y [nm]
x [nm]
b
d
f
h
B
5.0 deg
−5.0
−10.0
−15.0
−20.0
−25.0
−30.0
−35.0
−40.0
y [deg]
C
a
b
2.49 nm ± 0.11
3.98 nm ± 0.13
mica

2.3.4 Hepatocyte Response

To investigate whether physiochemical changes in the plasma membrane are capable of stimulating intracellular signaling in trout hepatocytes we examined the phosphorylation of PKA, PKC and AKT-substrate proteins in response to benzyl alcohol, a known membrane fluidizer, and cortisol. Our results demonstrate that benzyl alcohol (25 mM) significantly increases phosphorylation of PKC (Fig. 2-3A), PKA (Fig. 2-3B) and AKT (Fig. 2-3C) substrate proteins within 10 min. Similar to the response seen with benzyl alcohol, cortisol at 100 and 1000 ng/mL also significantly increased phosphorylation of putative substrate proteins for all three kinases at 10 min post-treatment (Figs. 2-3A–C).

Figure 2-3. Cortisol effect on rapid cell signaling in trout hepatocytes

Rainbow trout hepatocytes were incubated either with cortisol (0, 100 or 1000 ng/mL) or benzyl alcohol (BA; 25 mM) for 10 min. Cell homogenates (40 µg protein) were probed with polyclonal rabbit antibody (Cell Signaling Technology, Beverly, MA) to either phospho-(Ser) PKC substrate (A), phospho-PKA Substrate (RRXS/T) (B) or phospho-Akt substrate (RXXS/T) (C). Equal loading was confirmed with β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). A representative immunoblot for each is shown; values are plotted as % control and shown as mean ± S.E.M (n = 3 independent fish); bars with different letters are significantly different (repeated measures ANOVA, $p<0.05$). *significantly different from control (Paired Student's t-test; $p<0.05$).


A
Phosphorylation status (% control)
Control
Cort 100 ng/ml
Cort 1000 ng/ml
Benzyl Alcohol
300
200
100
0
a
b
b
*
Ctrl
C1
C2
BA
Ctrl C1 C2 BA
150
100
75
50
Region quantified
β - Actin
B
Phosphorylation status (% control)
Control
Cort 100 ng/ml
Cort 1000 ng/ml
Benzyl Alcohol
300
250
200
150
100
50
0
a
b
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C1
C2
BA
Ctrl C1 C2 BA
250
150
100
75
50
Region quantified
β - Actin
C
Phosphorylation status (% control)
Control
Cort 100 ng/ml
Cort 1000 ng/ml
Benzyl Alcohol
300
250
200
150
100
50
0
a
b
c
*
Ctrl
C1
C2
BA
Ctrl C1 C2 BA
150
100
75
50
Region quantified
β - Actin

2.3.5 Discussion

This study provides novel insight into the role of glucocorticoid in the acute stress response by demonstrating for the first time that stressed levels of this steroid rapidly alter liver plasma membrane fluidity and modulates cell signaling in a piscine model. Although physical changes to plasma membrane structure are known to be an important initiator of intracellular signaling events in response to stressors (Vigh et al., 2007), a role for steroids in this process is far from clear. Our results highlight a hitherto unknown role for cortisol in acute stress adaptation that is nonspecific and involves changes in membrane fluidity.

We demonstrate a rapid change in liver plasma membrane fluidity in response to stressed levels of cortisol *in vitro*. The fluidity changes seen with cortisol were not dose-related, but occurred above a certain threshold suggesting a receptor-independent mechanism likely associated with steroid incorporation into the lipid domain. This was supported by the inability of membrane impermeable cortisol-PEP to alter membrane fluidity. While changes in plasma membrane cholesterol levels alter lipid order (Lingwood and Simons, 2010) that appears unlikely in the present case as membrane cholesterol remained unchanged in response to cortisol treatment. The cortisol-induced fluidization of liver plasma membrane appears to be steroid specific, as neither 17β-estradiol nor testosterone treatment showed a similar response in trout plasma membrane. This agrees with the recent findings that the chemical structure of the steroid backbone affects interaction with the lipid bilayer and subsequent changes in plasma membrane fluidity (Rog et al., 2008). It remains to be determined whether the membrane biophysical effect is also seen with other corticosteroids and not just cortisol. However, cortisol is the primary corticosteroid that is released into the circulation in response to stress in trout. The membrane fluidizing effect of cortisol seen in liver may be a generalized response affecting all tissues in response to stress. Mammalian studies reported a fluidizing effect of glucocorticoid on fetal rat liver (Kapitulnik et al., 1986) and dog synaptosomal membranes (Deliconstantinos, 1985), whereas an ordering effect was observed in rat renal brush border (Levi et al., 1995) and rabbit cardiac muscle (Gerritsen et al., 1991). This suggests that stress-mediated cortisol effect on membrane order may be tissue-specific, but this remains to be determined in fish. Altogether, our results indicate that stress-induced elevation in cortisol levels rapidly fluidizes liver plasma membrane in rainbow trout.

AFM topographical and phase images further indicate that cortisol alters biophysical properties of liver plasma membranes. Specifically, cortisol exposure led to the reorganization of discrete

microdomains, likely gel phase (higher domains) and disordered fluid-phase (lower domains) in the lipid bilayer. These discrete domains differed in height, which increased after cortisol treatment. A recent study on erythrocytes also reported a glucocorticoid-induced domain reorganization, which involved formation of large protein-lipid domains by hydrophobic and electrostatic interactions leading to alteration in membrane structure and elasticity (Panin et al., 2010). Similar domain changes have also been reported for synthetic lipids in response to halothane exposures or melting transitions (Leonenko and Cramb, 2004), treatments that are known to increase membrane fluidity (Leonenko and Cramb, 2004), (Leonenko et al., 2004). Cortisol appears to have a greater effect on lower domains, as indicated by the greater change in surface adhesion (phase) following steroid treatment, compared to the higher lipid domains. Collectively, stressed levels of cortisol rapidly alter the biophysical properties of trout hepatic plasma membrane. We hypothesize that changes in membrane order by cortisol is the result of a non-uniform fluidization at the nanoscale among different membrane domains.

Rapid changes to membrane order by cortisol may play a role in triggering acute stress-related signaling pathways. Indeed membrane order perturbations lead to rapid activation of cell signaling pathways, including protein kinases (Vigh et al., 2007). In agreement, benzyl alcohol, a known membrane fluidizer, rapidly induced phosphorylation of PKA, PKC and AKT putative substrate proteins. The intracellular effect of benzyl alcohol has been attributed to its direct effect on plasma membrane structure. Interestingly, cortisol exposure also induced phosphorylation of PKA, PKC and AKT putative substrate proteins as seen with benzyl alcohol, supporting a rapid stress signaling event mediated by changes to membrane order. While membrane receptor mediated nongenomic glucocorticoid signaling has been reported before (Borski, 2000), to our knowledge this is the first report of membrane biophysical changes initiating a rapid signaling event induced by stressed levels of cortisol in any animal model.

To date, the genomic effects of cortisol have been the primary focus in establishing the role of this steroid in the acute stress response (Vegiopoulos and Herzig, 2007), (Aluru and Vijayan, 2009). In liver, stress-induced cortisol has been shown to modulate expression of genes involved in intermediary metabolism, including gluconeogenesis, which is essential for mobilizing glucose to cope with the enhanced energy demand (Aluru and Vijayan, 2009; Mommsen et al., 1999; Rose et al., 2010; Vegiopoulos and Herzig, 2007). This genomic response to cortisol is slow acting and, therefore, not considered to be important in the rapid glucose regulation associated with the fight-or-

flight response (McEwen, 2007). The PKA and AKT (Klover and Mooney, 2004) signaling pathways are both known to regulate hepatic glucose metabolism, while PKC has been implicated in hepatic insulin resistance (Samuel et al., 2007). Consequently, cortisol-mediated changes in membrane fluidity may be a key nonspecific stress response triggering the phosphorylation of putative protein kinase substrate proteins. This rapid activation of stress-related signaling pathways by cortisol may be playing an important role in the metabolic adjustments to the fight-or-flight response. As plasma membrane order can affect membrane receptor function (Chachisvilis et al., 2006), we hypothesize that cortisol-induced biophysical membrane changes may also modify hepatocyte responsiveness to other stress signals, including glucoregulatory hormone stimulation. In support, studies have shown a permissive effect of cortisol treatment on epinephrine-mediated glucose production in trout hepatocytes (Mommsen et al., 1999), (Reid et al., 1992).

Altogether, our results underscore a novel plasma membrane response to stressed levels of glucocorticoid exposure, leading to a nongenomic signaling event in trout hepatocytes. This rapid and nonspecific cortisol effect may act either alone and/or in concert with membrane receptor activation, to modulate stress-related signaling pathways. We propose that the rapid cortisol-mediated changes in membrane fluidity occur in a non-uniform domain-like manner and may have important consequences to the non-specific cellular stress response and adaptation to subsequent stressor insult in animals.

2.3.6 Acknowledgements

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Author's contributions: Conceived and designed the experiments: LD JM MMV. Performed the experiments: LD JM. Analyzed the data: LD JM EF ZL. Contributed reagents/materials/analysis tools: TLD ZL MMV. Wrote the paper: LD JM EF TLD ZL MMV.

Chapter 3: **Nongenomic cortisol signaling in response to acute stress involves changes in plasma membrane order in rainbow trout liver**

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3.1 Introduction

In response to an acute stressor a conserved physiological response is initiated, which involves elevation of stress hormones and subsequent metabolic adjustments, to ensure homeostasis (Sapolsky et al., 2000). The principal stress hormones, epinephrine and glucocorticoids, have critical functions in the stress adaptation process (Charmandari et al., 2005). The fight-or-flight response involves the activation of the sympathetic nervous system leading to the rapid release of epinephrine from chromaffin cells (Charmandari et al., 2005). This catecholamine plays a major role in the acute cardiovascular and metabolic adjustments associated with the fight-or-flight response. The synthesis and release of cortisol, the principal glucocorticoid in teleost, comprises the second phase of the neuroendocrine response, which has a longer-term effect on stress adaptation. A key target tissue involved in metabolic adjustments during stress adaptation is the liver. Within the liver, the genomic effects of cortisol involve changes in expression of genes involved in intermediary metabolic regulation (Aluru and Vijayan, 2009; Prunet et al., 2008). While several studies have examined the genomic effects associated with stressor-induced plasma cortisol elevation (Aluru and Vijayan, 2009; Prunet et al., 2008; Vegiopoulos and Herzig, 2007), few have investigated the rapid nongenomic effects of this steroid on peripheral tissues (Billing et al., 2012).

The plasma membrane is likely pivotal during the response to stress as the cell membrane is tightly linked to cellular physiology and controls ion transport, integral and peripheral membrane receptor activation and enzyme activity (Vigh et al., 2007). Mounting evidence indicate that subtle perturbations in the plasma membrane structure and/or composition can have important consequences to cellular physiology, including membrane transport (Melchior and Czech, 1979), endocytosis (Rhode et al., 2009), enzyme activity (Gordon et al., 1980), permeability (Lande et al., 1995) and apoptosis (Rodrigues et al., 2002). Indeed changes to membrane biophysical properties have been shown to modulate intracellular stress-activated signaling pathways in responses to temperature (Nagy et al., 2007), oxidative (Clement et al., 2010; Sergent et al., 2005) and mechanical stressors (Verstraeten et al., 2010). Despite evidence that changes in plasma membrane properties rapidly affect cellular stress response (Vigh et al., 2007), very little is known about the effect of acute physical stressor on membrane properties and associated signaling events in whole organisms.

Recently we reported that *in vitro* exposure of hepatic plasma membranes to cortisol rapidly alters fluidity, surface topography and elasticity in rainbow trout (*Oncorhynchus mykiss*) (Dindia et al., 2012). In addition to modulating plasma membrane properties, stress levels of cortisol rapidly

phosphorylated putative substrate protein of protein kinase C (PKC), protein kinase A (PKA) and AKT in hepatocytes. Benzyl alcohol, a well documented membrane fluidizer, also activated these kinase pathways suggesting that changes in membrane fluidity may be initiating stress signaling cascades in trout hepatic tissue (Dindia et al., 2012). This led us to hypothesize that acute stressor-induced plasma cortisol elevation rapidly alters hepatic membrane order *in vivo*, and may modulate the hepatic response to acute physical stressor. To test this hypothesis we carried out *in vivo* acute stress experiment either in the presence or absence of metyrapone, an 11β-hydroxylase inhibitor that blocks endogenous cortisol biosynthesis (Meikle et al., 1975). Liver plasma membrane fluidity, topography and composition along with phosphorylation of putative protein kinase C (PKC), protein kinase A (PKA) and AKT substrate proteins and ERK(1/2) MAPKs were measured after a 30 min physical stressor in rainbow trout. We report for the first time that an acute physical stressor fluidizes liver plasma membrane in rainbow trout and underscores a role for cortisol in rapidly modulating the membrane biophysical properties and evoking cellular responses.

3.2 Materials & Methods

3.2.1 Animals

Juvenile rainbow trout (100-300 g) purchased from Alma Aquaculture Research Station (Alma, Ontario) were maintained at the University of Waterloo aquatic facility exactly as described before (Sandhu and Vijayan, 2011). The tanks were supplied with a constant flow of aerated well water (12 ± 2°C) and were maintained under a 12hL:12hD photoperiod. Trout were acclimated for at least two weeks prior to experiments and were fed commercial trout feed (Martin Mills, Elmira, Ontario) to satiety once daily, 5 days a week. The animals were maintained according to Canadian Council on Animal Care guidelines and approved by the animal care committee at the University of Waterloo.

3.2.2 Experimental Protocol

There were four experimental tanks (100 L) with groups of 12-13 trout each maintained exactly as mentioned above. Fish in two tanks were injected with 1 mg/kg of metyrapone (Sigma, St. Louis, MO), while the other two tanks were injected with an equal volume of 0.9% saline (sham group). Prior to injection trout were sedated with 2-phenoxyethanol (1:10 000; Sigma). The fish were allowed to recover for an hour after which they were netted (5 min netting disturbance) and crowded together by pooling fish from the duplicate tanks into one tank (24-26 trout /tank). The sham fish or

metyrapone-treated fish were sampled 30 min after handling and crowding. All fish were euthanized with an overdose of 2-phenoxyethanol (1:1000) and were sampled either prior to (0 min) or 30 min of stressor exposure. Sampling consisted of quickly netting 6 fish into the anesthetic and bleeding all fish within 5 min. Fish were weighed and bled by caudal puncture into heparinized tubes followed by centrifugation (5 min at 6000Xg) for plasma collection. Plasma samples were frozen at -70°C for later determination of cortisol, glucose and lactate levels. Livers were quickly removed and flash frozen in liquid nitrogen and stored at -70°C for plasma membrane isolation.

3.2.3 Plasma cortisol, glucose and lactate analysis

Plasma cortisol levels were measured using a [^{3}H]-labeled cortisol radioimmunoassay as described previously (Alsop et al., 2009). Plasma glucose (Trinder method, Sigma) and lactate (Trinity Biotech, St. Louis, MO) levels were measured using commercially available colorimetric kits.

3.2.4 Liver plasma membrane

Liver plasma membranes were isolated using sucrose gradient as described previously (Sulakhe, 1987). The membrane pellet was resuspended in TCD buffer (300 mM sucrose, 10 mM Tris-HCl, 1 mM dithiothreitol (DDT), 0.5 mM $CaCl_2$, 1X protease inhibitor cocktail, pH 7.5; Sigma) and frozen at -70°C. All steps, including centrifugation, were carried out at 4°C. The enrichment of the membrane fraction was determined as described previously by measuring the activities of Na^+/K^+-ATPase (McGuire et al., 2010) and lactate dehydrogenase (Gravel et al., 2009). The five-fold Na^+/K^+-ATPase (H: 2.8 ± 0.31 vs. M: 10.4 ± 2.3; n = 23) and twelve-fold higher 5'- nucleotidase (H: 3.6 ± 0.56 vs. M: 41.7 ± 9.2; n = 12) activities (U/g protein) in the membrane (M) fraction compared to the initial tissue homogenate (H), confirm membrane enrichment. The ~97% drop in LDH activity (H: 5334.7 ± 386.2 vs. M: 163.7 ± 113.7; n = 23) in the membrane fraction compared to the homogenate further confirms enriched plasma membranes with negligible cytosolic contamination.

3.2.5 DPH anisotropy

The hepatic plasma membrane fluidity of sham and metyrapone-treated trout before and after acute stress was analyzed by measuring the steady-state fluorescence polarization using the membrane fluorescent probe, 1,6-diphenyl-1,3,5 hexatriene (DPH; Life Technologies Inc., Burlington, ON) exactly as described previously (Dindia et al., 2012). Membrane samples were added to 96-well opaque plates (Corning Incorporated, New York, USA; 100 µl with approximate protein

concentration of 0.3 mg /ml) and incubated with DPH (1:100 of 4.7 mM stock dissolved in tetrahydrafuran for 30 min in the dark. Readings were taken at various temperatures starting at 2°C thirty min post-treatment, followed by 12, 24 and 37 ± 1°C. The required temperature (reached within approximately 5-10 min) was maintained and this was confirmed by temperature monitoring within each well, using a digital thermometer, immediately prior to and after the anisotropy measurements.

3.2.6 Atomic force microscopy (AFM)

Atomic force microscopy (AFM) was used to compare topography and elasticity properties of hepatic plasma membranes. AFM measurements were carried out in a fluid cell (Molecular Imaging) using the Agilent Technologies 5500 Scanning Probe Microscope in tapping mode (MAC mode) as described before (Moores et al., 2011). Precise force regulation was obtained in MAC mode by using a magnetically coated cantilever (MacLevers Type II from Agilent Technologies; force constant: 2.8 N/m, tip radius: 7nm, and height: 10-15µm). Membrane samples were transferred onto a freshly cleaved piece of mica placed within the liquid cell and equilibrated for 10 min followed by a quick rinse with nanopure water. Supported plasma membranes were scanned in water at 0.7 ln/s immediately following the rinse, which took approximately 15 min. Quantitative analysis of topography and phase images was conducted using Gwyddion (Czech Republic, http://gwyddion.net/). Difference in membrane height (membrane roughness) and viscoelastic properties between different membrane regions was calculated by taking the average difference in height or phase between lower and higher membrane regions, respectively.

3.2.7 Lipid extraction

Membrane samples were isolated as described above and extracted as previously described (Chung et al., 2011) and stored at -20°C for gas chromatography-flame ionization detection (GC-FID) and thin layer chromatography- flame ionization detection (TLC-FID) analysis as described before (THOMAS et al., 2010). Thin layer chromatography (TLC) was used to separate the individual phospholipids present in the sample and was preformed exactly as described previously (Chung et al., 2011). The dried samples (separated phospholipids) were derivatized for gas chromatography (GC) analysis as previously explained (Chung et al., 2011).

3.2.8 Immunoblotting

Liver tissue was homogenized and protein concentration was measured using the bicinchoninic acid (BCA) method using bovine serum albumin as the standard. All samples were diluted in Laemmli's sample buffer (1M tris-HCl, pH 6.8, 60 mM, glycerol 25%, SDS 2%, β-mercaptoethanol 14.4 mM, bromophenol blue 0.1%) and the immunodetection carried out exactly as described in (Dindia et al., 2012). Briefly, total protein (40 μg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane and blocked with 5% solution of non-fat dry milk in 1 X TTBS (2 mM Tris, 30 mM NaCl, 0.01% Tween, pH 7.5) for 1 h at room temperature. This was followed with an overnight incubation (1:1000 dilution) with either phospho-ERK(1/2) (Thr202/Tyr204) or total ERK(1/2) monoclonal rabbit antibodies or phospho-(Ser) PKC substrate, phospho-Akt substrate (RXXS/T) or phospho-PKA Substrate (RRXS/T) polyclonal rabbit antibodies (Cell Signaling Technology, Beverly, MA). Blots were incubated for 1 h at room temperature with anti-rabbit horseradish peroxidase (HRP)-labeled secondary antibody (Bio-rad; 1:3300 dilutions in 5% skim milk). Protein bands were detected with ECL Plus™ chemiluminescence (GE Health Care, Baie d'Urfe, QC) and imaged using either the Typhoon 9400 (Amersham Biosciences) or the Pharos FX Molecular Imager (Bio-rad). Total lane or protein band intensity was quantified using AlphaImager HP™ (Alpha Innotech, CA) and values were normalized to a standard trout liver sample run on each blot for inter-blot comparisons. Each immunoblot was subsequently probed with Cy3™ conjugated monoclonal mouse β-actin antibody (Sigma, Cy3™ conjugated monoclonal mouse; 1:1000) for 1 h at room temperature. Densitometric values were then normalized to β-actin to control for protein loading differences between samples.

3.2.9 Statistical analysis

A two-way analysis of variance was used to compare time and treatment effects. A least significant differences (LSD) *post hoc* test was used to determine within factor effects. For AFM analysis, Student's t-test was used to compare surface topography and phase parameters differences between 0 and 30 min of stressor exposure. A linear regression analysis was carried out between plasma cortisol values and the phosphorylation intensity of active ERK(1/2) (ratio of phosphorylated to total) and PKC, PKA and AKT substrate proteins. Statistics were performed either on raw or log transformed data (when necessary to meet normality and equal variance assumptions). A probability level of $p < 0.05$ was considered significant. All statistical analyses were performed using SigmaPlot 11 software (Systat Software Inc., San Jose, CA, USA).

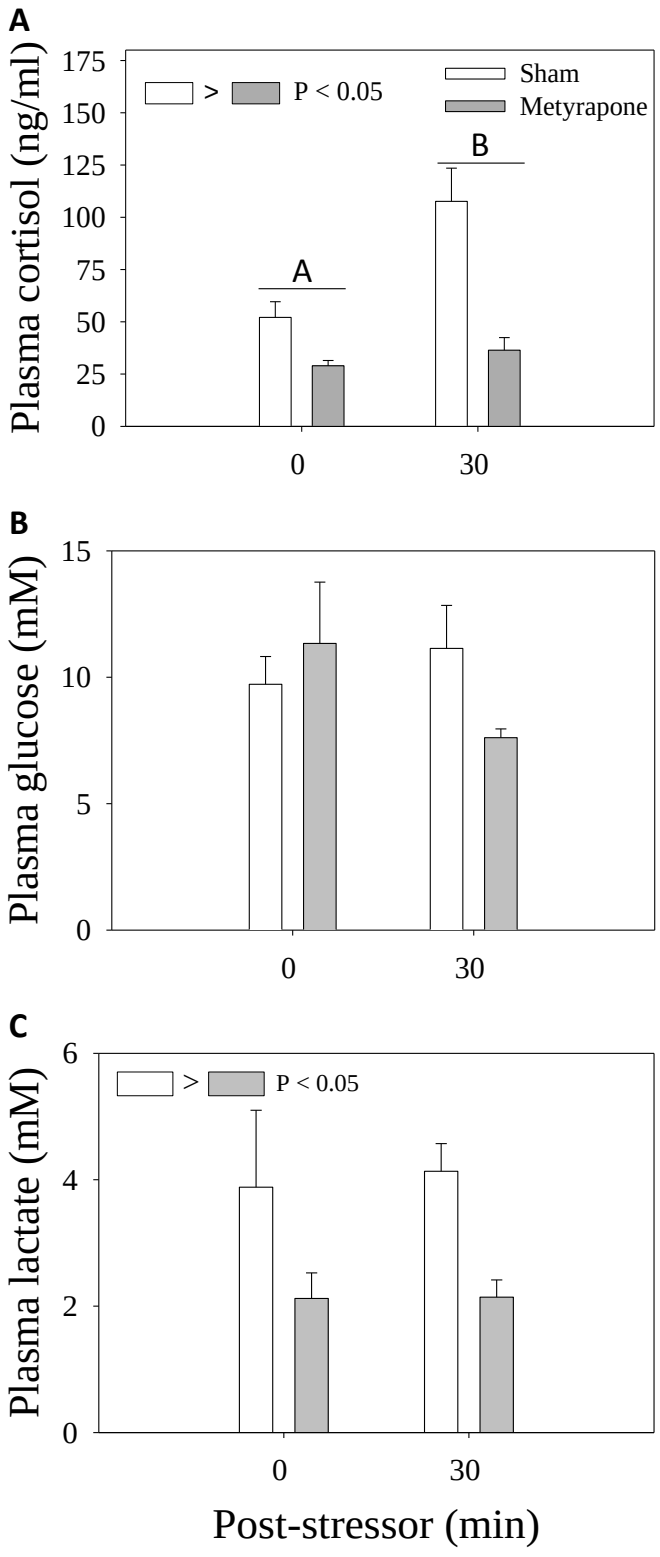
3.3 Results

3.3.1 Plasma cortisol, glucose and lactate levels

Plasma cortisol levels were significantly higher 30 min after stressor exposure, while prior metyrapone treatment inhibited this response (Fig.3-1A). Plasma glucose levels were not significantly affected by the stressor exposure in both groups (Fig. 3-1B). Plasma lactate levels were not significantly different 30 min after stressor exposure. However, metyrapone-treated trout had significantly lower plasma lactate levels compared to sham trout at both 0 h and 30 min post- stressor exposure (Fig. 3-1C).

Figure 3-1. Effect of acute stressor exposure in the presence or absence of metyrapone on plasma cortisol

(A), glucose (B) and lactate (C) levels in rainbow trout. Data represents mean ± S.E.M (n = 4-6). Different upper case letters indicate significant time effects and inset indicates significant treatment effects (two-way ANOVA, $p < 0.05$).


A
Plasma cortisol (ng/ml)
175
150
125
100
75
50
25
0
> P < 0.05
Sham
Metyrapone
B
A
0
30
B
Plasma glucose (mM)
15
10
5
0
0
30
C
Plasma lactate (mM)
6
4
2
0
> P < 0.05
0
30
Post-stressor (min)

3.3.2 Stress effect on hepatic plasma membrane order

As expected DPH anisotropy (reciprocal to fluidity) decreased with increasing temperatures, indicating that plasma membrane fluidity increased with temperature (Fig. 3-2). In addition to a temperature effect, there was a significant plasma membrane fluidization in response to acute stress such that plasma membranes were significantly more fluid (lower DPH anisotropy) compared to the pre-stressor group. Metyrapone-injection did not significantly affect plasma membrane fluidity in response to stress compared to sham treated trout. The stress-induced fluidization in the sham group was significant at 2, 11, and 24°C (Fig. 3-2).

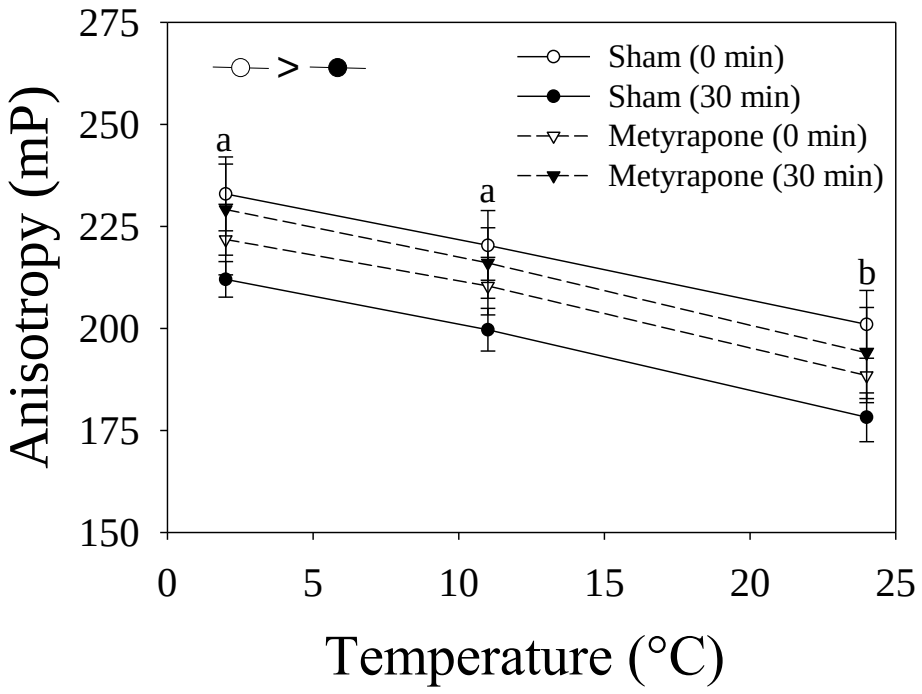


Figure 3-2. Effect of acute stress on 1,6-Diphenyl-1,3,5-hexatriene (DPH) fluorescence

DPH fluorescence polarization was measured in enriched hepatic plasma membranes isolated from sham or metyrapone-treated trout before and 30 min after a stressor exposure. Data represents mean ± S.E.M (n = 4-6). Different upper case letters and inset indicate significant time and treatment effects, respectively (two-way ANOVA, $p < 0.05$).

3.3.3 Stress alters membrane biophysical properties

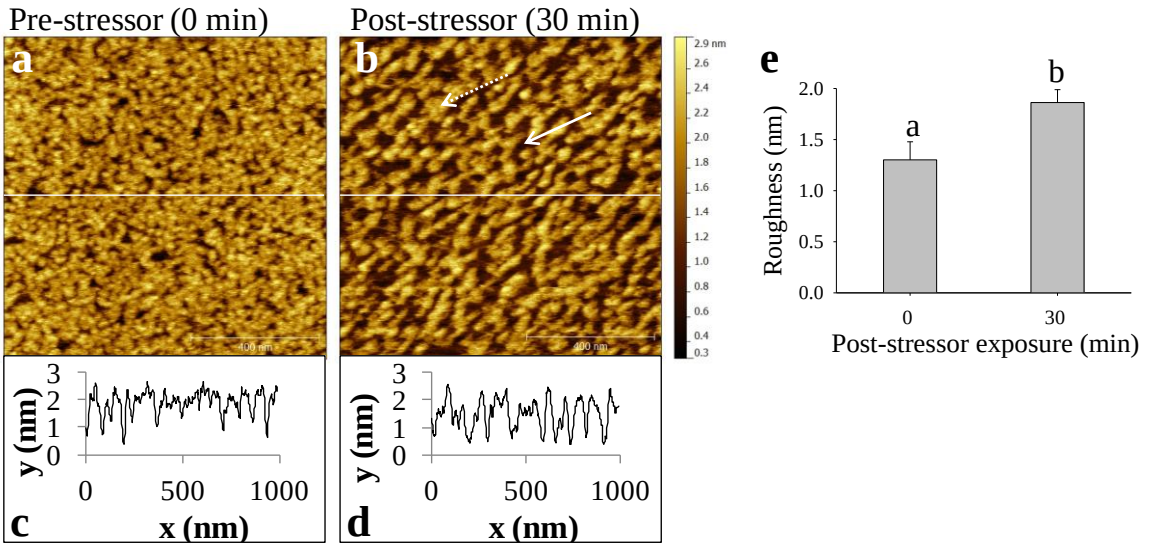
Atomic force microscopy was used to image the surface properties of liver plasma membranes isolated from sham trout before and 30 min after the handling and crowding stressor. In both topography (Fig. 3-3A [a,b]) and phase (Fig. 3-3B [a,b]) images there are two plasma regions (light versus dark regions, which likely represent different plasma membrane domains). These two plasma membrane domains differ in height (Fig. 3-3A [c,d]), with the solid arrow pointing to the lower domain, while the dotted arrow denotes a higher domain (Fig. 3-3A[b]). In addition to height, the two plasma membrane domains also differ in their viscoelasticity properties (relative hardness or softness), as seen in the phase image (Fig. 3-3B [a,b]). In the phase image (Fig. 3-3B[b]), the solid arrow indicates a harder domain, while the dotted arrow indicates a softer domain.

The acute stressor significantly altered membrane topography and domain organization (Fig. 3-3A), such that membrane roughness (difference between lower and higher domains) significantly increased following acute stress (Fig. 3-3A[e]). In addition, as evident in the corresponding cross-section plots, the width of lower microdomains increased after stressor exposure (Fig. 3-3A[d]) compared to pre-stressor membranes (Fig. 3-3A[c]). The degree to which the two domains differed in phase also tended to increase following acute stress; however, the difference was not statistically significant (Fig. 3-3B[e]).

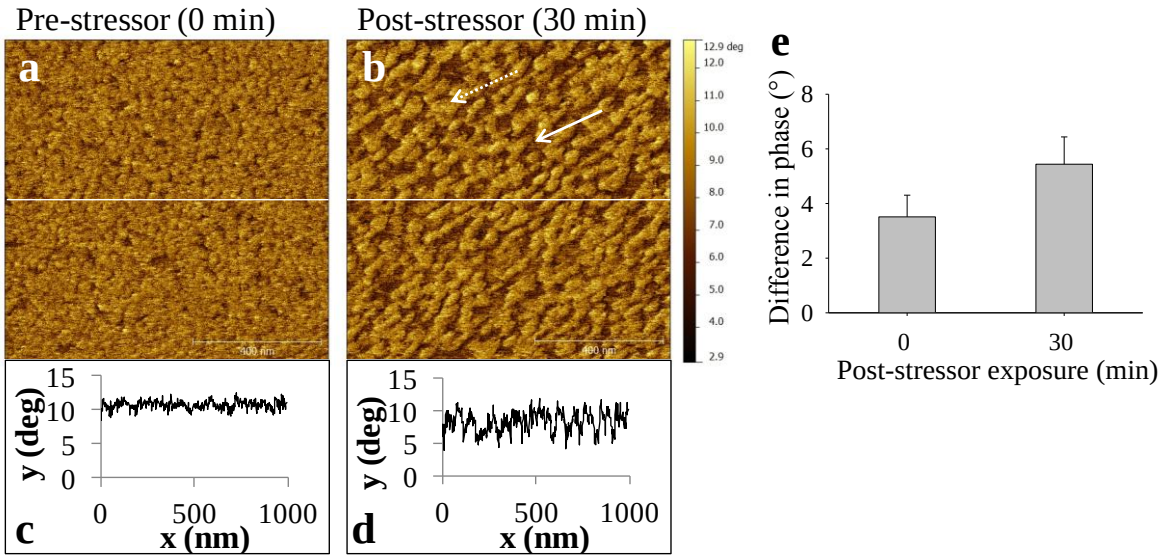
Figure 3-3. Figure 3. Effect of acute stress on liver plasma membrane topography and surface adhesion.

(A) Representative atomic force microscopy (AFM) topography images of supported hepatic plasma membrane before (a) and 30 min after stressor exposure (b) Two distinct domains, which differ in height, are visible in both unstressed and stressed plasma membranes. A representative higher domain is indicated by the dotted arrow, while the lower domain is indicated by the solid arrow (b). The cross-section graph featured below each image (c and d) was calculated from points along the white horizontal line. The y-axis represents vertical height (nm), whereas the x-axis represents the horizontal distance (nm). The average membrane roughness (difference in height between the lower and higher membrane regions) before and after acute stressor exposure is shown in the corresponding histogram (e). (B) Representative AFM phase images (surface adhesion properties) of supported hepatic plasma membrane before (a) and 30 min after stressor exposure (b). Overall there are two distinct phases within the hepatic plasma membrane, which differ in their surface adhesion. A region with higher surface adhesion is indicated by the dotted arrow while the solid arrow denotes a region with lower surface adhesion. The cross-section graph featured below each image (c and d) was calculated from points along the white horizontal line. The y-axis represents degree of deflection (degrees), whereas the x-axis represents the horizontal distance (nm). The average difference in phase between the lower and higher membrane regions before and after acute stressor exposure is shown in the corresponding histogram (e).

A) Topography



B) Phase



3.3.4 Stress effect on membrane lipid composition

Plasma membrane lipid composition was measured to evaluate whether stress-induced physical and structural changes were due to changes in plasma membrane lipid composition. Quantitative thin layer chromatography was used to analyze the individual lipid classes in isolated hepatic membranes. The classes analyzed were phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), cholesterol (C), sphingomyelin (SM), free fatty acids (FFA), and the ratios of C/PL, PE/PC and PC+SM/PE+PS (Table 3-1). Overall, there was no significant change in lipid composition in response to acute stress; however metyrapone-treated trout had significantly higher levels of PE 30 min after acute stressor exposure compared to all other groups and lower levels of FFA compared to sham trout. Within sham trout, there was no change in the total levels of saturates, monounsaturates, polyunsaturates, unsaturates, and plasmalogens after stress compared to values prior to stress (Table 3-1).

Levels of saturated fatty acids (FA) significantly decreased in response to acute stress in metyrapone-treated trout but not sham trout, whereas levels of unsaturates, and in particular polyunsaturates, increased significantly in response to acute stress in the metyrapone-treated but not sham trout. Although there was no difference in overall saturation level within the sham, the levels of saturated and the saturated/polyunsaturated fatty acid level within PC were significantly higher 30 min post-stressor compared to pre-stressor values (Table 3-1). In comparison, there were no changes in FA type within PC for metyrapone-treated trout in response to acute stress; however, the levels of monounsaturates and plasmalogens were significantly lower whereas the levels of polyunsaturates were significantly higher in metyrapone group compared to sham trout. Within PE, there were no significant changes in fatty acid composition in response to acute stress or metyrapone treatment.

Table 3-1. Lipid components of liver plasma membrane

	Sham		Metyrapone	
Components	**Pre-stressor 0 min**	**Post-stressor 30 min**	**Pre-stressor 0 min**	**Post-stressor 30 min**
PC	37.98 ± 4.61[a]	41.92 ± 3.96[a]	39.69 ± 6.38[a]	46.96 ± 7.39[a]
PE	13.78 ± 0.41[a]	14.40 ± 0.68[a]	14.76 ± 0.75[a]	17.83 ± 1.30[b]
PS	13.44 ± 2.19[a]	9.91 ± 1.02[a]	12.19 ± 2.24[a]	9.55 ± 3.19[a]
SM	11.03 ± 1.57[a]	9.56 ± 1.32[a]	10.20 ± 1.75[a]	8.51 ± 2.98[a]
C	13.89 ± 0.7[a]	12.59 ±1.38[a]	13.29 ± 1.56[a]	12.63 ± 1.86[a]
FFA	12.07 ± 1.24[a]	11.46 ± 1.40[a]	9.84 ± 1.59[ab]	5.98 ± 1.43[b]
PC+SM/PE+PS	1.79 ± 0.27[a]	2.12 ± 0.14[a]	1.92 ± 0.30[a]	2.16 ± 0.37[a]
PE/PC	0.38 ± 0.03[a]	0.35 ± 0.01[a]	0.40 ± 0.04[a]	0.42 ± 0.10[a]
C/PL	0.18 ± 0.0[a]	0.16 ± 0.02[a]	0.17 ± 0.02[a]	0.15 ± 0.02[a]
Total fatty acid				
Saturates	36.99 ± 0.8[a]	35.00 ± 1.54[a]	41.45 ± 1.06[b]	34.43 ± 1.12[a]
Monounsaturates	23.71 ± 0.65[a]	23.17 ± 1.31[a]	23.49 ± 1.07[a]	21.08 ± 0.75[a]
Polyunsaturates	39.29 ± 1.0[b]	41.82 ± 1.60[ab]	35.05 ± 1.27[c]	44.47 ± 1.85[a]
Unsaturates	63.00 ± 0.81[a]	65.00 ± 1.54[a]	58.54 ± 1.06[b]	65.56 ± 1.12[a]
Plasmalogens	2.49 ± 0.52[a]	1.54 ± 0.26[a]	2.69 ± 0.31[a]	1.51 ± 0.37[a]
Saturates/polyunsaturates	0.94 ± 0.04[b]	0.84 ± 0.05[bc]	1.19 ± 0.06[a]	0.77 ± 0.05[c]
Saturates/unsaturates	0.58 ± 0.02[a]	0.54 ± 0.02[a]	0.71 ± 0.03[b]	0.52 ± 0.02[a]
PC fatty acid				
Saturates	37.19 ± 1.24[a]	42.45 ± 1.55[b]	44.43 ± 1.28[b]	45.07 ± 0.73[b]
Monounsaturates	58.05 ± 1.51[a]	50.90 ± 2.00[b]	45.09 ± 1.86[c]	45.12 ± 0.06[c]
Polyunsaturates	4.75 ± 0.26[a]	6.64 ± 0.68[a]	10.47 ± 1.27[b]	9.79 ± 0.33[b]
Unsaturates	62.68 ± 1.20[a]	57.01 ± 1.81[b]	53.37 ± 0.29[b]	53.64 ± 0.58[b]
Plasmalogens	50.59 ± 1.11[a]	42.98 ± 2.27[a]	33.05 ± 2.98[b]	32.47 ± 0.33[b]
Saturates/polyunsaturates	7.83 ± 0.18[a]	6.51 ± 0.64[a]	4.36 ± 0.54[b]	4.63 ± 0.42[b]
Saturates/unsaturates	0.59 ± 0.03[a]	0.74 ± 0.05[b]	0.83 ± 0.02[b]	0.84 ± 0.02[b]
PE fatty acid				
Saturates	37.15 ± 1.18[a]	39.45 ± 0.45[a]	39.48 ± 0.16[a]	33.26 ± 9.76[a]
Monounsaturates	57.77 ± 1.58[a]	54.20 ± 2.34[a]	56.00 ± 0.73[a]	62.03 ± 11.90[a]
Polyunsaturates	5.07 ± 1.48[a]	6.34 ± 1.89[a]	4.51 ± 0.90[a]	4.70 ± 2.14[a]
Unsaturates	62.85 ± 1.18[a]	60.54 ± 0.45[a]	60.52 ± 0.16[a]	66.59 ± 9.90[a]
Plasmalogens	50.88 ± 0.12[a]	45.09 ± 1.28[a]	43.86 ± 4.19[a]	59.54 ± 14.35[a]
Saturates/polyunsaturates	9.67 ± 4.01[a]	6.79 ± 1.95[a]	8.29 ± 1.35[a]	7.73 ± 1.45[a]
Saturates/unsaturates	0.59 ± 0.02[a]	0.65 ± 0.01[a]	0.65 ± 0.01[a]	0.53 ± 0.22[a]

Values (percent by weight) represent means ± standard errors. Means in the same row accompanied by different superscript letters *(e.g. a and b)* are significantly different between treatments at LSD = 0.05, n = 5 for all components except for PC and PE fatty acid components analysis where n= 3. PC = phosphatidylcholine, PE = phosphatidylethanolamine, PS = phosphatidylserine, SM=sphingomyelin, C= cholesterol, FFA=free fatty acids, PL=phospholipids, unsaturates=mono+polyunsaturates

3.3.5 Stress effect on protein phosphorylation

To investigate whether acute stressor exposure alters intracellular signaling, we examined the phosphorylation of putative PKA, PKC and AKT-substrate proteins. Our results demonstrate that there is a significant positive correlation between phosphorylation status of PKC (Fig. 3-4) and PKA (Fig. 3-5) but not AKT (Fig. 3-6) substrate proteins with plasma cortisol levels. In addition to these protein kinase pathways, the ratio of phosphorylated to total ERK (1/2) MAPK was also significantly correlated with plasma cortisol levels (Fig. 3-7).

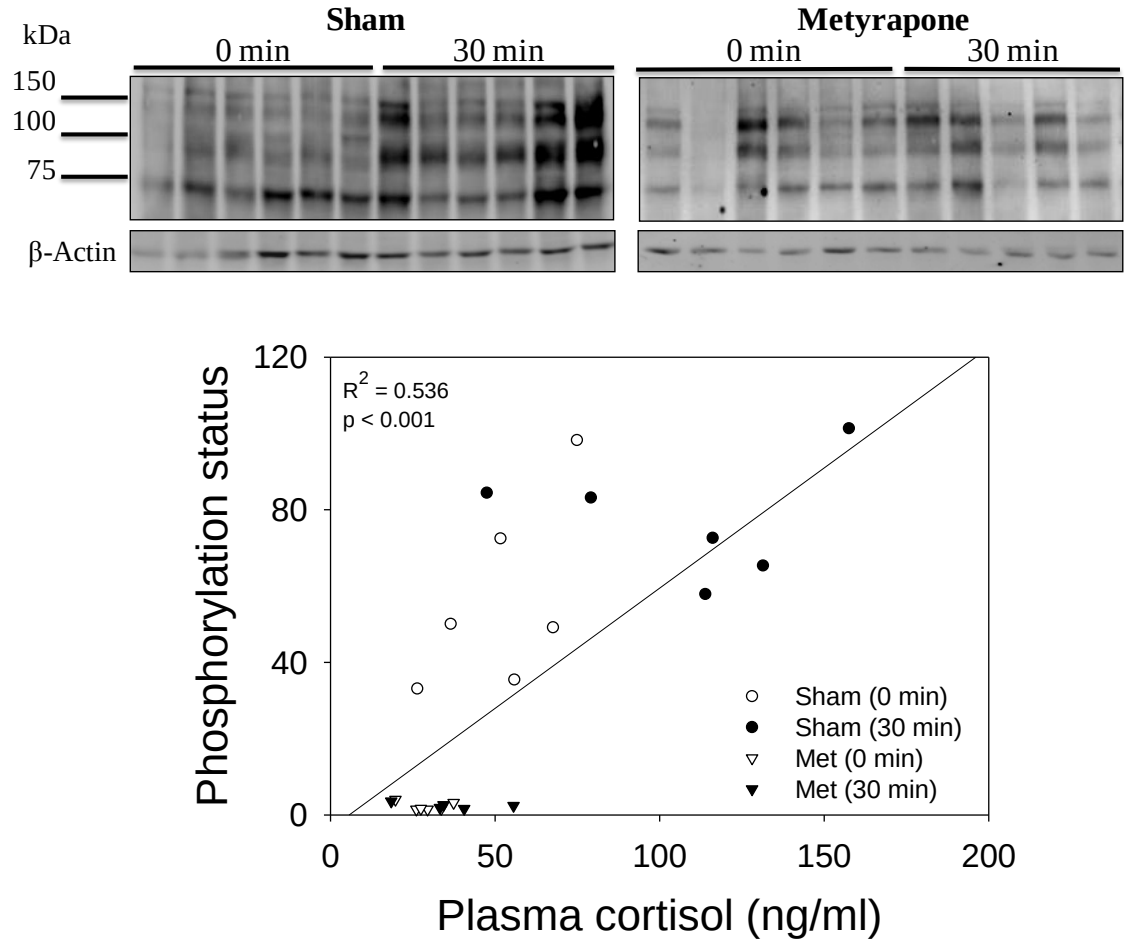


Figure 3-4. Effect of acute stress on hepatic protein phosphorylation of protein kinase C (PKC) substrate proteins.

Rainbow trout liver homogenates (40 µg protein) from sham and metyrapone-treated trout, before and after acute stress were probed with phospho-(Ser) PKC substrate polyclonal rabbit antibody (Cell Signaling Technology, Beverly, MA), which detects proteins which are phosphorylated within the phosphorylation motif for PKC. Protein loading was controlled for by normalizing to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometry values were obtained by quantifying the total phosphorylation intensity of each lane (region shown). The corresponding scatter plot of the plasma cortisol values against the total lane densitometry values is shown. There is a significant positive relationship between plasma cortisol values and PKC substrate phosphorylation ($R^2 = 0.607$, $p < 0.001$, $n = 23$).

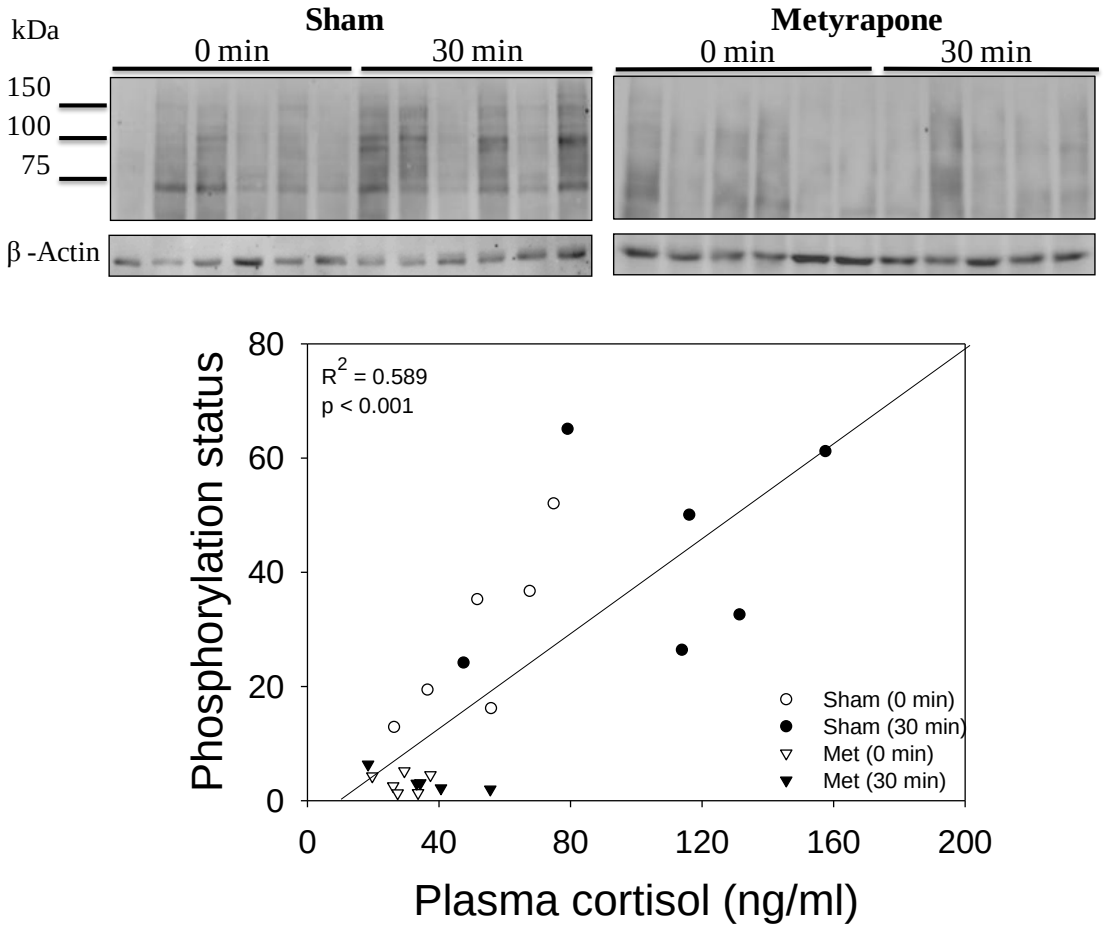


Figure 3-5. Effect of acute stress on hepatic protein phosphorylation of protein kinase A (PKA) substrate proteins.

Effect of acute stress on hepatic protein phosphorylation of protein kinase A (PKA) substrate proteins. Rainbow trout liver homogenates (40 µg protein) from sham and metyrapone-treated trout, before and after acute stress were probed with phospho-PKA Substrate (RRXS/T) polyclonal rabbit antibody (Cell Signaling Technology, Beverly, MA), which detects proteins that are phosphorylated within the phosphorylation motif for PKA. Protein loading was controlled for by normalizing to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometry values were obtained by quantifying the total phosphorylation intensity of each lane (region shown). The corresponding scatter plot of the plasma cortisol values against the total lane densitometry values is shown. There is a significant positive relationship between plasma cortisol values and PKA substrate phosphorylation ($R^2 = 0.615$, $p < 0.001$, $n = 23$).

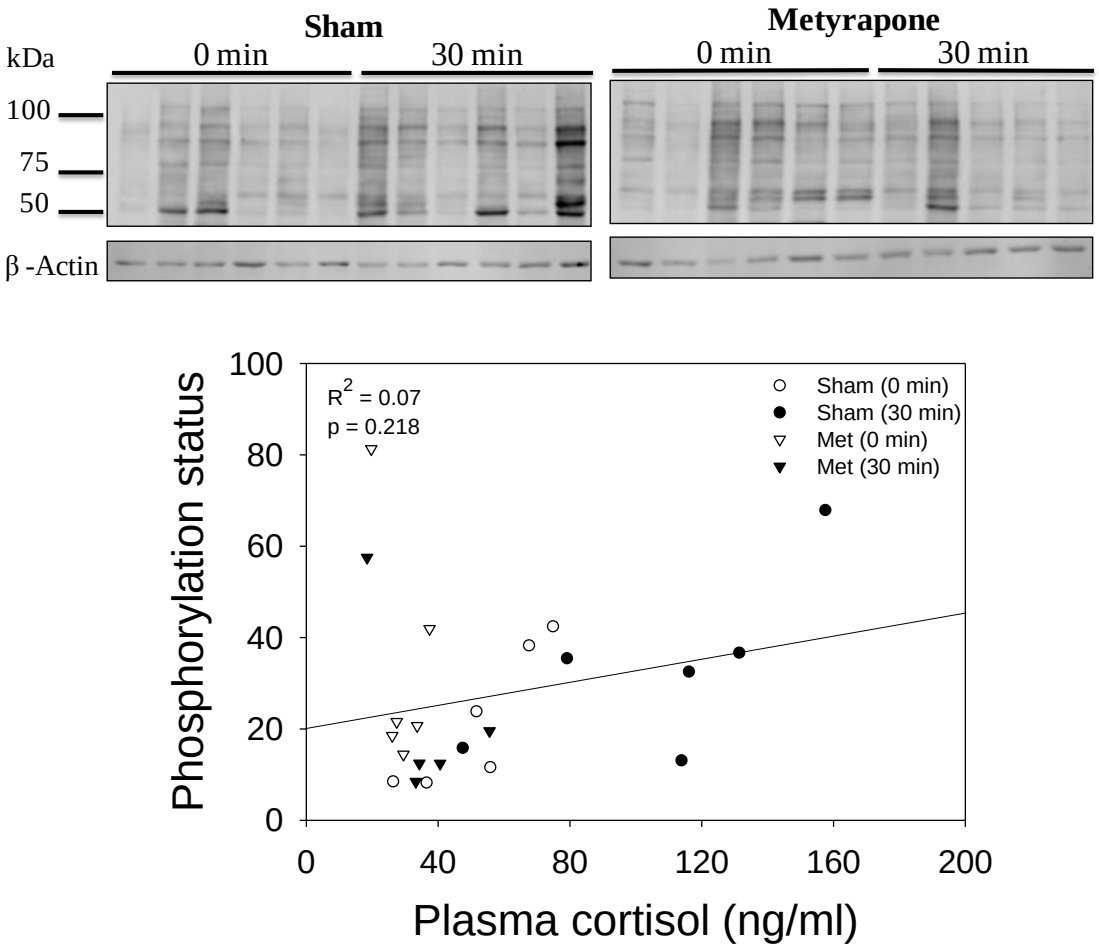


Figure 3-6. Effect of acute stress on hepatic protein phosphorylation of AKT substrate proteins.

Rainbow trout liver homogenates (40 µg protein) from sham and metyrapone-treated trout, before and after acute stress were probed with phospho-Akt substrate (RXXS/T) polyclonal rabbit antibody (Cell Signaling Technology, Beverly, MA), which detects proteins that are phosphorylated within the AKT phosphorylation motif. Protein loading was controlled for by normalizing to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometry values were obtained by quantifying the total phosphorylation intensity of each lane (region shown). The corresponding scatter plot of the plasma cortisol values against the total lane densitometry values is shown. There is no significant relationship between plasma cortisol values and AKT substrate phosphorylation ($p > 0.05$, $n = 23$).

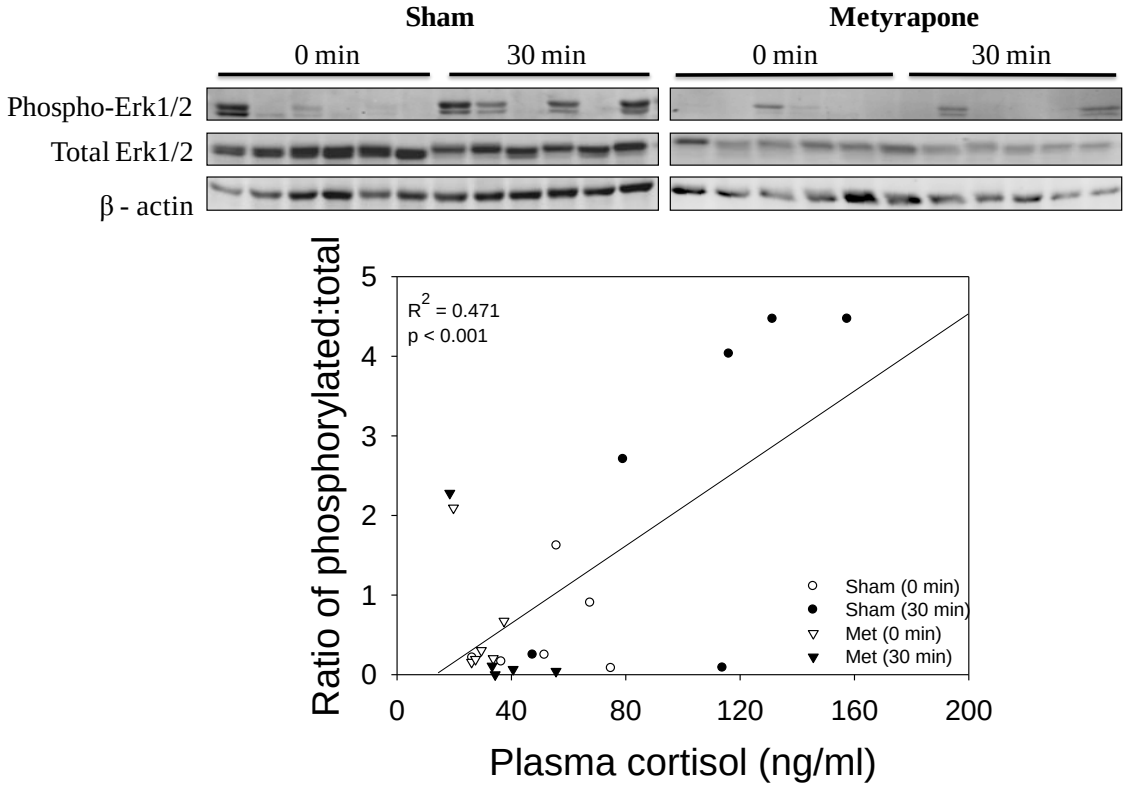


Figure 3-7. Activation of ERK(1/2) mitogen-activated protein kinase in response to acute stress.

Rainbow trout liver homogenates (40 µg protein) from sham and metyrapone-treated trout, before and after acute stress were probed with total or phospho-specific ERK(1/2).monoclonal rabbit antibody (Cell Signaling Technology, Beverly, MA). Protein loading was controlled for by normalizing to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). A scatter plot of the plasma cortisol values plotted against the ratio of phosphorylated ERK(1/2) to the total ERK(1/2), quantified by measuring the densitometry of the protein band intensity, is shown. There is a significant positive relationship between plasma cortisol values and activation of ERK(1/2) MAPK (ratio of densitometry values of phosphorylated to total ERK(1/2) ($R^2 = 0.338$, $p < 0.01$, $n = 23$).

3.4 Discussion

The novel finding from the present study is that stressor-induced plasma cortisol elevation alters the biophysical properties of plasma membranes in rainbow trout and evokes cellular stress responses. This is in agreement with our recent finding that cortisol treatment in vitro rapidly fluidizes liver plasma membrane, leading to nongenomic activation of stress-related signaling pathways in trout hepatocytes (Dindia et al., 2012). Most studies have focused on the genomic actions of cortisol in stress adaptation (Aluru and Vijayan, 2009), while this study highlights a novel nongenomic action of cortisol that is rapid and involves membrane structure alterations and modulation of stress-related signaling pathways in trout liver.

The plasma membrane physicochemical properties are highly sensitive to the surrounding physical and chemical environment, leading to the proposal of the membrane-mediated stress sensing model, which theorizes that the plasma membrane is the primary detector of stress stimuli and activator of the cellular stress response (Verstraeten et al., 2010). Subsequently, recent studies suggest that the plasma membrane is central in mediating the cellular stress response and aiding in cellular tolerance to a variety of stressors, including osmotic (Rodriguez-Vargas et al., 2006), temperature (Thomas et al., 2010) and oxidative stress (Berzosa et al., 2010). This study provides additional evidence for this model, as we show for the first time that a physical stressor rapidly fluidizes plasma membranes. More importantly, the stress-induced fluidization was abolished in trout injected with metyrapone, suggesting that the stress-induced elevation of plasma cortisol mediates the plasma membrane effect. Further confirmation arises from our recent finding that stress levels of cortisol *in vitro* increases liver plasma membrane fluidity in rainbow trout (Dindia et al., 2012). While studies have shown that longer-term glucocorticoid exposure has a membrane fluidizing effect *in vivo* (Kapitulnik et al., 1986), this is the first report of a rapid effect of endogenous cortisol on membrane order in any animal model.

Plasma membrane fluidization in response to stress in the present study was accompanied by membrane microdomain reorganization as detected with atomic force microscopy, including significantly higher membrane roughness (average difference in height between high and low domains). Microdomains within the plasma membrane are regions which differ in their lipid phase primarily due to their lipid and protein composition (Lingwood and Simons, 2010). These microdomains are commonly referred to as lipid rafts, which generally have a high percentage of

sphingolipid, cholesterol and membrane proteins (Lingwood and Simons, 2010). This stressor-induced microdomain reorganization likely has important functional consequences as lipid rafts play a critical role in influencing receptor activity and intracellular signaling (Lingwood and Simons, 2010; Nagy et al., 2007; Simons and Ikonen, 1997). The increase in plasma membrane roughness detected in response to stressor exposure, associated with microdomain reorganization, may play a role in activating the liver cellular response in trout. As cortisol treatment *in vitro* also increases plasma membrane roughness (Dindia et al., 2012), our results suggest that stressor-mediated elevation of plasma steroid hormone levels facilitate domain reorganization in rainbow trout.

Membrane order changes may aid in the hepatic response to acute stress by regulating cellular processes important for stress adaptation. For instance, studies have shown that glucose transport systems are sensitive to the surrounding lipid environment (Melchior and Czech, 1979) and are markedly affected by temperature-induced changes in membrane fluidity (Pilch et al., 1980). In addition to metabolite transport, alterations to the plasma membrane can directly activate several stress-signaling pathways (Lasserre et al., 2008; Raghavendra et al., 2007; Slater et al., 1994) leading to cellular stress response. Also, stress-mediated alterations in membrane biophysical properties may have important permissive or suppressive effects on other hormonal cell signaling pathways (Makara and Haller, 2001). Altogether, our results suggest that stressor exposure, and the associated elevation of plasma cortisol level rapidly alters membrane order and this may modulate cellular stress response in rainbow trout.

Plasma membrane properties, such as fluidity and domain structure are highly dependent on membrane composition (Van Meer et al., 2008). However, there were no stress-induced changes in the proportion of each lipid component, except the FFA composition within PC phospholipids. Although significant, the increase in saturation and decrease in monounsaturates was minor (5 and 8%, respectively), and only occurred within PC class. Therefore, it is unlikely that the minor shift in saturation level affected the fluidity of the bulk plasma membrane fraction. Metyrapone by itself affected PC fatty acid composition and, therefore, was not a useful tool for identifying cortisol-mediated effects on membrane composition in trout.

The higher levels of saturated fatty acids and decreased monounsaturates in response to acute stress would imply a reduction in membrane fluidity, but that was not the case in the present study. This suggests that the stressor-mediated membrane fluidization seen in the present study was independent of changes in lipid composition and related to alteration in biophysical properties. We

recently demonstrated that stress levels of cortisol, but not a membrane impermeable form of this steroid (cortisol bound to a peptide moiety), was able to fluidize trout liver plasma membrane in vitro (Dindia et al., 2012). While the mechanism remains unknown, this clearly points to membrane order changes associated with incorporation of the stress steroid within the lipid bilayer, at levels above a certain threshold ($\geq$ 100 ng/ml). In the present study, the stressor-mediated membrane fluidity changes corresponded with plasma cortisol levels > 100 ng/ml, leading us to propose that the rapid membrane order changes observed may be due to cortisol incorporation into the lipid bilayer.

In addition to modulating physicochemical plasma membrane properties, our results provide evidence that stressor-induced cortisol elevation activates intracellular stress-related signaling cascades in the liver. Plasma cortisol levels, which were higher in stressed animals, positively correlated with higher phosphorylation of putative PKC and PKA but not AKT substrate proteins. Furthermore, activation of ERK(1/2) MAPK (ratio of phosphorylated to total) was also positively correlated with elevated plasma cortisol levels. This supports our recent finding *in vitro* showing that stress levels of cortisol rapidly phosphorylates PKC and PKA substrate proteins, further supporting a nongenomic role for cortisol in the liver response to acute stress.

Although the mechanism of PKC, PKA and MAPK activation in trout hepatocytes is not known, we hypothesize that cortisol-mediated biophysical changes to the plasma membrane may be playing a role in this nongenomic signaling event. Perturbations to the plasma membrane biophysical properties have been shown to regulate PKC, PKA, AKT and MAPK signaling (Butler et al., 2002; Lei et al., 2009; Sangwan et al., 2002). Furthermore, benzyl alcohol, which mediates its effects at the level of the plasma membrane (Nagy et al., 2007), also activated these signaling pathways in trout hepatocytes (Dindia et al., 2012), suggesting that these signaling pathways are sensitive to membrane perturbations in trout liver. However, we cannot rule out the possibility that the cell signaling response seen in the present study may also be due to activation of GR or other membrane receptors. For instance, PKC and MAPK pathways modulation is thought to play a role in the central nongenomic GR-dependent inhibition of ACTH secretion (Solito et al., 2003). Also, dexamethasone (a synthetic glucocorticoid) modulated the T cell phosphorylation profile in a GR-dependent manner (Boldizsar et al., 2010) suggesting a role for this receptor in rapid cellular action. Similarly in T lymphocytes and adipocytes, glucocorticoid-mediated inhibition of insulin signaling involves rapid GR-dependent down regulation of several insulin-related kinases (Löwenberg et al., 2006). However, glucocorticoid-mediated PKC, PKA and ERK(1/2) phosphorylation have also been reported in the

absence of GR activation in the hippocampus of mammalian species (reviewed in (Groeneweg et al., 2012)), suggesting multiple modes of nongenomic signaling by this stress steroid. Although rapid nongenomic signaling by glucocorticoid have been reported in teleosts (Borski et al., 2002), a role for nongenomic signaling by GR has not been established (Aluru and Vijayan, 2009). Together, the activation of phosphorylation of putative PKC and PKA substrate proteins and ERK(1/2) MAPK, and its association with elevated plasma cortisol levels suggest a novel nongenomic role for cortisol in liver stress response, while the mechanisms involved remains to be elucidated. We propose that modulation of membrane biophysical properties by stress levels of cortisol in trout may be a novel mechanism leading to modulation of cell signaling pathways.

In conclusion, the structural changes to the plasma membrane in response to stressor exposure provide evidence for a novel plasma membrane-mediated mechanism of stress adaptation in hepatic tissue. Importantly, our results indicate that the rapid stress-induced membrane perturbations are likely mediated by cortisol, underscoring a nongenomic stress signaling by this steroid during stress adaptation. Plasma cortisol elevation was also correlated with activation of stress-associated protein kinases further supporting a key role for nongenomic cortisol signaling in cellular stress adaptation. Thus, in addition to GR-activated effects, our results suggest that stressor-mediated plasma cortisol elevation also rapidly activate nongenomic stress signaling pathways by altering the membrane biophysical properties. We propose that this nonspecific stress response, mediated by cortisol incorporation into the lipid bilayers and modulation of stress signaling pathways, plays an important role in acute stress adaptation in animals.

3.5 Acknowledgements

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Chapter 4 **Rapid action of cortisol on hepatic signaling pathways: involvement of PKA, PKC and MAPK**

Dindia, L and Vijayan, M.M. Rapid action of cortisol on hepatic signaling pathways: involvement of PKA, PKC and MAPK. Prepared for scientific journal submission.

4.1.1 Introduction

Glucocorticoids (GC), are important regulators of metabolism, immune responses and development, mediate many of its effects *via* the glucocorticoid receptor (GR (Stahn and Buttgereit, 2008)). The cytosolic GR is a ligand-dependent transcription factor, which regulates transcription by binding to the glucocorticoid response element in the promoter region of target genes (Stahn and Buttgereit, 2008). In addition to the classical genomic pathway, GC, like other steroid hormones are also capable of eliciting rapid cellular effects within seconds to minutes of steroid exposure (Lösel and Wehling, 2003). Along with being rapid, the cellular responses are independent of gene transcription and, therefore, referred to as nongenomic effects. The reported nongenomic effects of GC, and their mechanism of action, are extremely diverse and have proven to be tissue- and context-dependent (Groeneweg et al., 2012).

The liver is critical for mediating the metabolic changes that support stress adaptation, and the genomic signaling by GC play a key role in this process (Hoene and Weigert, 2010; Mommsen et al., 1999). For instance, in mammalian (Oishi et al., 2005) and non-mammalian vertebrates (Aluru and Vijayan, 2009), GC alters the expression of key metabolic enzymes, including those involved in gluconeogenesis (Yabaluri and Bashyam, 2010). While very little is known about the rapid GC effects in the liver, studies have demonstrated nongenomic GC signaling events in the brain and pituitary (Borski, 2000, Andrews et al., 2012). Peripherally, GC have been shown to rapidly modulate catecholamine secretion from chromaffin cells (Park et al., 2008). The precise mechanisms mediating the rapid GC effects are not completely understood. However, the protein kinase C (PKC), protein kinase A (PKA), protein kinase B/AKT and mitogen-activated protein kinase pathways are commonly implicated in nongenomic steroid signaling (Dindia et al., 2012; Tasker et al., 2006).

In teleosts, cortisol is the predominant circulating corticosteroid and the majority of studies on the action of this hormone have focused on genomic signaling (Aluru and Vijayan, 2009). However, modulation of cyclic AMP (cAMP) signaling has been shown to be a key component of rapid cortisol inhibition of pituitary prolactin release in the tilapia, *Oreochromis mossambicus* (Borski et al., 2002). In the tilapia, cortisol has also been shown to rapidly reduce activates of hepatic lipogenic enzymes (Sunny et al., 2002) and increase branchial Na^+/K^+-ATPase and Ca^{2+}-ATPase (Sunny and Oommen, 2001) independent of protein synthesis. Also, cAMP-PKA pathway has been implicated in the rapid inhibition of cortisol on phagocytosis in the freshwater catfish (*Channa punctatus*; Roy and Rai, 2009). In rainbow trout (*Oncorhynchus mykiss*) cortisol rapidly (10 min)

induces coronary vasoconstriction, whereas 17-β-estradiol causes significant dilation (Agnisola et al., 2004), while the mechanism mediating these rapid coronary effects are currently unknown.

In mammalian studies, the PKC pathway appears to be an important mediator of rapid GC action, particularly in the brain (Di et al., 2003; Qiu et al., 2003; Zhang et al., 2012), whereas the phosphatidylinositol 3-kinase (PI3K)/protein kinase Akt pathway is a prominent mediator of nongenomic GC action in the cardiovascular system (Lee et al., 2012). Also, MAPKs (particularly p38 and ERK1/2), which act on numerous transcription factors, appear to be significant players regulating rapid GC effects in central and peripheral tissues. For instance, like PKA and PKC, MAPK pathways are involved in the GC inhibition of adrenocorticotropic hormone (ACTH) secretion from the pituitary (Solito et al., 2003) and recent research suggests that MAPK pathways play a notable role in the rapid immunosuppressive effect of GCs (Ayroldi et al., 2012).

We recently demonstrated for the first time that cortisol treatment *in vitro* rapidly fluidizes liver plasma membrane of rainbow trout (see Chapter 2; Dindia et al., 2012). Also, stress-mediated cortisol elevation *in vivo* altered liver plasma membrane order in trout supporting a rapid effect of this steroid in stress adaptation (see Chapter 3; Dindia et al., in review). Furthermore, we provided evidence that phosphorylation of substrate proteins by PKA and PKC occurred in parallel to plasma membrane alterations in response to endogenous cortisol elevation (see Chapter 3; Dindia et al., in review). Therefore the aim of the current study was to further confirm a role for cortisol in the rapid induction of PKA, PKC, AKT and MAPK signaling pathways. To determine whether the rapid cortisol effects were GR independent, studies were conducted using the GR antagonist, mifepristone (also known as RU486). An attempt was also made to examine the possibility of membrane-receptor mediated by using a membrane impermeable peptide-cortisol conjugate (PEP-C). Unfortunately the unconjugated peptide activated cell signaling pathways in liver and, therefore, could not be used to elucidate membrane-mediated effects (see Appendix B). The effect of benzyl alcohol, a membrane fluidizer, was also investigated to evaluate the possibility that the rapid effects due to cortisol may involve changes in plasma membrane fluidity in trout hepatocytes.

4.2 Material and Methods

4.2.1 Animals & sampling

Juvenile rainbow trout (100-300 g), purchased from Alma Aquaculture Research Station (Alma, Ontario), were maintained at the University of Waterloo aquatic facility exactly as described before

(Sandhu and Vijayan, 2011). The tanks were supplied with a constant flow of aerated well water (12 ± 2°C) and were maintained under a 12hL:12hD photoperiod. Trout were acclimated for at least two weeks prior to experiments and were fed commercial trout feed (Martin Mills, Elmira, Ontario) to satiety once daily, 5 days a week. Experiments were approved by the University of Waterloo Animal Care Protocol Review Committee and adhere to guidelines established by the Canadian Council on Animal Care for the use of animals in teaching and research.

4.2.2 Liver tissue slices

Trout liver slices were utilized to mimic the intracellular phosphorylation events in response to cortisol in the intact liver. Rainbow trout were sampled as mentioned above. Livers were quickly excised and washed in ice cold modified Hank's buffer (110 mM NaCl, 3 mM KCl, 1.25 mM K_2HPO_4, 5 mM $NaHCO_3$, 0.6 mM $MgSO_4$, 1 mM $MgCl_2$ and 10 mM Hepes; 1.5 mM $CaCl_2$, 5 mM glucose; pH 7.63 at room temperature). Livers were then sliced into 8-10 mm pieces (500 µm maximum width) using a MD-1100 tissue slicer (Munford, USA), washed three times with modified Hank's buffer containing 1.5mM $CaCl_2$ and were placed in six-well tissue culture plates (approximately 50 mg of tissue/well) with L15 media. Liver slices were maintained at 13°C and were gently rocked during the incubation. Media was changed after an hour after slicing and tissue was incubated for an additional hour prior to treatment. Liver slices were then treated with either hydrocortisone (0, 100, or 1000 ng/ml), Mifepristone (RU486; 1000 ng/ml), or a combination of cortisol (1000 ng/ml) and Mifepristone. In the combination group Mifepristone was added 30 min prior to the addition of cortisol. Treatments were stopped at the indicated time (10 or 30 min post-treatment) by washing the liver slices in modified Hank's buffer containing 1.5 mM $CaCl_2$ and were immediately frozen on dry ice and stored at -70°C.

4.2.3 Isolated trout hepatocytes

The rapidly signaling events associated with cortisol treatment were also examined in primary hepatocytes, which are the parenchyma cells of the liver. Rainbow trout hepatocytes were isolated using *in situ* collagenase perfusion and maintained exactly as described previously (Dindia et al., 2012). Trypan blue dye exclusion method was used to determine hepatocyte viability and >95% cells were viable. Isolated cells in suspension were used to measure cAMP production exactly as described previously (Ings et al., 2011). Briefly, cells were thoroughly suspended (20 mg of cells/ml) in modified Hank's buffer containing 1.5 mM $CaCl_2$ and were transferred in 500 µl aliquots to 1.5 mL

centrifuge tubes. Cells were maintained in suspension by gentle rocking at 13°C in the dark. Cells were treated exactly as mentioned above. Each treatment was quickly terminated at the appropriate time interval (10 and 30 min) with the addition of perchloric acid (2% final concentration). The supernatant was collected for cAMP measurements after centrifugation at 10 000 X g for 2 min. cAMP levels were measured using a commercially available competitive ELISA kit. Procedures were carried out exactly as outlined in the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, Michigan).

For protein phosphorylation experiments, hepatocytes were suspended in L-15 (sigma, St. Louis, MO) medium and plated in six-well tissue culture plates (Sarstedt, Inc., Newton, NC) at a density of 1.5 million cells/well (0.75 million cells/ml). Primary cell culture was established over a 24 h incubation period at 13°C. The L-15 media was then replaced and the cells were allowed to recover for an additional 2 h before the start of experiments. Hepatocytes were then treated as described above. The reaction was stopped by replacing L-15 media with 100 µl ice cold lysis buffer (50 mM Tris, 0.25 M sucrose, 1 % SDS, 10 mM NaF, 5 mM EDTA, 5 mM NEM, 0.1 % Nonidet-P40). Lysed cells were quickly heated at 95°C for 5 min followed by brief sonication (sonic dismembrator, Fisher Scientific) and centrifuged at 10 000 X g for 5 min. The supernatant was collected and stored at -80°C.

4.2.4 Immunoblotting

Liver tissue was homogenized and protein concentration was measured using the bicinchoninic acid (BCA) method using bovine serum albumin as the standard. All samples were diluted in Laemmli's sample buffer (1M tris-HCl, pH 6.8, 60 mM, glycerol 25%, SDS 2%, β-mercaptoethanol 14.4 mM, bromophenol blue 0.1%) and the immunodetection carried out exactly as described before (Dindia et al., 2012). Briefly, total protein (40 µg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane and blocked with 5% solution of non-fat dry milk in 1 X TTBS (2 mM Tris, 30 mM NaCl, 0.01% Tween, pH 7.5) for 1 h at room temperature. This was followed with an overnight incubation with either phospho-ERK(1/2) (Thr202/Tyr204), phosphor-p38 MAPK (Thr180/Tyr182), phospho-(Ser) PKC substrate, phospho-Akt substrate (RXXS/T) or phospho-PKA Substrate (RRXS/T) polyclonal rabbit antibodies or monoclonal rabbit phospho-cAMP regulatory element binding protein (CREB) (ser133) and total CREB (Cell Signaling Technology, Beverly, MA). All antibodies were diluted 1:1000 in 5% BSA in TTBS. Blots were incubated for 1 h at room temperature with anti-rabbit horseradish peroxidase (HRP)-labeled

secondary antibody (Bio-rad; 1:3300 dilutions in 5% skim milk in TTBS). Protein bands were detected with ECL Plus™ chemiluminescence (GE Health Care, Baie d'Urfe, QC) and imaged using either the Typhoon 9400 (Amersham Biosciences) or the Pharos FX Molecular Imager (Bio-rad). Total lane protein band intensity was quantified using AlphaImager HP™ (Alpha Innotech, CA) and values were normalized to a standard trout liver sample run on each blot for inter-blot comparisons. Each immunoblot was subsequently probed with Cy3™ conjugated monoclonal mouse β-actin antibody (Sigma, Cy3™ conjugated monoclonal mouse; 1:1000) for 1 h at room temperature. Densitometric values were then normalized to β-actin to control for protein loading differences between samples.

4.2.5 Statistical analysis

A two-way repeated analysis of variance was used to compare time and treatment effects. A least significant differences (LSD) *post hoc* test was used to determine within factor effects. Statistics were performed either on raw or log transformed data (when necessary to meet normality and equal variance assumptions). A probability level of $p < 0.05$ was considered significant. All statistical analyses were performed using SigmaPlot 11 software (Systat Software Inc., San Jose, CA, USA).

4.3 Results

4.3.1 cAMP levels

Cortisol rapidly and transiently altered cAMP levels in trout hepatocytes (Fig. 4-1A). Stress levels of cortisol rapidly (5 min) induced cAMP levels, which returned to control values within 15 min. High cortisol levels (1000 $ng.ml^{-1}$) also rapidly increased cAMP values within 5 min of treatment, which was followed by a significant inhibition at 15 min. To address whether cortisol-induced cAMP regulation is receptor mediated, suspended hepatocytes were also treated with mifepristone (RU486 - GR antagonist; Fig.4-1B). Mifepristone by itself and when treated with cortisol increased cAMP production at 1 and 5 min (31 and 35 min, respectively, after mifepristone addition), but had no effect at 15 min (45 min after mifepristone addition).

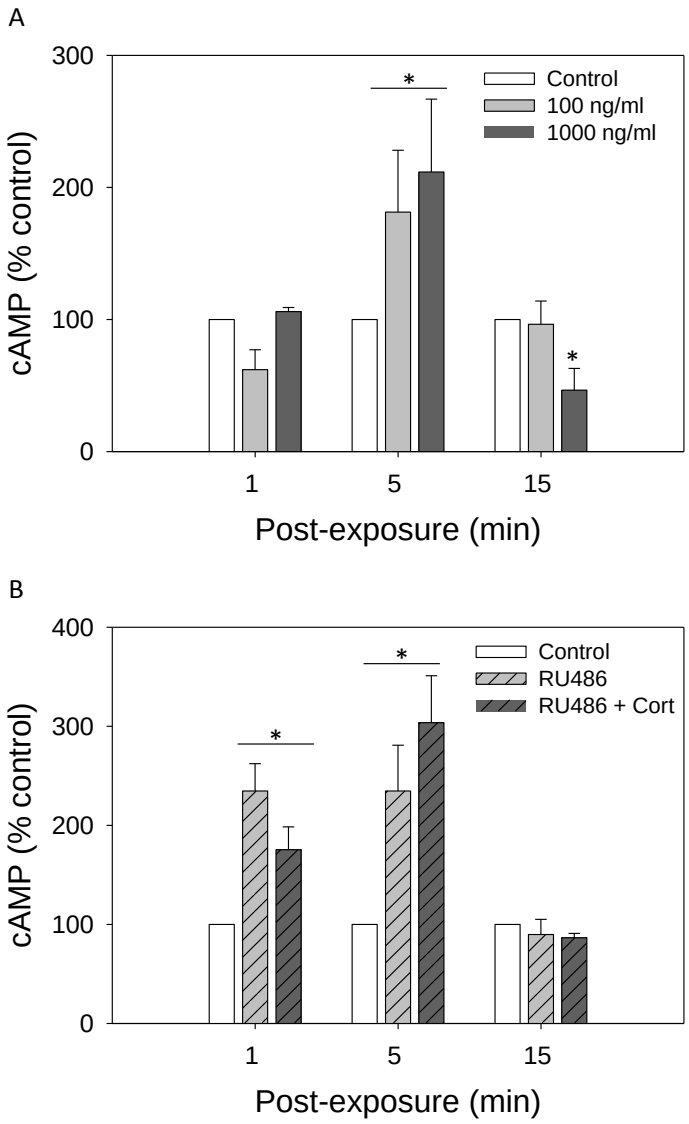


Figure 4-1. Rapid effect of cortisol on cAMP accumulation in trout hepatocytes.

(A) The dose and time response of cAMP levels in response to cortisol treatment (100 and 1000 ng/ml) and (B) Mifepristone (1000 ng/ml) alone and in combination with cortisol treatment (1000 ng/ml). Values are plotted as % cortisol and show mean ± S.E.M (n = 4 independent fish); bars with different symbols are significantly different within that time point (repeated measures ANOVA, $p < 0.05$).

4.3.2 Phosphorylation by PKA, PKC and AKT

Transient cortisol induction of cAMP accumulation was accompanied by a rapid increase in the phosphorylation status of PKA substrate proteins in trout liver slices, which was dose and time-dependent (Fig. 4-2A). Specifically, treatment with stress levels of cortisol (100 and 1000 ng/ml) resulted in a rapid and transient rise in phosphorylated PKA substrate proteins by approximately two- and three-fold, respectively. A similar transient rise in phosphorylation within 10 min of cortisol treatment was also observed in trout hepatocytes (Fig. 4-2A inset), although the magnitude of the response was blunted compared to the effect in liver tissue. In hepatocytes, activation of CREB, a key substrate for PKA, was phosphorylated 10 min after cortisol treatment, particularly in response to high levels of cortisol, which was maintained even at 30 min post-exposure (Fig. 4-3A). Treatment with mifepristone had no effect on CREB activation, and successfully blocked cortisol-mediated activation (Fig. 4-3A and B).

Cortisol significantly increased PKC substrate protein phosphorylation levels in both liver slices (Fig.4-4) and hepatocytes (Fig.4-4 inset). However the effect was transient in trout hepatocytes (only occurred 10 min after steroid treatment), while it was prolonged (significant at 10 and 30 min) in liver slices. In slices and cultured hepatocytes, both concentrations of cortisol significantly increased PKC substrate protein phosphorylation.

A rapid increase in AKT substrate protein phosphorylation was also detected in response to cortisol treatment (100 and 1000 ng/ml); however, this effect was only significant in trout hepatocytes (Fig. 4-5 inset) and not in liver slices (Fig. 4-5).

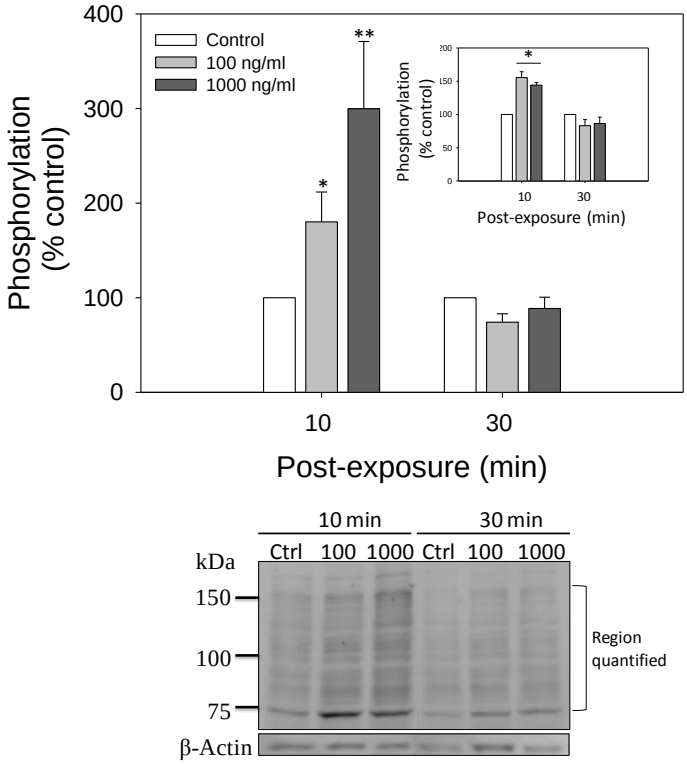


Figure 4-2. Rapid effect of cortisol on phosphorylation status of protein kinase A (PKA) substrate proteins.

Rapid effect of cortisol on phosphorylation status of protein kinase A (PKA) substrate proteins. Histogram and corresponding representative immunoblot of the time and dose response of PKA substrate protein phosphorylation in rainbow trout liver slices and hepatocyte culture (inset, modified from Dindia et al., 2012) following cortisol (100 and 1000 ng/ml) treatment. Liver samples (40 µg of protein) were probed with phospho-PKA Substrate (RRXS/T) polyclonal rabbit antibody (Cell Signaling Technology, Beverly, MA), which detects proteins that are phosphorylated within the phosphorylation motif for PKA. Protein loading was controlled for by normalizing to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometry values were obtained by quantifying the total phosphorylation intensity of each lane (region shown). Values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); bars with different symbols are significantly different within the time point (repeated measures ANOVA, $p < 0.05$).

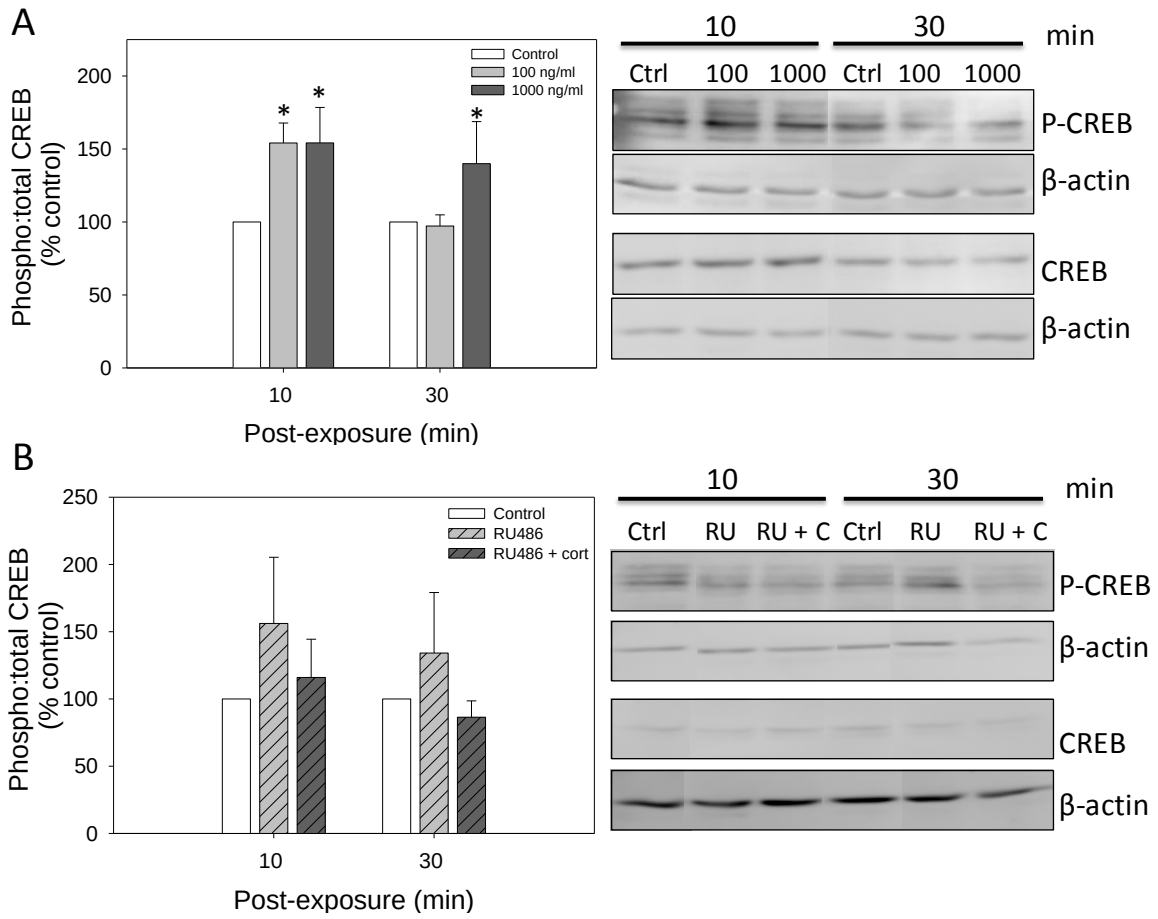


Figure 4-3. Rapid activation of CREB in response to cortisol treatment.

Histogram and corresponding representative immunoblot of the time and dose response of CREB activation in rainbow trout primary hepatocytes following treatment with (A) cortisol (100 and 1000 ng/ml) (B) mifepristone (RU486; 1000 ng/ml), mifepristone in combination with cortisol treatment (1000 ng/ml). Cell lysates (40 µg of protein) were probed with phospho-CREB (ser133) or total CREB monoclonal rabbit antibody (Cell Signaling Technology, Beverly, MA). Protein loading was controlled for by normalizing to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Values plotted are the ratio of the phosphorylated to total CREB densitometric values. Values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); *indicates treatment is significantly different compared to the control (repeated measures ANOVA, $p < 0.05$).

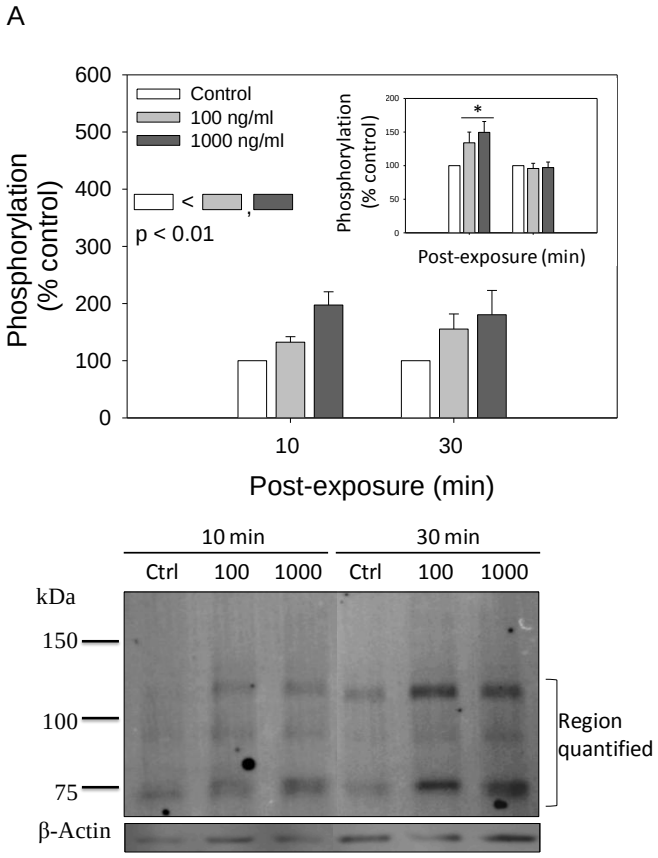


Figure 4-4. Phosphorylation status of protein kinase C (PKC) substrate proteins.

Histogram and corresponding representative immunoblot of the time and dose response of PKC substrate protein phosphorylation in rainbow trout liver slices and hepatocyte culture (inset, modified from Dindia et al., 2012) following cortisol (100 and 1000 ng/ml) treatment. Liver samples (40 µg of protein) were probed with phospho-(Ser) PKC substrate polyclonal rabbit antibody (Cell Signaling Technology, Beverly, MA), which detects proteins which are phosphorylated within the phosphorylation motif for PKC. Protein loading was controlled for by normalizing to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometry values were obtained by quantifying the total phosphorylation intensity of each lane (region shown). Values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); inset indicates significant treatment effect (repeated measures ANOVA, $p < 0.05$).

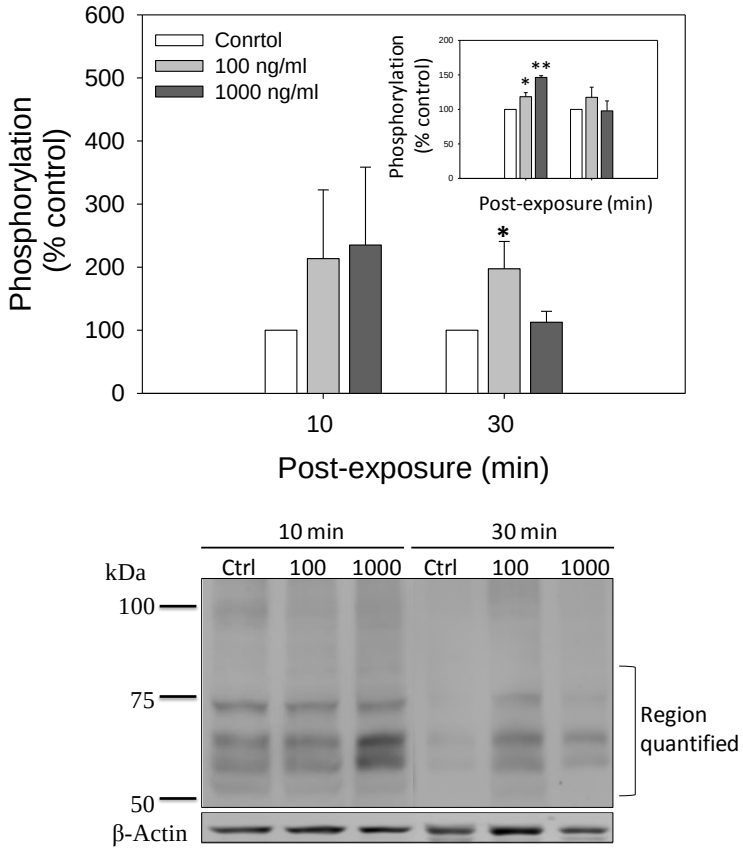


Figure 4-5. Phosphorylation status of AKT substrate proteins.

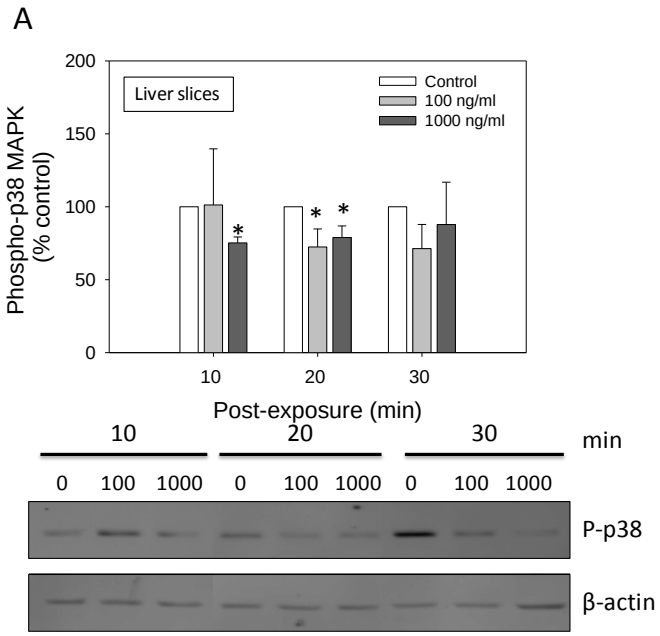
Histogram and corresponding representative immunoblot of the time and dose response of AKT substrate protein phosphorylation in rainbow trout liver slices and hepatocyte culture (inset, modified from Dindia et al., 2012) following cortisol (100 and 1000 ng/ml) treatment. Liver samples (40 µg of protein) were probed with phospho-Akt substrate (RXXS/T) polyclonal rabbit antibody (Cell Signaling Technology, Beverly, MA), which detects proteins that are phosphorylated within the AKT phosphorylation motif. Protein loading was controlled for by normalizing to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometry values were obtained by quantifying the total phosphorylation intensity of each lane (region shown). Values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); bars with different symbols are significantly different within the time point (repeated measures ANOVA, $p < 0.05$).

4.3.3 P38 MAPK and ERK (1/2) phosphorylation

Overall, cortisol treatment resulted in a rapid dephosphorylation of p38 MAPK in tissue slices (Fig. 4-6A and B) and decreased phosphorylation levels in cultured cells ($P < 0.07$ Fig.4-6C) within 30 min. Cortisol did not significantly alter the phosphorylation status of ERK (1/2) (Fig. 4-7A and B).

Figure 4-6. Regulation of p38 mitogen-activated protein kinase (MAPK) phosphorylation in trout liver slices and hepatocyte culture in response to cortisol.

Histogram and corresponding representative immunoblot of the time and dose response of p38 MAPK phosphorylation following treatment with cortisol (100 and 1000 ng/ml) in liver slices (A) or primary hepatocytes (B). Liver slices or hepatocytes cell homogenates (40 µg protein) were probed with phospho-specific p38 MAPK monoclonal rabbit antibody (Cell Signaling Technology, Beverly, MA). Protein loading was controlled for by normalizing to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometric values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); bars with different symbols are significantly different within the time point (repeated measures ANOVA, $p < 0.05$).


A
Liver slices
Control
100 ng/ml
1000 ng/ml
Phospho-p38 MAPK
(% control)
200
150
100
50
0
10
20
30
Post-exposure (min)
10
20
30
min
0 100 1000 0 100 1000 0 100 1000
P-p38
β-actin

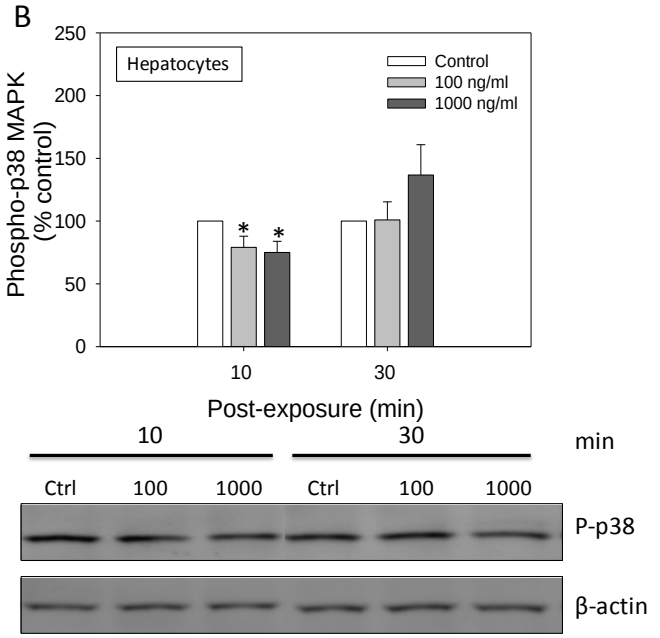
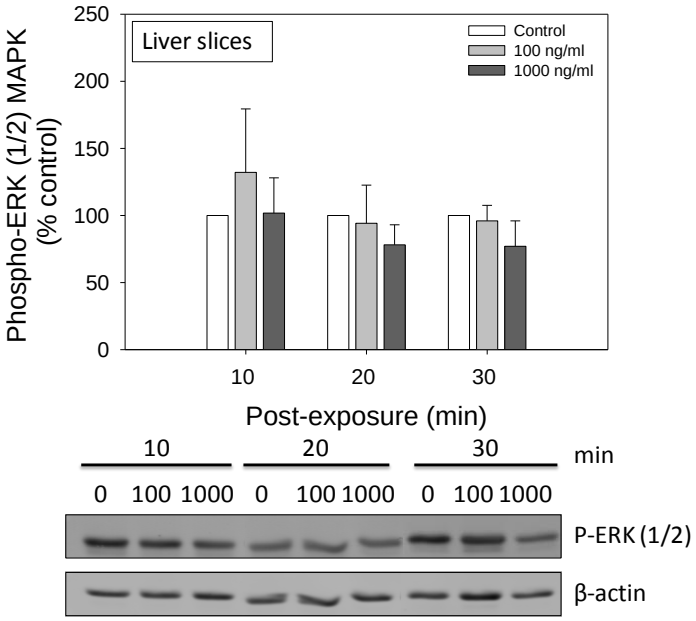

B
Hepatocytes
Control
100 ng/ml
1000 ng/ml
Phospho-p38 MAPK
(% control)
250
200
150
100
50
0
10
30
Post-exposure (min)
10
30
min
Ctrl 100 1000 Ctrl 100 1000
P-p38
β-actin

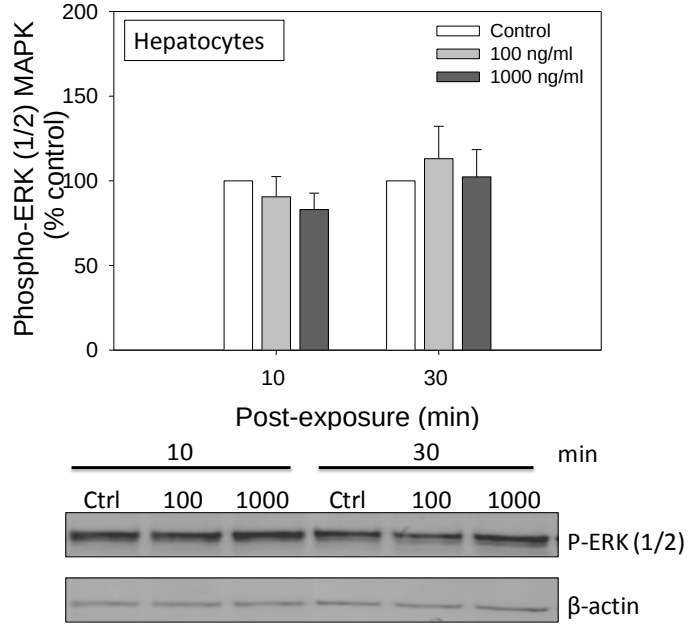
Figure 4-7. ERK (1/2) mitogen-activated protein kinase (MAPK) phosphorylation in trout liver slices and hepatocyte culture in response to cortisol.

(A) Histogram and corresponding representative immunoblot of the time and dose response of ERK (1/2) MAPK phosphorylation following treatment with cortisol (100 and 1000 ng/ml) in rainbow trout liver slices (A) or primary hepatocytes (B). Liver slices or hepatocytes cell homogenates (40 µg protein) were probed with phospho-specific ERK(1/2) MAPK monoclonal rabbit antibody (Cell Signaling Technology, Beverly, MA). Protein loading was controlled for by normalizing to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometric values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); bars with different symbols are significantly different within the time point (repeated measures ANOVA, $p < 0.05$).

A


Liver slices
Control
100 ng/ml
1000 ng/ml
Phospho-ERK (1/2) MAPK (% control)
250
200
150
100
50
0
10
20
30
Post-exposure (min)
10
20
30
min
0 100 1000 0 100 1000 0 100 1000
P-ERK (1/2)
β-actin

B

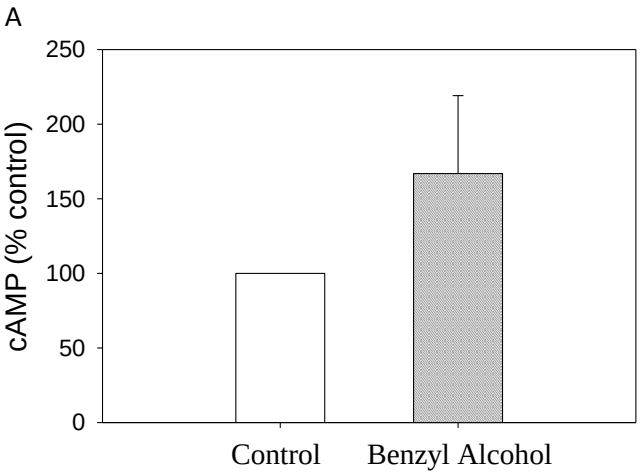

Hepatocytes
Control
100 ng/ml
1000 ng/ml
Phospho-ERK (1/2) MAPK (% control)
200
150
100
50
0
10
30
Post-exposure (min)
10
30
min
Ctrl 100 1000 Ctrl 100 1000
P-ERK (1/2)
β-actin

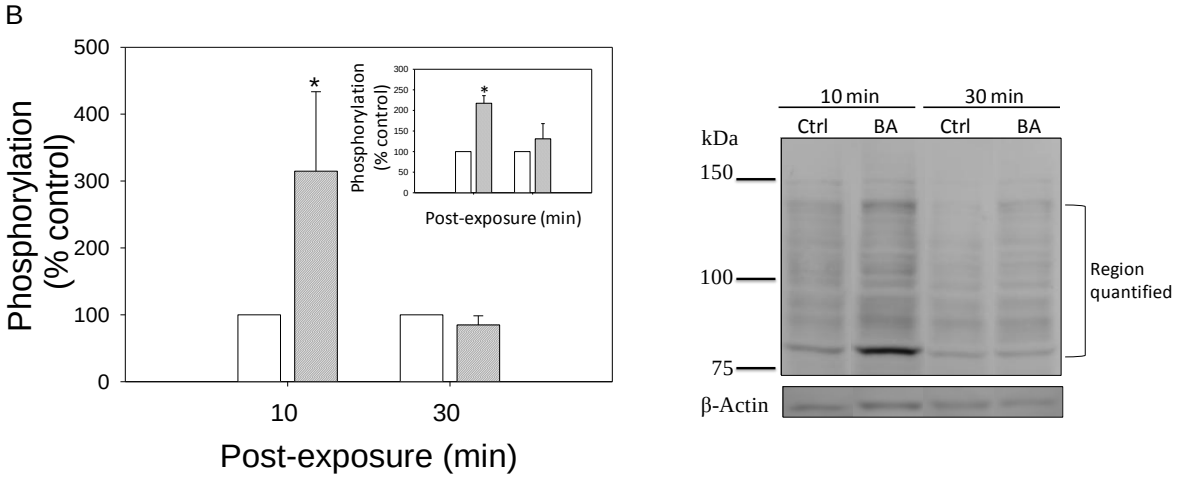
4.3.4 Benzyl alcohol rapidly activates multiple signaling pathways in liver

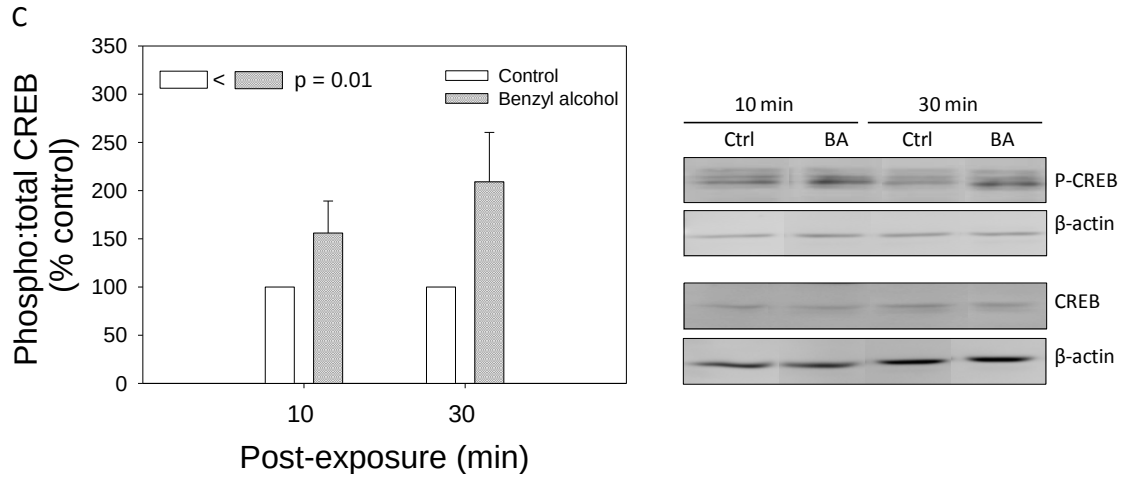
Benzyl alcohol, a known chemical fluidizer of cellular membranes, was used to investigate whether the above signaling pathways are responsive to plasma membrane perturbations, such as fluidization. Similar to cortisol, benzyl alcohol rapidly (10 min) stimulated cAMP production (Fig. 4-8A), and resulted in an increase in PKA substrate phosphorylation (Fig. 4-8B), including CREB activation (Fig. 4-8C). The effect on PKA substrate proteins occurred within 10 min and returned to control levels within 30 min (Fig. 4-8B), while CREB was activated at 10 and 30 min post-treatment (Fig. 4-8C). Benzyl alcohol also rapidly and transiently increased PKC (Fig. 4-9) and AKT (Fig. 4-10) substrate protein phosphorylation, however the effect on AKT proteins was only significant in trout hepatocytes (Fig. 10 inset). Lastly, in trout hepatocytes (Fig. 4-11A) but not in liver slices (Fig. 4-11B), benzyl alcohol strongly induced p38 MAPK phosphorylation, which was sustained 30 min post-treatment. In contrast, benzyl alcohol caused a dephosphorylation of ERK (1/2) in liver slices (Fig. 4-12A) but had no effect on ERK (1/2) MAPK in trout hepatocytes (Fig. 4-12B).

Figure 4-8. The effect of benzyl alcohol on cAMP-PKA-CREB signaling in rainbow trout liver.

(A) cAMP levels 10 min after benzyl alcohol (25 mM) treatment in cultured primary hepatocytes. Values are plotted as % cortisol and show mean ± S.E.M (n = 6 independent fish). (B) Histogram and corresponding representative immunoblot for the time and dose response of protein kinase A (PKA) substrate protein phosphorylation in rainbow trout liver slices and primary hepatocyte culture (inset, modified from Dindia et al., 2012) in response to benzyl alcohol (25 mM) treatment. Liver slice or hepatocyte homogenates (40 µg of protein) were probed with phospho-PKA Substrate (RRXS/T) polyclonal rabbit antibody (Cell Signaling Technology, Beverly, MA). Densitometric values were obtained by quantifying the total phosphorylation intensity of each lane (region shown). Values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); bars with different symbols are significantly different (repeated measures ANOVA, $p < 0.05$). (C) Time and dose response of CREB activation following benzyl alcohol (25 mM) treatment. Primary hepatocyte homogenates (40 µg of protein) were probed with phosphor-CREB (ser133) or total CREB monoclonal rabbit antibody (Cell Signaling Technology, Beverly, MA). Values plotted are the ratio of phosphorylated to total CREB which are plotted as % control and show mean ± S.E.M (n = 5 independent fish); inset indicates a significant treatment effect (repeated measures ANOVA, $p < 0.05$). Protein loading was controlled for by normalizing to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO).


A
cAMP (% control)
250
200
150
100
50
0
Control
Benzyl Alcohol


B
Phosphorylation (% control)
500
400
300
200
100
0
*
Phosphorylation (% control)
Post-exposure (min)
10
30
Post-exposure (min)
10 min
30 min
Ctrl
BA
Ctrl
BA
kDa
150
100
75
β-Actin
Region quantified


C
Phospho:total CREB (% control)
350
300
250
200
150
100
50
0
< p = 0.01
Control
Benzyl alcohol
10
30
Post-exposure (min)
10 min
30 min
Ctrl
BA
Ctrl
BA
P-CREB
β-actin
CREB
β-actin

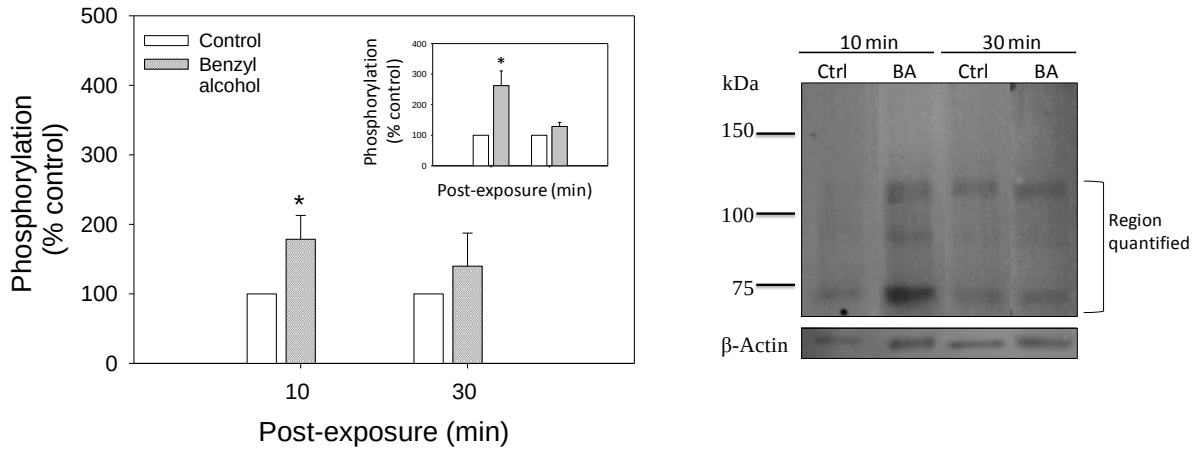


Figure 4-9. Benzyl alcohol-induced phosphorylation of protein kinase C (PKC) substrate proteins.

(A) Histogram and corresponding representative immunoblot of the time and dose response of PKC substrate protein phosphorylation in rainbow trout liver slices and hepatocyte culture (inset, modified from Dindia et al., 2012) following benzyl alcohol treatment (25 mM). Liver homogenates (40 µg of protein) were probed with phospho-(Ser) PKC substrate polyclonal rabbit antibody (Cell Signaling Technology, Beverly, MA), which detects proteins which are phosphorylated within the phosphorylation motif for PKC. Protein loading was controlled for by normalizing to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometric values were obtained by quantifying the total phosphorylation intensity of each lane (region shown). Values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); bars with different symbols are significantly different within the time point (repeated measures ANOVA, $p < 0.05$).

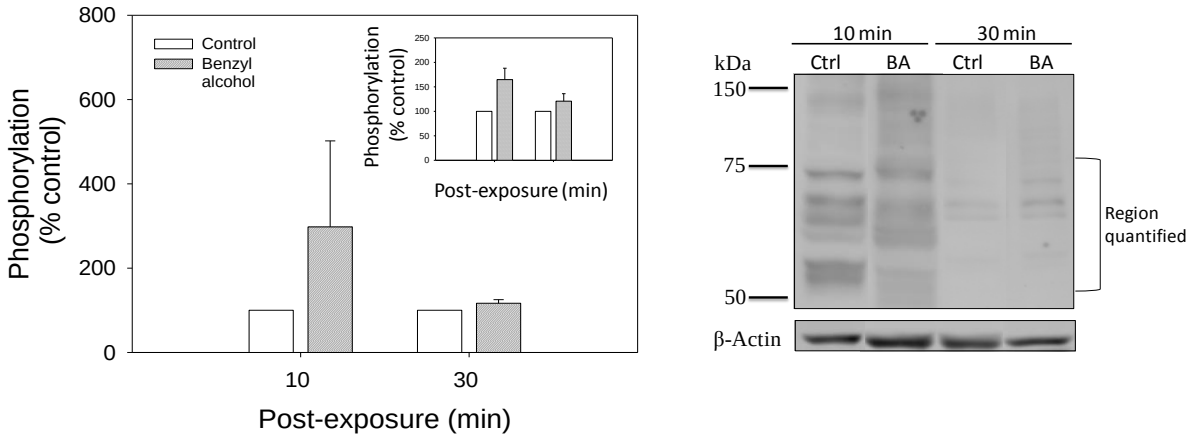


Figure 4-10. Phosphorylation of AKT substrate proteins in response to benzyl alcohol.

(A) Histogram and corresponding representative immunoblot of the time and dose response of AKT substrate protein phosphorylation in rainbow trout liver slices and hepatocyte culture (inset, modified from Dindia et al., 2012) following benzyl alcohol treatment (25 mM). Liver homogenates (40 µg of protein) were probed with phospho-Akt substrate (RXXS/T) polyclonal rabbit antibody (Cell Signaling Technology, Beverly, MA), which detects proteins that are phosphorylated within the AKT phosphorylation motif. Protein loading was controlled for by normalizing to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometric values were obtained by quantifying the total phosphorylation intensity of each lane (region shown). Values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); bars with different symbols are significantly different within the time point (repeated measures ANOVA, $p < 0.05$).

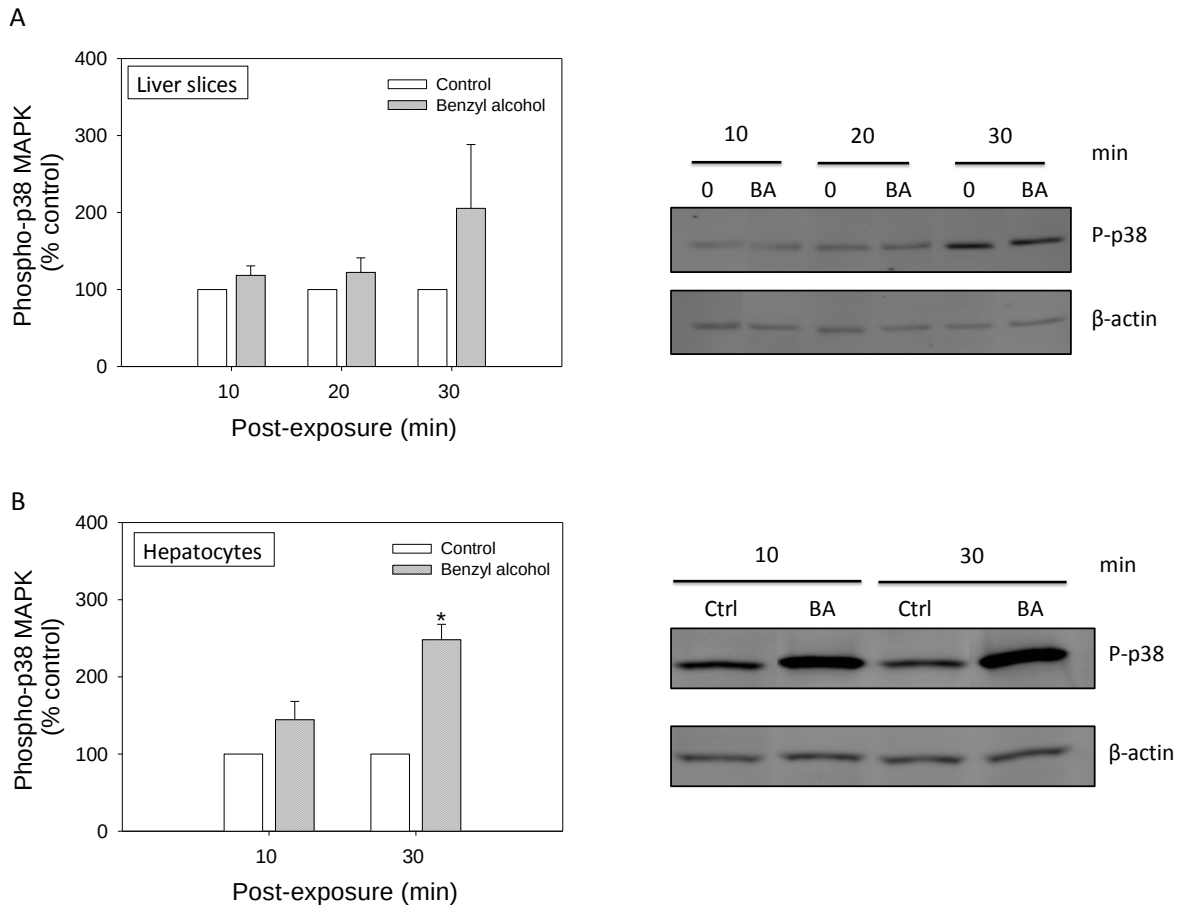


Figure 4-11. Phosphorylation of p38 mitogen-activated protein kinase (MAPK) in trout liver slices

Phosphorylation of p38 MAPK in liver slices (A) and hepatocyte culture (B) in response to benzyl alcohol (25 mM). Liver slices or hepatocytes homogenates (40 µg protein) were probed with phospho-specific p38 MAPK monoclonal rabbit antibody (Cell Signaling Technology, Beverly, MA). Protein loading was controlled for by normalizing to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometric values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); bars with different symbols are significantly different within the time point (repeated measures ANOVA, $p < 0.05$).

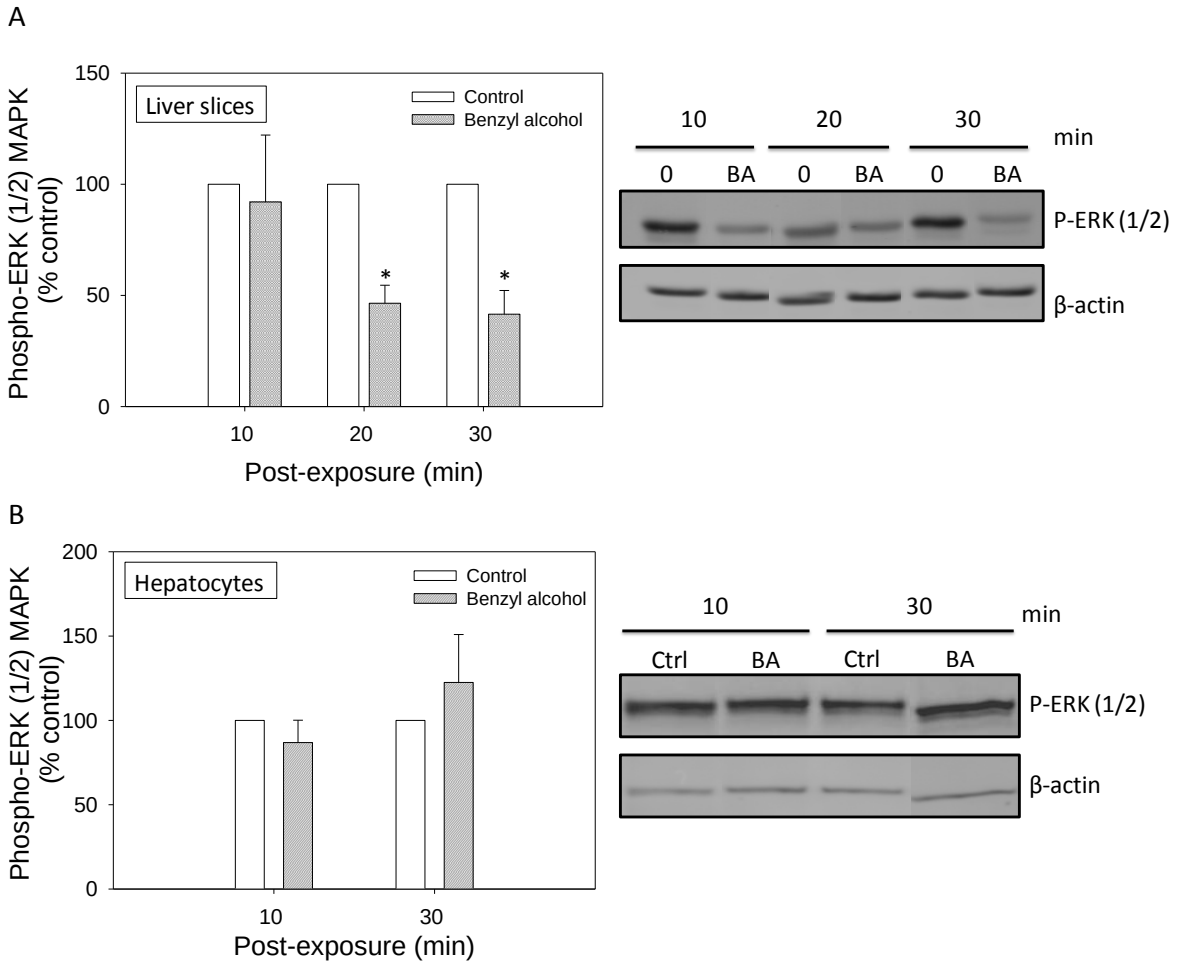


Figure 4-12. Phosphorylation of ERK (1/2) mitogen-activated protein kinase (MAPK) in trout liver slices

Phosphorylation of ERK (1/2) MAPK in liver slices (A) and hepatocyte culture (B) in response to benzyl alcohol (25 mM). Liver slices or hepatocytes homogenates (40 µg protein) were probed with phospho-specific ERK(1/2) MAPK monoclonal rabbit antibody (Cell Signaling Technology, Beverly, MA). Equal loading was confirmed with β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometric values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); bars with different symbols are significantly different within the time point (repeated measures ANOVA, $p < 0.05$).

4.4 Discussion

The results presented here demonstrate for the first time that cortisol rapidly activates cAMP-PKA, PKC, and MAPK pathways in trout hepatic tissue. Cortisol is known to elicit numerous transcription-dependent effects in liver tissue (Rose et al., 2010), but a nongenomic role for this steroid through activation of intracellular signaling proteins is largely uncharacterized in vertebrates. The finding that cortisol modulates multiple intracellular signaling pathways, both rapidly and transiently, underscores a novel nongenomic mechanism of stress adaptation by this steroid in trout liver.

While studies using non-piscine models have reported specific binding sites for glucocorticoid on hepatic cellular membranes, little is known about rapid intracellular effects of glucocorticoid in hepatocytes (Borski, 2000). Earlier studies conducted with rat hepatocytes reported both transient and sustained activation of glycogen phosphorylase and synthase enzymes within minutes of dexamethasone treatment (Gomez-Munoz et al., 1989). The glucocorticoid-induced activation of glycogen-metabolizing enzymes occurred independently of protein synthesis and appeared to be mediated via Ca^{2+} signaling (Baqué et al., 2004; Gomez-Munoz et al., 1989). Cortisol was also shown to rapidly alter lipid metabolism by nongenomically inhibiting key lipogenic enzymes in piscine liver tissue, but the mechanism is unknown (Sunny et al., 2002). Together, these studies support a role for nongenomic cortisol signaling in the liver, while the mechanism of action remains unclear.

Our results provide clear evidence that cortisol rapidly up-regulates hepatic cAMP levels, a key second messenger, leading to activation of PKA substrate proteins, including CREB. As reported previously (Beck et al., 1993), mifepristone on its own rapidly and transiently induced cAMP production, and, therefore, was not a useful tool to differentiate GR-independent effects. In addition to altering cAMP levels, there was a corresponding increase in phosphorylated PKA substrate proteins in response to cortisol treatment. The rapid and transient increase in cAMP production and increased PKA substrate phosphorylation suggests a rapid effect of cortisol on hepatic stress response that is nongenomic. Indeed, rapid activation of the PKA pathway in response to cortisol is an important finding, as this signaling pathway is prominent in hormone-mediated hepatic glucose homeostasis (Ramnanan et al., 2011). Epinephrine and glucagon are key activators of hepatic PKA, which ultimately promotes glucose mobilization through the direct stimulation of enzymes involved in glycogenolysis and gluconeogenesis (Jiang and Zhang, 2003).

In addition to rapid phosphorylation and modulation of metabolic enzymes (Berridge et al., 2003), activation of PKA has important genomic consequences. For instance, it is a primary activator of CREB, a key transcriptional regulator of various metabolic genes in the liver (Mayr and Montminy, 2001). Rapid, nongenomic activation of CREB has been reported in response to various steroids and is a key interactive point for crosstalk between nongenomic and genomic steroid signaling (Lösel and Wehling, 2003). The present study provides additional evidence for CREB mediated steroid action, as cortisol transiently induced CREB phosphorylation, indicating a role for PKA in nongenomic and genomic cortisol signaling. Nongenomic CREB activation has been reported for other steroid hormones, including mineralocorticoids (Christ et al., 1999). However, the role of CREB in nongenomic GC signaling has not been extensively investigated and this is the first study to report rapid steroid CREB activation in the liver. Moreover, a well established genomic effect of GC is the transactivation of the rate-limiting enzyme in gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK). Indeed, CREB is also involved in the upregulation of PEPCK transcription (Altarejos and Montminy, 2011), and several studies have indicated that PKA activation of CREB supports the hormonal regulation of PEPCK transcription by GC (Herzig et al., 2001; Imai et al., 1993; Liu et al., 1991). Co-operative action between GR and the cAMP-PKA pathway has been implicated in several GR mediated transcriptional events (Kassel and Herrlich, 2007), but has never been reported in fish.

In addition to PKA, our results support a role for PKC in mediating rapid cortisol action in the liver. The PKC substrate protein phosphorylation in response to cortisol treatment in liver slices occurred within 10 min and this response was sustained over a 30 min period. PKC signaling is involved in the regulation of carbohydrate and lipid metabolism, although limited studies have been conducted in piscine models. In rainbow trout, PKC activation is a prominent regulator of the shift from anabolic to catabolic activity during the fasted state in several metabolic tissues, including the liver (Bergan et al., 2011). Rapid activation of PKC substrate proteins may not only reflect direct effects on liver metabolism (Samuel et al., 2010), but also indirect effects by regulating other signaling pathways, including MAPKs (Cote-Vélez et al., 2008; Roberts et al., 2000). In addition PKC is often activated in concert with PKA, in response to diverse stimuli (Grafer et al., 2009). For instance, in mammalian adipocytes activation of hormone-sensitive lipase involves a synergetic activation of PKA and PKC-mediated MAPK activation (Duncan et al., 2007), leading to enhanced lipolysis. Therefore, cortisol mediated activation of both PKA and PKC pathways may synergistically regulate rainbow trout liver metabolism during stress adaptation. In addition to PKA and MAPK, in

teleost PKC also interacts with heat shock proteins to regulate cytochrome P450 (CYP450), which involved in the oxidation of metabolites and xenobiotics (Ghosh and Ray, 2012). Moreover, GR is a known regulator of CYP450 protein family (Aluru and Vijayan, 2007), leading to the possibility that PKC and GR activation in response to cortisol may co-regulate CYP450 in trout hepatocytes.

Cortisol, in addition to modulating PKA and PKC substrate phosphorylation, also rapidly reduced p38 MAPK phosphorylation levels in trout hepatocytes. Previous studies have reported GC-mediated nongenomic MAPK regulation, while this is the first report in liver tissue. MAPK nongenomic regulation by GC has been well documented in PC12 cells, a rat adrenal medulla cell line, although a functional role is still being investigated. Specifically, corticosterone rapidly phosphorylates p38 and ERK (1/2) MAPK in PC12 cells in a dose-dependent manner, which is PKC-dependent and independent of the intracellular GR activation (Qi et al., 2005). In addition to nongenomic effects, the activation of MAPK by GC may lead to genomic signaling (Ayroldi et al., 2012). For instance, in mammalian species, the anti-inflammatory actions of GC involve rapid induction of MAPK phosphatase-1 (MKP-1) synthesis, which is a dual-specific phosphatase responsible for the dephosphorylation MAPKs (Kassel et al., 2001). However, the induction of MKP-1 protein synthesis by cortisol was observed under an hour in a rat mast cell line (Kassel et al., 2001), and unlikely to be the cause for the steroid-mediated dephosphorylation of p38 detected in the present study, as this effect occurred within 10 min of steroid treatment. Lastly, cortisol-mediated p38 inactivation reported in this study might facilitate mRNA destabilization induced by cortisol, as p38 MAPK is involved in stabilizing mRNA transcripts (Clark et al., 2003). Although the exact function of rapid p38 MAPK inactivation in rainbow trout liver awaits further investigation, the rapid action of cortisol on MAPK signaling suggests a the metabolic response of the liver during stress adaptation involves a cross-talk between these two stress activated pathways.

We demonstrate for the first time that cortisol increased the phosphorylation status of AKT substrate protein in cultured hepatocytes. Within the liver, the PI3K-Akt pathway is a well defined component of the insulin pathway, which mediates anabolic metabolism within the liver (Taniguchi et al., 2006). Since cortisol's action is mainly catabolic it remains to be determined whether Akt plays a role in the metabolic response mediated by cortisol. Activation of Akt in response to insulin acts as a negative feedback circuit by down regulating the expression of insulin receptor (Puig and Tjian, 2005), therefore Akt activation by cortisol may modulate hepatocyte responsiveness to other hormonal stimulation during acute cortisol elevation. In the liver, acute cortisol activation of Akt

signaling may also promote cell survival, as Akt signaling is involved in cell survival by inhibiting apoptosis (Song et al., 2007). Support for this argument comes from the cardiovascular system, as glucocorticoids nongenomically activate the PI3K/AKT kinase pathway, leading to cellular protective effects (Liao, 2004). In human lung epithelial cells, glucocorticoids rapidly activate Akt leading to increased phosphorylation of downstream targets (Matthews et al., 2008). Disruption of glucocorticoid Akt activation resulted in growth inhibition, supporting a cell survival effect of dexamethasone-induced Akt pathway activation (Matthews et al., 2008). Similarly in human fibroblasts, dexamethasone mediates anti-apoptotic effect by activating the PI3K-Akt pathway (Nieuwenhuis et al., 2010). Lastly, long-term glucocorticoid exposure is associated with growth inhibition and increased apoptosis, and more importantly is ameliorated by increased Akt signaling (Poulsen et al., 2011). Therefore, while other studies have suggest chronic cortisol elevation leads to Akt inactivation (Poulsen et al., 2011), our results suggest that acute cortisol elevation rapidly activates Akt signaling. We propose that this may modulate hepatocyte responsiveness to other hormones and provide cellular stress protection and survival.

In addition to receptor-mediated mechanism, GC can potentially modulate intracellular pathways via mechanotransduction as cortisol integration into the lipid bilayer induces functional biophysical changes to the plasma membrane (Dindia et al., 2012). Since plasma membrane perturbation is known to induce several intracellular signaling pathways (Huang et al., 2004), we examined the effects of benzyl alcohol, which has well documented effects on the plasma membrane biophysical architecture and is commonly used to determine functional effects of membrane hyperfluidization (Vigh et al., 1998). Moreover, benzyl alcohol (20-50 mM) has previously been used to study fluidization effect of liver Golgi (Mitranic et al., 1982), mitochondrial (Kolodziej and Zammit, 1990) and plasma membrane enzyme activity(Gordon et al., 1980), including rainbow trout liver (McKinley and Hazel, 2000). The concentration of benzyl alcohol used in the current study (25 mM) is comparable to those used in the above studies, which ranged from 10-50 mM, with effects most commonly reported between 20-40 mM. In the present study, benzyl alcohol induced phosphorylation of PKA and PKC substrate proteins; however, unlike cortisol, benzyl alcohol strongly induced phosphorylation of p38 MAPK. Although not tested in this study, the divergent effects of benzyl alcohol and cortisol on p38 MAPK activity may be linked to their differential effects on the cellular heat shock response. For instance, although not confirmed in teleost, p38 MAPK is responsive to activators of the heat shock response in lower vertebrates (Aggeli et al., 2002). Moreover, previous studies have shown that fluidization induced by benzyl alcohol (30 mM),

independently of protein denaturation, activates the heat shock response in mammalian cells (Balogh et al., 2005), whereas cortisol has been shown to attenuate the heat shock response in teleost (Basu et al., 2001; Iwama et al., 1999; Sathiyaa et al., 2001). Therefore it will be interesting to conduct future studies to determine whether benzyl alcohol induces the heat shock response in trout liver and whether p38 MAPK is implicated in the teleost response to thermal stress. Benzyl alcohol-induced hyperfluidization of liver plasma membrane induces intracellular signaling cascades in trout liver, suggesting that teleost stress-activated signaling cascades are sensitive to perturbation in lipid order. Although we cannot directly compare the effects of cortisol and benzyl alcohol, both of which fluidize trout membranes, it does support the idea that membrane alterations induced by cortisol may at least partially mediate nongenomic effects in trout liver. It is important to emphasize that in addition to membrane-mediated effects there is strong evidence that nongenomic GC action is initiated by membrane and intracellular GRs (Borski, 2000), therefore it is also possible that the cellular responses to cortisol reported here are partly receptor-mediated. In fact, mifepristone, a GR antagonist, blocked cortisol-mediated CREB activation suggesting involvement of the classical GR activation in this nongenomic response, but the mechanism is unknown.

In conclusion, cortisol rapidly and nongenomically activates cAMP-PKA, PKC and p38 MAPK pathways in liver. Moreover, this is the first study to report cortisol-mediated nongenomic signaling in the liver and supports the emerging view that these rapid glucocorticoid actions may involve complex signaling networks with multiple interactions. The results presented not only reveals a possible novel direct mechanism of rapid cortisol-mediated effect on hepatic function that is nongenomic, but also highlight the possibility of indirect effect by modulation of transcription factors and the associated regulation of genomic signaling. Importantly, now that a rapid cellular response to cortisol has been established in trout hepatocytes, future studies can focus on elucidating the physiological implications of this response on cell function during stress.

4.5 Acknowledgements

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Chapter 5: **Cortisol acutely alters liver metabolite levels possibly through a nongenomic pathway**

Dindia, L., Jordan, J., Habibi, H., and Vijayan, M.M. Cortisol acutely alters liver metabolites levels possibly through a nongenomic pathway. Prepared for scientific journal submission

5.1 Introduction

During both basal and stress-activated states, the liver is a vital organ for whole-body energy homeostasis as it is a primary regulator of carbohydrate, lipid and protein metabolism (Orman et al., 2011). Among other hormones, Glucocorticoids (GC) are well known for their regulatory roles on metabolic processes, particularly within the liver (Vegiopoulos and Herzig, 2007). GC also mediates both genomic and nongenomic effects, while the involvement of non-transcriptional GC effects in peripheral tissues, including the liver, remain poorly characterized in all vertebrate species (Borski, 2000). In rainbow trout (*Oncorhynchus mykiss*) liver, stress levels of cortisol rapidly modulate phosphorylation of protein kinase A (PKA), protein kinase C (PKC), and AKT substrate proteins *in vitro* (Dindia et al., 2012). Furthermore, as mentioned previously (Chapter 2), endogenous plasma cortisol levels positively correlate with phosphorylation of PKA and PKC substrate proteins *in vivo*, strongly suggesting a role for nongenomic cortisol signaling in liver. Although cortisol rapidly activates important signaling cascades in the liver, a role for nongenomic signaling in altering liver metabolism in teleosts is unknown.

The cortisol-mediated metabolic regulation in the liver has been examined in mammalian (McMahon et al., 2009) and teleost species. It is well accepted that GC play a pivotal role in carbohydrate metabolism, primarily through the activation of gluconeogenesis (Vijayan et al., 1994a). The rate-limiting enzyme in the gluconeogenic pathway is phosphoenolpyruvate carboxykinase (PEPCK), which is upregulated in response to GC in various species, including rainbow trout (Vijayan et al., 2003). In addition to genomic effects on carbohydrate metabolism, there is emerging evidence that GC nongenomically regulate carbohydrate substrate availability. For instance, in rat hepatocyte, dexamethasone activated glycogen phosphorylase (GP) within 10 min, independent of protein synthesis (Gomez-Munoz et al., 1989). GC are also potent regulators of protein and lipid metabolism. In the liver, glucocorticoids are known to induce transcription of enzymes involved in amino acid metabolism, such as glutamine synthetase (Abcouwer et al., 1995; McDonald et al., 2009), tyrosine aminotransferase (Schmid et al., 1987) and arginase (Vijayan et al., 1996). Overall, GC are characterized as being lipolytic, but can also induce lipogenesis as well as suppress lipogenic enzyme activity (Peckett et al., 2011). In teleosts, cortisol activates hepatic triacylglycerol lipase, leading to a decrease in triacylglycerol and phospholipid levels (Lidman et al., 1979). More recently, cortisol was shown to nongenomically decrease malic enzyme, glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase activity in the liver of *Oreochromis mossambicus* (Sunny et al., 2002).

We have recently reported that cortisol nongenomically activates key protein kinase pathways, including protein kinase A (PKA), protein kinase C (PKC) and AKT, in rainbow trout (Dindia et al., 2012). However, whether cortisol nongenomically alters metabolic endpoints in trout liver is unknown. We tested the hypothesis that cortisol exerts effects on liver metabolism independently of protein synthesis by examining the liver metabolite profile, using nuclear magnetic resonance approach, response to cortisol treatment in the presence of either cycloheximide, a protein synthesis inhibitor, or mifepristone, a GR antagonist.

5.2 Material and Methods

5.2.1 Animals & sampling

Juvenile rainbow trout (100-300 g) purchased from Alma Aquaculture Research Station (Alma, Ontario) were maintained at the University of Waterloo aquatic facility exactly as described before (Sandhu and Vijayan, 2011). The tanks were supplied with a constant flow of aerated well water (12 ± 2°C) and were maintained under a 12hL:12hD photoperiod. Trout were acclimated for at least two weeks prior to experiments and were fed commercial trout feed (Martin Mills, Elmira, Ontario) to satiety once daily, 5 days a week. Experiments were approved by the University of Waterloo Animal Care Protocol Review Committee and adhere to guidelines established by the Canadian Council on Animal Care for the use of animals in teaching and research.

5.2.2 Liver tissue culture

Trout liver slices were used to investigate the effect of cortisol on liver intermediary enzyme activity and metabolites levels. Rainbow trout were sampled as mentioned above. Livers were quickly excised and washed in ice cold modified Hanks buffer (110 mM NaCl, 3 mM KCl, 1.25 mM K_2HPO_4, 5 mM $NaHCO_3$, 0.6 mM $MgSO_4$, 1 mM $MgCl_2$ and 10 mM Hepes; 1.5 mM $CaCl_2$, 5 mM glucose; pH 7.63 at room temperature). Livers were then sliced into small pieces (500 µm maximum width), using a Krumdieck MD-1100 tissue slicer (Munford, USA). The slices were washed three times with modified Hank's buffer and were placed in six-well tissue culture plates (approximately 50 mg of tissue/well) with L15 media. Liver slices were maintained at 13°C with gentle rocking. Media was changed after an hour after slicing and tissue was incubated for an additional hour prior to treatment. Liver slices were then exposed to either hydrocortisone (cortisol; 0, 100, or 1000 ng/ml) alone or cortisol (1000 ng/ml) was added in the presence of cycloheximide or Mifepristone (RU486).

Cycloheximide (10 µg/µl) and RU486 (1000 ng/ml) were added 60 min, prior to cortisol treatment. Slices were washed twice in ice cold modified Hank's buffer and frozen at -70°C for later determination of enzyme activities and metabolite levels. Liver slice incubation media was collected 30 and 120 min after cortisol addition and immediately frozen at -70°C for determination of media glucose and lactate levels.

5.2.3 Enzyme and Metabolite Analysis

Liver tissue was homogenized and stored in 50% glycerol buffer (50% glycerol, 21 mM Na^2HPO^4, 0.5 mM EDTA-Na, 0.2% BSA, 5 mM β-mercaptoethanol pH 7.5) for enzyme determination exactly as described previously (Vijayan et al., 2006). Liver and media lactate and glucose levels were measured exactly as described previously (Ings et al., 2012). Measurement of metabolites including free amino acids was carried out by nuclear magnetic resonance (NMR) spectroscopy exactly as described previously (Jordan et al., 2011). Enzyme activities were measured in 50 mM imidazole-buffered enzyme reagent (pH 7.4) at 22 °C by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA, USA) using the conditions below and described in detail previously (Vijayan et al., 2006). Activity of Na^+/K^+-ATPase was determined by measuring ouabain-sensitive ATPase activity as described previously (McCormick, 1993).

Alanine aminotransferase (AlaAT: EC 2.6.1.2): 0.12 mM NADH, 200 mM L-alanine, 0.025 mM pyridoxal 5-phosphate, and 12 U/mL LDH; reaction started with 10.5 mM α-ketoglutarate.

Aspartate aminotransferase (AspAT: EC 2.6.1.1): 7 mM α-ketoglutarate, 0.025 mM pyridoxal 5-phosphate, 0.12 mM NADH, and 8 U/mL malate dehydrogenase; reaction started with 40 mM aspartate.

Glucose 6-phosphate dehydrogenase (G6PDH: EC 1.1.1.49): 7 mM $MgCl_2$, 0.4 mM NADP, reaction started with1 mM glucose-6-phosphate.

Glucokinase (GK:EC 2.7.1.2): 15 mM glucose, 5 mM $MgCl_2$, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 5 U/mL lactate dehydrogenase (LDH) and 2.5 U/mL pyruvate kinase; reaction started with 1 mM ATP.

Hexokinase (HK: EC 2.7.1.1): 1 mM glucose, 5 mM $MgCl_2$, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 5 U/mL lactate dehydrogenase (LDH) and 2.5 U/mL pyruvate kinase; reaction started with 1 mM ATP.

Isocitrate dehydrogenase (ICDH: EC1.1.1.42): 7 mM $MgCl_2$, 0.4 mM NADP, reaction started with 0.6 mM isocitrate.

Lactate dehydrogenase (LDH: EC 1.1.1.27): 0.12 mM NADH and reaction initiated with 1 mM pyruvate.

Malate dehydrogenase (MDH: EC 1.1.1.37): 0.12 mM NADH and reaction initiated with 1 mM oxaloacetate.

Malic enzyme (ME: EC 1.1.1.40): 7 mM $MgCl_2$, 0.4 mM NADP, reaction started with1 mM malate.

Pyruvate kinase (PK: EC 2.7.1.40): 3 mM KCl, 10 mM $MgCl_2$, 0.12 mM NADH, 2.5 mM ADP, 20 U/mL LDH; reaction started with 2.5 mM PEP.

5.2.4 Statistical Analysis

For clarity purposes, all values are graphically presented as the percentage of the control (all treatments were paired comparisons). The error bars represent standard error of the mean (S.E.M). A one-way repeated measure analysis of variance (ANOVA) was used to determine significant differences between experimental treatments. Statistics were performed either on raw or log transformed data when necessary to meet normality and equal variance assumptions. Statistics were performed on ranks when transformations were not effective at meeting parametric assumptions. A probability level of $p < 0.05$ was considered significant. All statistical analyses were performed using SigmaPlot 11 software (Systat Software Inc., San Jose, CA, USA).

5.3 Results

5.3.1 Rapid effects of cortisol on liver glucose and lactate levels

Short-term (30 min) cortisol treatment did not significantly alter liver lactate or glucose levels (Fig. 5-1A) and no changes were detected in the incubation media (Fig. 1B). In contrast, both lactate and glucose levels significantly decreased in liver slices 2 hours after cortisol treatment (100 and 1000 ng/ml; Fig.5- 2A), however there were no significant changes to media levels (Fig. 5-2B). The depletion of liver lactate and glucose levels in response to cortisol was not affected by cycloheximide treatment (Fig. 5-2C). Liver glucose levels were also significantly lower compared to control in the presence of Mifepristone and cortisol treatment, while cortisol treatment did not significantly reduce lactate levels in the presence of Mifepristone (Fig. 5-2D).

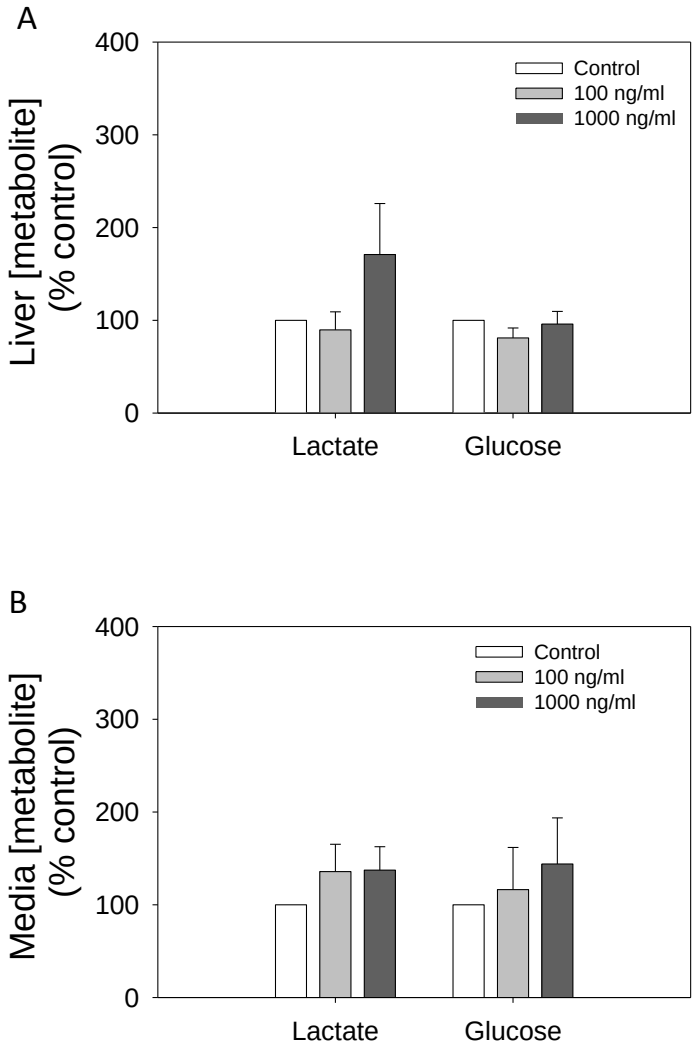


Figure 5-1. Glucose and lactate levels in the liver and media.

Glucose and lactate levels were measured in liver tissue (A) and media (B) 30 min after treatment with cortisol (100 and 1000 ng/ml; A and B). Glucose and lactate levels are plotted as percent control for each time point, and all values represent mean ± SEM of the percent control values, (n = 5-6).

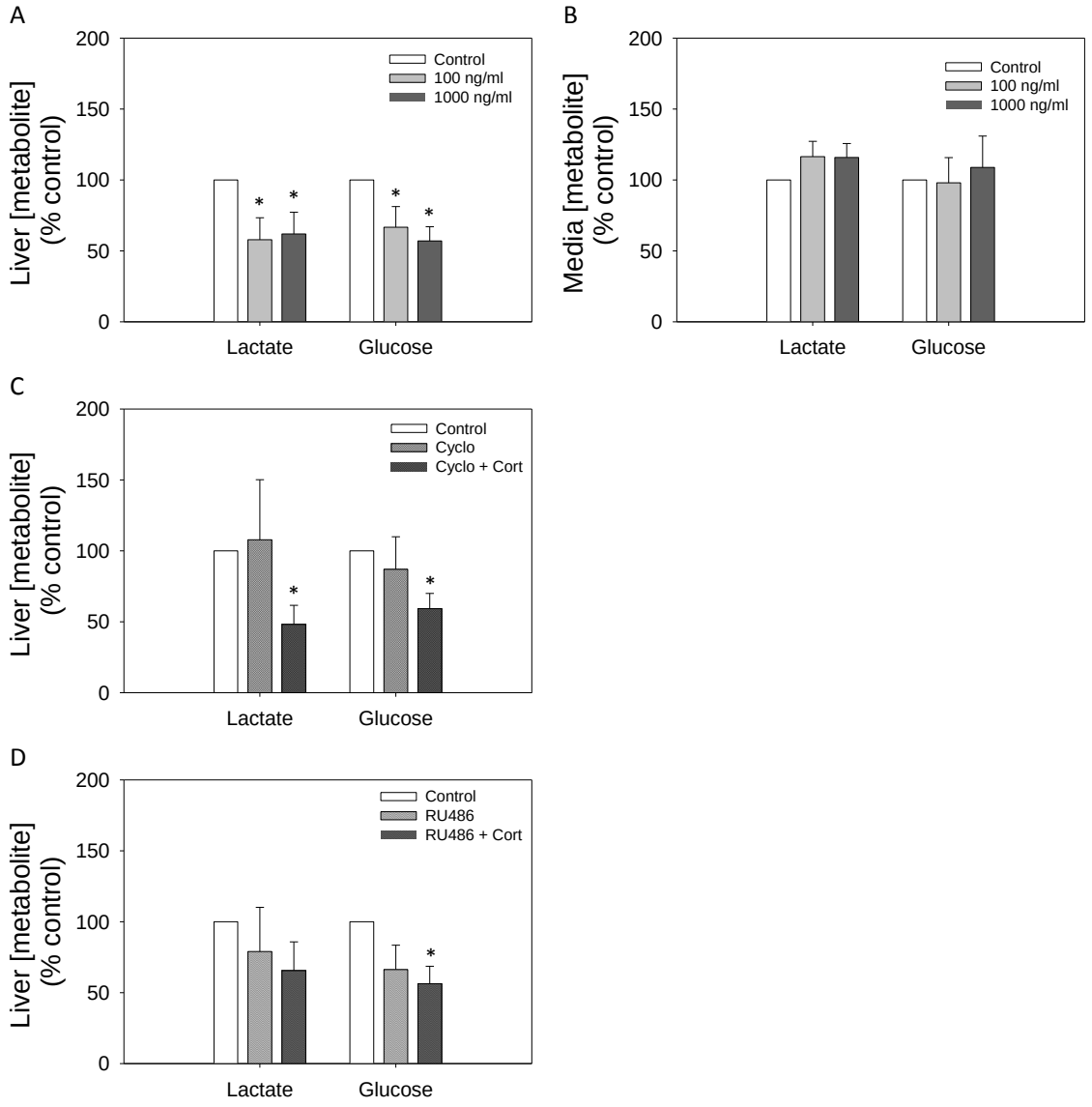


Figure 5-2. Liver and media glucose and lactate levels 120 min after cortisol, cycloheximide and RU486 treatment.

Glucose and lactate were measured in liver tissue (A, C, D) and surrounding incubation media (B) 2 hours following treatment with cortisol (100 and 1000 ng/ml; A and B), cycloheximide (cyclo), with and without cortisol (1000 ng/ml; C) or RU486, with and without cortisol (1000 ng/ml; D). Glucose levels are plotted as percent control for each time point, and all values represent mean ± SEM of the percent control values, (n = 5-6). An * indicates treatment is significantly different compared to the control ($p < 0.05$, One-way repeated ANOVA).

5.3.2 Rapid effects of cortisol on intermediary metabolism

Overall short-term (30 min) cortisol treatment did not affect the activities of enzymes involve in intermediary metabolism (Table 5-1). Only aspartate aminotransferase showed a tendency to increase ($p<0.07$) with cortisol (1000 ng/ml). Neither cycloheximide nor RU486 had any significant effects on liver enzyme activities (Table 5-1).

Table 5-1. Intermediary enzyme activity in response to short-term (30 min) cortisol treatment.

	Enzyme activity (µmoles/min/g)		
Enzyme	**Control**	**100 ng/ml**	**1000 ng/ml**
AspAT	71.3 ± 15.3	89.1 ± 5.0	109.7 ± 9.7*
AlaAT	60.5 ± 9.9	49.4 ± 4.0	50.9 ± 7.1
G6PD	183.9 ± 12.4	160.0 ± 10.9	178.9 ± 21.6
GK	20.1 ± 2.4	20.4 ± 3.8	18.3 ± 4.3
HK	32.9 ± 2.9	34.9 ± 4.1	33.5 ± 4.0
IDH	87.0 ± 9.4	102.2 ± 19.7	91.9 ± 12.7
LDH	46.5 ± 6.1	55.4 ± 6.6	50.9 ± 10.2
MDH	42.2 ± 3.6	31.1 ± 4.6	31.0 ± 4.5
ME	24.2 ± 3.3	22.7 ± 3.5	26.5 ± 6.9
PK	20.4 ± 4.6	20.0 ± 5.4	21.9 ± 5.6
Na^+K^+-ATPase	12.2 ± 1.7	15.9 ± 2.5	19.5 ± 2.7

* indicates treatment is different compared to control (p = 0.068, One-way repeated ANOVA). AspAT = aspartate aminotransferase; AlaAT = alanine aminotransferase; G6PDH = glucose-6-phosphate dehydrogenase; GK = glucokinase; HK = Hexokinase; IDH = isocitrate dehydrogenase; LDH = lactate dehydrogenase; MDH = malate dehydrogenase; ME = malic enzyme; PK = pyruvate kinase; NaK = Na^+/K^+-ATPase.

5.3.3 Cortisol reduces free amino acid levels in liver tissue

In general, cortisol treatment reduced liver amino acid levels. Specifically, there was a significant decrease in the levels of 2-aminobutyrate, alanine, aspartate, glycine, isoleucine, sarcosine, threonine and valine (Fig. 5-3A) at the higher cortisol concentration (1000 ng/ml), but not at 100 ng/ml; the only exception was taurine and it was significantly lower at both cortisol concentrations. Cortisol treatment had no effect on asparagines, glutamate, glutamine, leucine methionine, phenylalanine, or tyrosine (Table 5-2). In general, the reduction in liver amino acid levels in response to cortisol treatment was unaffected by cycloheximide, with the only exceptions of 2-aminobutyrate, leucine and sarcosine (Fig. 5-3B). In contrast, the changes in liver amino acid levels seen with cortisol were not evident in the presence of RU486 (Fig. 5-3C).

Figure 5-3. The effect of cortisol, cycloheximide and RU486 on intracellular free amino acid level in trout liver.

Free amino acid concentrations were measure in liver slice homogenates following 2 h after cortisol (100 and 1000 ng/ml; A), cycloheximide (cyclo), with and without cortisol (1000 ng/ml; B) or RU486, with and without cortisol (1000 ng/ml; C) treatment. Amino acid levels are plotted as percent control, and all values represent mean ± SE, (n = 6). Raw values for amino acid levels are provided in Appendix C (Table C1). * indicates treatment is significantly different compared to the control ($p < 0.05$, One-way repeated ANOVA). Ala; alanine, 2-AB; 2-aminobutyrate, Asp; aspartate, Gly; glycine, Ile; isoleucine; Sar; sarcosine, Taur; taurine, Thr; Threonine, Val; valine.

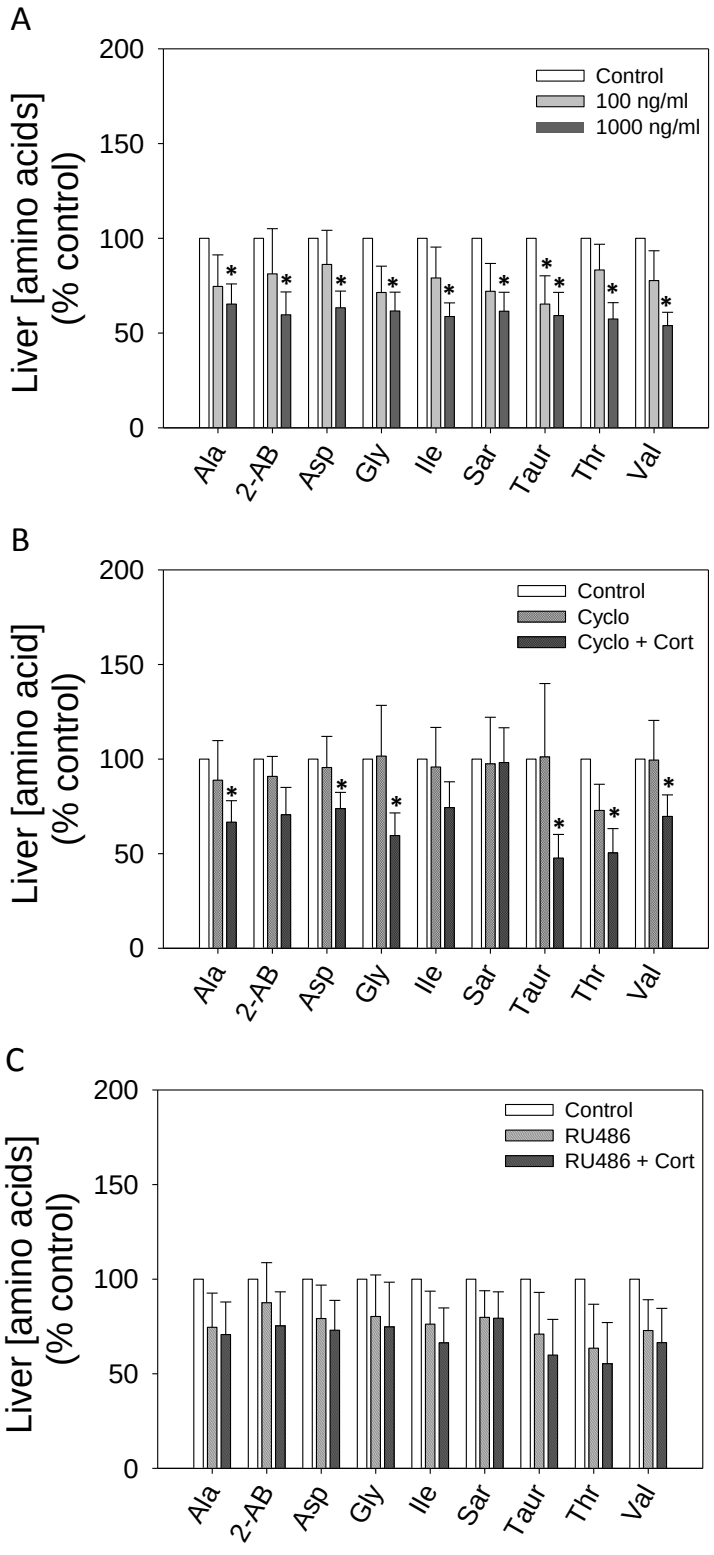

A
Liver [amino acids]
(% control)
200
150
100
50
0
Control
100 ng/ml
1000 ng/ml
Ala
2-AB
Asp
Gly
Ile
Sar
Taur
Thr
Val
B
Liver [amino acid]
(% control)
Control
Cyclo
Cyclo + Cort
C
Liver [amino acids]
(% control)
Control
RU486
RU486 + Cort

Table 5-2. Amino acid levels (μM) that were not significantly modulated by cortisol treatment.

Amino acid	Control	100 ng/ml	1000 ng/ml
Asn	1.295 ± 0.241	1.141 ± 0.289	0.828 ± 0.128
Glu	6.092 ± 1.235	4.331 ± 0.545	3.758 ± 0.529
Gln	2.91 ± 0.652	2.098 ± 0.455	1.648 ± 0.346
Leu	1.539 ± 0.321	1.008 ± 0.273	0.824 ± 0.179
Met	0.670 ± 0.142	0.497 ± 0.150	0.377 ± 0.094
Phe	0.725 ± 0.106	0.607 ± 0.092	0.569 ± 0.085
Tyr	1.514 ± 0.246	1.346 ± 0.332	0.978 ± 0.184

* indicates treatment is significantly different compared to the control ($p < 0.05$, One-way repeated ANOVA). Asn; asparagine, Glu; glutamate, Gln; glutamine, Leu; leucine, Met; methionine, Phe; phenylalanine, Tyr; tyrosine.

5.3.4 Energy substrates and intermediate metabolites are reduced in response to cortisol treatment

Cortisol treatment significantly lowered intracellular levels of acetate, creatine, creatinine, and succinate (Fig. 5-4A). The levels of creatine, creatinine and succinate were altered only response to higher cortisol concentration (1000 ng/ml), whereas acetate was significantly reduced at both the low (100 ng/ml) and high cortisol concentrations. The cortisol-mediated effects on metabolites were not modified by cycloheximide treatment (Fig.5-4B). On the other hand, RU486, successfully blocked cortisol's effect on acetate, creatine, and succinate but not creatinine levels in trout liver slices (Fig. 5-4C).

In addition to modulating energy substrate levels, cortisol also lowered levels several other prominent liver metabolites, including choline, glycerophosphocholine (GPC), phosphocholine (PC), and xanthine (Fig.5-5A). Whereas lower cortisol levels (100 ng/ml) altered choline and PC levels, only the higher dose of cortisol (1000 ng/ml) reduced levels GPC and xanthine (Fig. 5-5A). Cortisol effect on liver choline, GPC and xanthine levels were not modified by either cycloheximide (Fig.5-5B) or RU486 (Fig. 5-5C), while PC effect was completely abolished by the two drugs.

Figure 5-4. The effect of cortisol, cycloheximide and RU486 on intracellular energy metabolites in trout liver. .

Liver levels of acetone, acetate, creatine, creatinine, formate, fumarate, citrate and succinate were measure in liver slice homogenates following 2 h incubation with cortisol (100 and 1000 ng/ml; A), cycloheximide (cyclo), with and without cortisol (1000 ng/ml; B) or RU486, with and without cortisol (1000 ng/ml; C). Metabolite levels are plotted as percent control, and all values represent mean ± SEM, (n = 6). Raw values are provided in Appendix C (Table 2C).* indicates treatment is significantly different compared to the control ($p < 0.05$, One-way repeated ANOVA).

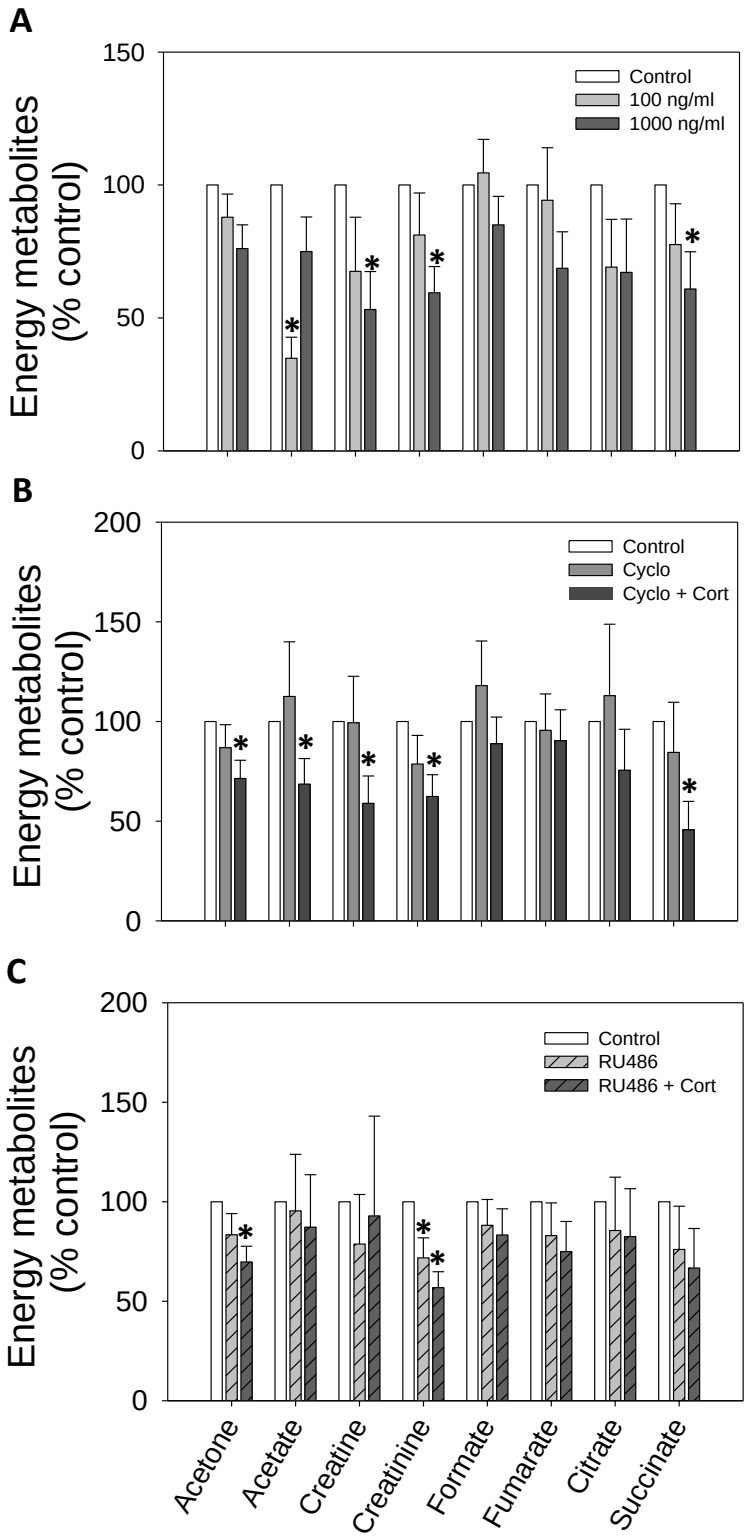
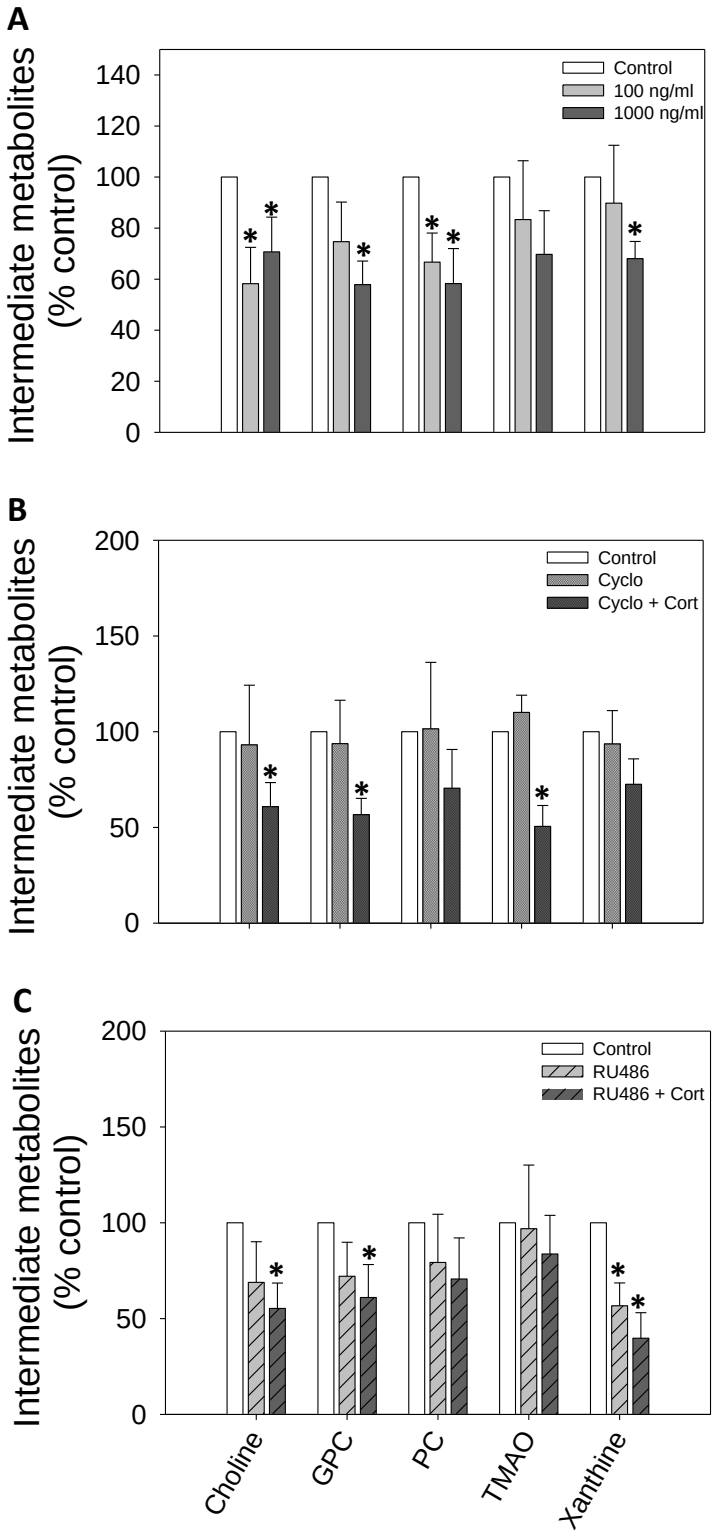

A
Energy metabolites (% control)
150
100
50
0
Control
100 ng/ml
1000 ng/ml
B
Energy metabolites (% control)
200
150
100
50
0
Control
Cyclo
Cyclo + Cort
C
Energy metabolites (% control)
200
150
100
50
0
Control
RU486
RU486 + Cort
Acetone
Acetate
Creatine
Creatinine
Formate
Fumarate
Citrate
Succinate

Figure 5-5. The effect of cortisol, cycloheximide and RU486 on intracellular intermediate metabolites in trout liver.

Liver levels of choline, glycerophosphocholine (GPC), phosphocholine (PC), trimethylamine N-oxide (TMAO) and xanthine were measure in liver slice homogenates following 2 hr incubation with cortisol (100 and 1000 ng/ml; A), cycloheximide (cyclo), with and without cortisol (1000 ng/ml; B) or RU486, with and without cortisol (1000 ng/ml; C). Metabolite levels are plotted as percent control, and all values represent mean ± SE, (n = 6). Raw values are provided in Appendix C (Table 2C). * indicates treatment is significantly different compared to the control ($p < 0.05$, One-way repeated ANOVA).


A
Intermediate metabolites (% control)
Control
100 ng/ml
1000 ng/ml
B
Intermediate metabolites (% control)
Control
Cyclo
Cyclo + Cort
C
Intermediate metabolites (% control)
Control
RU486
RU486 + Cort
Choline
GPC
PC
TMAO
Xanthine

5.3.5 The adenylate energy charge ratio is not affected by cortisol treatment

To evaluate whether cortisol modulates the overall energy status of the liver, levels of adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP) were measured along with the adenylate energy charge ratio (Fig. 5-6). Cortisol (100 and 1000 ng/ml) had no effect on AMP, ADP, or ATP and subsequently did not alter the adenylate energy charge ratio (Fig. 5-6A).

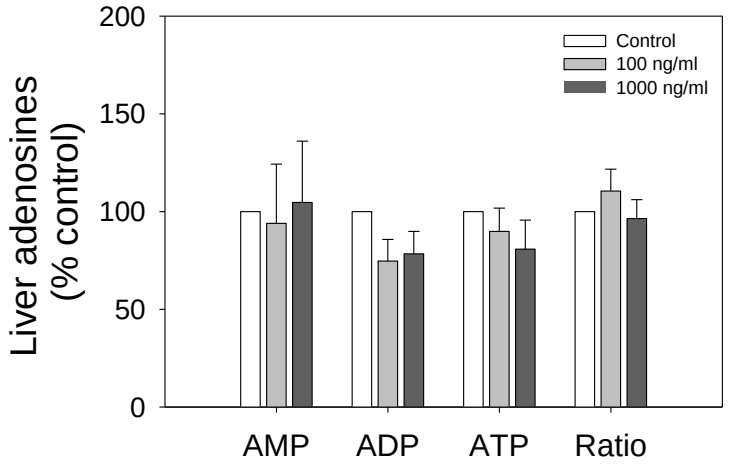


Figure 5-6. The effect of cortisol on intracellular adenosine levels and the adenylate energy charge ratio.

Levels of adenosine monophosphate (ATP), adenosine diphosphate (ADP), and adenosine triphosphate as well as the adenylate charge ratio ([ATP] + 0.5[ADP]/[ATP] + [ADP] + [AMP]) were measure in liver slice homogenates following 2 hr incubation with cortisol (100 and 1000 ng/ml). Adenosine levels and the energy charge ratio are plotted as percent control, and all values represent mean ± SE, (n = 6). Raw values are provided in Appendix C (Table 2C). * indicates treatment is significantly different compared to the control ($p < 0.05$, One-way repeated ANOVA).

5.4 Discussion

Previous studies have investigated cortisol's role in liver metabolism, but the majority of studies have focused on the transcriptional regulation of metabolism (Aluru and Vijayan, 2009; Yabaluri and Bashyam, 2010). The important finding from this study is that cortisol rapidly regulates liver metabolism by nongenomic action. To our knowledge, this is the first study to examine global metabolite changes in response to cortisol. In general, our results reveal that cortisol depletes several liver metabolites, including amino acids, citric acid intermediates, as well as intracellular glucose and lactate levels over a 2 h period after steroid treatment. Overall, the majority of cortisol-mediated changes in metabolite levels occur independently of protein synthesis, underscoring a nongenomic role for cortisol in hepatic metabolism.

Cortisol is known to enhance metabolic capacity of the liver, particularly by augmenting gluconeogenic potential (Aluru and Vijayan, 2009; Vijayan et al., 1994b) and amino acid oxidation (Vijayan et al., 1994b). However, the current study provides novel evidence that stress-induced elevation in cortisol rapidly affects tissue metabolite levels, and this response is independent of *de novo* protein synthesis. For instance, cortisol strongly reduced intracellular glucose and lactate levels 2 h after steroid treatment and this response was insensitive to cycloheximide (protein synthesis inhibitor) and RU486 (GR antagonist) treatment. More importantly, glucose and lactate levels in the incubation media was unaltered by cortisol treatment suggesting intracellular depletion was associated with endogenous usage for oxidation. In rat liver, corticosterone was shown to rapidly stimulate glycogenolysis by rapidly activating glycogen phosphorylase (Gomez-Munoz et al., 1989). Although it remains to be tested whether cortisol rapidly activates glycogen phosphorylase in trout, enhanced glycogen breakdown may provide glucose for endogenous use by the liver. Interestingly, cortisol also acutely suppresses epinephrine-stimulated glucose secretion from trout liver (Chapter 6). Therefore, we propose a novel role for cortisol during the immediate response to acute stress, which involves maintaining the energy balance by stimulating glucose utilization and modulating hormone-stimulated glucose output from the liver. This rapid cortisol response involves nongenomic signaling, but the mechanisms remains to be elucidated.

In addition to carbohydrate metabolism, it is well accepted that glucocorticoids affect amino acid metabolism (Rose et al., 2010). However, the effect of cortisol on liver amino acid metabolism, especially in teleosts, is largely uncharacterized (Mommsen et al., 1999). Within the muscle, GCs are associated with increased proteolysis (mainly in the muscle), increased transaminase activity, and

increases ammonia output due to amino acid degradation (Mommsen et al., 1999). In the current study, cortisol reduced the intracellular pool of amino acids in trout liver tissue, along with the lack of change to cycloheximide, suggesting that nongenomic cortisol signaling is involved in this enhanced amino acid turnover. Few studies have directly examined the effect of GC on intracellular amino acid levels. An earlier study showed that endogenous glucocorticoids are associated with lower intracellular amino acid levels in rat liver (Viru and Éller, 1976). In fibroblasts, glucocorticoids inhibit amino acid production, thereby affecting the turnover of amino acids (Murota et al., 1976). Although the changes in intracellular amino acid pools have been attributed to GC induction of protein synthesis, this has not been directly assessed (Malmberg and Adams, 2008; Viru and Éller, 1976). Our results support a rapid effect of cortisol in modulating the amino acid pool in trout liver and may be a key stress response that is essential for metabolically adapting the animal to stress. This may involve enhancement of amino acid turnover rates by nongenomic cortisol signaling, thereby providing oxidative substrates to meet the increased energy demand associated with acute stress.

Amino acid degradation produces substrates which can be shuttled into the citric acid cycle to increase ATP production (Mommsen et al., 1999). Cortisol treatment significantly affected intermediates of the citric acid cycle, which included significant depletion of acetate and succinate levels, and this occurred in the presence of cycloheximide. In addition to these intermediate energy substrates, cortisol reduced liver creatine and creatinine levels, which act as an energy shuttle system that quickly replenishes ATP levels by the dephosphorylation of phosphocreatine (Longo et al., 2011), further indicating that cortisol initially stimulates ATP producing pathways. In addition to ATP supporting pathways, several metabolites of phospholipid metabolism were also responsive to cortisol treatment. Some of the above effects appear to be mediated by a GR-independent pathway (acetone and creatinine), whereas co-treatment with the GR antagonist did abolish other metabolite responses (creatine, acetate, and succinate,) suggesting partial involvement of GR signaling. Most of the metabolite responses were not associated with changes in intermediary enzyme activities suggesting rapid cortisol effect on metabolite turnover in trout liver.

The overall depletion of liver metabolites in response to cortisol treatment is possibly the result of energy repartitioning in order to meet increased energetic demands associated with stress adaptation (Sapolsky et al., 2000). In response to metabolic stress the energy status of the cell, which is often measured by the adenylate energy charge ratio, is strictly regulated to meet increased energy demands (Oakhill et al., 2011). To access the overall energy status of liver in response to cortisol

treatment, the intracellular levels of adenosines and the adenylate charge ratio were measured. Despite the large depletion of intracellular metabolites, cortisol did not alter the energy charge ratio and there was no change in adenosine levels. Maintenance of the adenylate pool, and more importantly the relative amounts of different adenosines is critical during metabolic stress (Oakhill et al., 2011). Therefore, cortisol-stimulated changes in intracellular metabolite levels might act to maintain the overall energy balance within the liver during the initial phase of the acute stress response.

The biochemical adjustments mediated by stress hormones, such as cortisol, function to maintain homeostasis by regulating energetic pathways. Therefore, depletion of liver metabolites in response to cortisol might stabilize intracellular energy stores, which is supported by the unaltered adenylate energy charge ratio. Taken together, the results highlight that cortisol mediates acute effects on liver metabolism, which were largely insensitive to cycloheximide, suggesting the involvement of nongenomic signaling in rainbow trout. Although the mechanism is unknown, our results indicate that the classical GR might be involved as mifepristone inhibited some of the cortisol-mediated metabolite changes. Although not directly tested here, the altered metabolite pool might also be mediated by mGR or signaling events initiated by biophysical changes at the plasma membrane (Borski, 2000; Dindia et al., 2012). Overall stress adaptation is an energetic process, therefore the short-term metabolite changes in response to acute cortisol treatment is likely adaptive immediately following acute stressor exposure by enhancing endogenous metabolic turnover and substrate utilization within the liver in order to meet the enhanced energy demands of this important metabolic tissue.

5.5 Acknowledgements

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Chapter 6: **Rapid hepatic action of cortisol on epinephrine-stimulated signaling and glucose production**

Dindia, L., Sanjar, S., and Vijayan, M.M. Rapid hepatic action of cortisol on epinephrine-stimulated signaling and glucose production. Prepared for scientific journal submission

6.1 Introduction

When an animal encounters a stressor a highly complex and co-ordinated physiological response is mounted involving the rapid release of catecholamines from chromaffin cells, along with the activation hypothalamus-pituitary-adrenal axis leading to plasma cortisol elevations (Sapolsky et al., 2000). Since plasma GC elevation is dependent on HPA activation and *de novo* steroid synthesis the plasma GC response is delayed (several minutes) compared to the immediate sympathetic-stimulated release of catecholamines (within seconds to minutes). Most notably, stress hormones facilitate energy substrate mobilization to help meet increased energy demands during acute stress (Kyrou and Tsigos, 2009).

Traditionally, the immediate catabolic actions in response to stress are attributed to catecholamines (primarily epinephrine), which bind to adrenergic G-protein coupled receptors (GPCR) linked to adenylate cyclase (AC). Studies in fish indicate the presence of both α- and β-adrenergic receptors in hepatocytes; however, only β-adrenergic responses play a key role in stress-mediated metabolic adjustments in teleost hepatocytes (Fabbri et al., 1998). The β-adrenoreceptor is coupled to stimulatory G-proteins, resulting in cyclic AMP (cAMP) accumulation through the activation of AC. The spike in cAMP levels then activates cAMP-dependent protein kinase (PKA), which directly activates enzymes important for glycogenolysis (Reid et al., 1992). In contrast, GCs are considered to co-ordinate long-term or sustained effects by modulating gene transcription and translation, particularly those involved in gluconeogenesis (Aluru and Vijayan, 2009). GCs regulate gene expression by binding to the intracellular glucocorticoid receptor (GR), a ligand-activated transcription factor, which binds to glucocorticoid response elements (GRE) in target genes, resulting in the activation or repression of gene transcription (Mommsen et al., 1999). In addition to transcriptional modifications, GC also elicits rapid cellular effects, termed nongenomic GC signaling (Borski, 2000).

In addition to independently regulating liver function, GC and catecholamines also interact to modulate cellular responses to cope with stress. While most studies have focused on mammalian cell systems (Evinger et al., 2007; Hwang and Joh, 1993; Sakaue and Hoffman, 1991), a cross-talk between GC and catecholamine signaling pathways have also been reported in teleosts (Dugan and Moon, 1998; Perry and Reid, 1993; Reid et al., 1992; Takahashi and Iizuka, 1991), but the mechanisms are far from clear. Beyond the GC transcription-dependent effects on adrenergic signaling, there is also evidence suggesting nongenomic interplay between glucocorticoids and

catecholamine cellular effects. For instance, GC enhance memory consolidation via nongenomic interaction with noradrenergic activation within the rat amygdala (Roozendaal et al., 2002; Roozendaal et al., 2006) and a similar enhancement in GC-mediated β-adrenergic signaling has also been reported in airway vasculature (Mendes et al., 2008).

Although long-term cortisol exposure is known to alter adrenergic signaling in piscine liver, it is unknown whether cortisol acutely regulates adrenergic signaling. Considering both catecholamines and GC rapidly rise in response to acute stress, and cortisol is known to have rapid effects on liver metabolism (see Chapter 5) there is potential that nongenomic cortisol action plays an acute role in modulating adrenergic responses. Therefore in the present study, we tested the hypothesis that acute stressor-induced cortisol elevation enhances epinephrine-stimulated liver responses in rainbow trout. This hypothesis was tested by examining the effects of acute cortisol treatment on epinephrine-mediated effects on cAMP accumulation, cAMP response element binding protein (CREB) phosphorylation, p38 mitogen-activated protein kinase (MAPK) phosphorylation and glucose production in trout hepatocytes *in vitro*.

6.2 Material and Methods

6.2.1 Animals & sampling

Juvenile rainbow trout (*Oncorhynchus mykiss*; 100-300 g) purchased from Alma Aquaculture Research Station (Alma, Ontario) were maintained at the University of Waterloo aquatic facility exactly as described before (Sandhu and Vijayan, 2011). The tanks were supplied with a constant flow of aerated well water (12 ± 2°C) and were maintained under a 12hL:12hD photoperiod. Trout were acclimated for at least two weeks prior to experiments and were fed commercial trout feed (Martin Mills, Elmira, Ontario) to satiety once daily, 5 days a week. Experiments were approved by the University of Waterloo Animal Care Protocol Review Committee and adhere to guidelines established by the Canadian Council on Animal Care for the use of animals in teaching and research.

6.2.2 Trout hepatocyte isolation

The rapidly signaling events associated with cortisol treatment were examined using trout hepatocytes either as suspended cells (Ings et al., 2011) or in primary cultures exactly as described previously (Sathiyaa et al., 2001). Hepatocytes were isolated using *in situ* collagenase perfusion and maintained exactly as described previously (Ings et al., 2011). Isolated hepatocytes were suspended in L-15

media, supplemented with antibiotic and antimycotic agents, and Trypan blue dye exclusion method was used to determine hepatocyte viability (>95% cells were viable).

6.2.3 Suspended hepatocyte experiment

Experiments measuring cAMP levels were conducted using suspended hepatocytes as previously described (Ings et al., 2011). Cells were thoroughly suspended in modified Hank's buffer (110 mM NaCl, 3 mM KCl, 1.25 mM K_2HPO_4, 5 mM $NaHCO_3$, 0.6 mM $MgSO_4$, 1 mM $MgCl_2$ and 10 mM Hepes; 1.5 mM $CaCl_2$) and transferred in 500 µl aliquots to 1.5 mL centrifuge tubes. Cells were maintained in suspension by gentle rocking at 13°C in the dark. Cells were treated with hydrocortisone (0, 100, or 1000 ng/ml), or benzyl alcohol (25 mM) in the presence or absence of epinephrine (10^{-5} M). For clarity, the order of treatment and duration is indicated on each figure in the results section. Each treatment was quickly terminated at the appropriate time interval with the addition of perchloric acid (2% final concentration). The supernatant was collected for cAMP measurements after immediate centrifugation at 10 000 X g for 2 min. cAMP levels were measured using a commercially available competitive ELISA kit exactly as outlined in the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, Michigan).

6.2.4 Hepatocytes in primary culture

Since cAMP was measured using hepatocytes, phosphorylated and total CREB was measured using primary cultured hepatocytes. Cultured hepatocytes, as opposed to suspended hepatocytes, were used to minimize basal activation of CREB in response to the collagenase-perfusion, as primary cultures are incubated for 24 hours prior to treatment. Hepatocytes were isolated as described above and were suspended in L-15 (sigma, St. Louis, MO) medium after initial washing steps and plated in six-well tissue culture plates (Sarstedt, Inc., Newton, NC) at a density of 1.5 million cells/well (0.75 million cells/ml). Primary cell culture was established over a 24 h incubation period at 13°C. The L-15 media was then replaced and the cells were allowed to recover for an additional 2 h before the start of experiments. Hepatocytes were then treated with epinephrine (10^{-5} M) followed by treatment with hydrocortisone (100 and 1000 ng/ml). The reaction was stopped by replacing L-15 media with 100 µl ice cold lysis buffer (50 mM Tris, 0.25 M sucrose, 1 % SDS, 10 mM NaF, 5 mM EDTA, 5 mM NEM, 0.1 % Nonidet-P40). Lysed cells were quickly heated at 95°C for 5 min followed by brief sonication (sonic dismembrator, Fisher Scientific).

6.2.5 Liver slices

Trout liver slices were utilized to investigate the effects of cortisol on epinephrine-stimulated p38 MAPK activity and glucose production. Liver slices were used as cultured hepatocytes typically produce less glucose when stimulated with epinephrine compared to fresh tissue (Weber and Shanghavi, 2000). Livers were prepared by quickly excising and washing the liver in ice cold modified Hank's buffer (110 mM NaCl, 3 mM KCl, 1.25 mM K_2HPO_4, 5 mM $NaHCO_3$, 0.6 mM $MgSO_4$, 1 mM $MgCl_2$ and 10 mM Hepes; 1.5 mM $CaCl_2$, 5 mM glucose; pH 7.63 at room temperature). Livers were then sliced into 8-10 mm pieces (500 µm maximum width) using a MD-1100 tissue slicer (Munford, USA), washed three times with modified Hank's buffer containing 1.5mM $CaCl_2$ and were placed in six-well tissue culture plates (approximately 50 mg of tissue/well) with 2 mL of L15 media. Liver slices were maintained at 13°C with constant rocking. Media was changed an hour after slicing and tissue was incubated for an additional hour prior to treatment. Liver slices were then exposed to either hydrocortisone (0, 10, 100, or 1000 ng/ml) or benzyl alcohol (5, 10 or 25 mM) for 30 min and were then treated with epinephrine (10^{-5}M). Treatment was stopped 10 min or 60 min after addition of epinephrine. Liver slices were collected 10 min following epinephrine treatment for p38 MAPK phosphorylation quantification. Slices were washed twice in ice cold modified Hank's buffer and frozen at -70°C. Media was collected 60 min post-epinephrine treatment and immediately frozen at -70°C for glucose analysis at a later date. Glucose production in response to epinephrine is detectable after an hour, which is sufficient time for genomic activation, therefore when measuring glucose production cycloheximide (an inhibitor of protein synthesis) was used in combination with cortisol and benzyl alcohol to differentiate between genomic and nongenomic effects. For cycloheximide treatments 10 µg/ml was added 1 hour prior to cortisol or benzyl alcohol.

6.2.6 Glucose Analysis

Media glucose levels were measured by monitoring NAD reduction by the hexokinase (HK)-glucose-6-phosphate dehydrogenase (G6PDH) assay method in Tris buffer (120 mM Tris-base, 80 mM Tris-HCl, 5 mM NAD, 2 mM MgS04, 5 mM ATP). The reaction was started with G6PDH (0.4 U/ml) and HK (0.5 U/ml). The reaction was measured at 22 °C by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA, USA).

6.2.7 Immunoblotting

Tissue and cell lysates were homogenized and protein concentration was measured using the bicinchoninic acid (BCA) method using bovine serum albumin as the standard. All samples were diluted in Laemmli's sample buffer (1M tris-HCl, pH 6.8, 60 mM, glycerol 25%, SDS 2%, β-mercaptoethanol 14.4 mM, bromophenol blue 0.1%) and the immunodetection carried out exactly as described before (Dindia et al., 2012). Briefly, total protein (40 μg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane and blocked with 5% solution of non-fat dry milk in 1 X TTBS (2 mM Tris, 30 mM NaCl, 0.01% Tween, pH 7.5) for 1 h at room temperature. This was followed with an overnight incubation with phospho-p38 MAPK (Thr180/Tyr182) polyclonal rabbit antibodies or monoclonal rabbit phospho- cAMP regulatory element binding protein (CREB) (ser133) and total CREB (Cell Signaling Technology, Beverly, MA). All antibodies were diluted 1:1000 in 5% BSA in TTBS. Blots were incubated for 1 h at room temperature with anti-rabbit horseradish peroxidase (HRP)-labeled secondary antibody (Bio-rad; 1:3300 dilutions in 5% skim milk in TTBS). Protein bands were detected with ECL Plus™ chemiluminescence (GE Health Care, Baie d'Urfe, QC) and imaged using either the Typhoon 9400 (Amersham Biosciences) or the Pharos FX Molecular Imager (Bio-rad). Total lane or protein band intensity was quantified using AlphaImager HP™ (Alpha Innotech, CA). Each immunoblot was subsequently probed with Cy3™ conjugated monoclonal mouse β-actin antibody (Sigma, Cy3™ conjugated monoclonal mouse; 1:1000) for 1 h at room temperature. Densitometric values were then normalized to β-actin values to control for protein loading differences between samples.

6.2.8 Statistical analysis

For clarity, all values are presented as percentage of the control with error bars representing standard error of the mean of percent control values. Therefore, where appropriate, either a repeated measure analysis of variance (ANOVA) or paired t-tests were used to determine significant differences between experimental treatments. A two-way repeated measures ANOVA was carried out to determine time and treatment differences, where applicable. A least significant differences (LSD) *post hoc* test was used to determine within factor effects. Statistics were performed either on raw or log transformed data, when necessary to meet normality and equal variance assumptions. Statistics were performed on ranks when transformations were not effective at meeting parametric assumptions. A probability level of $p < 0.05$ was considered significant. All statistical analyses were performed using SigmaPlot 11 software (Systat Software Inc., San Jose, CA, USA).

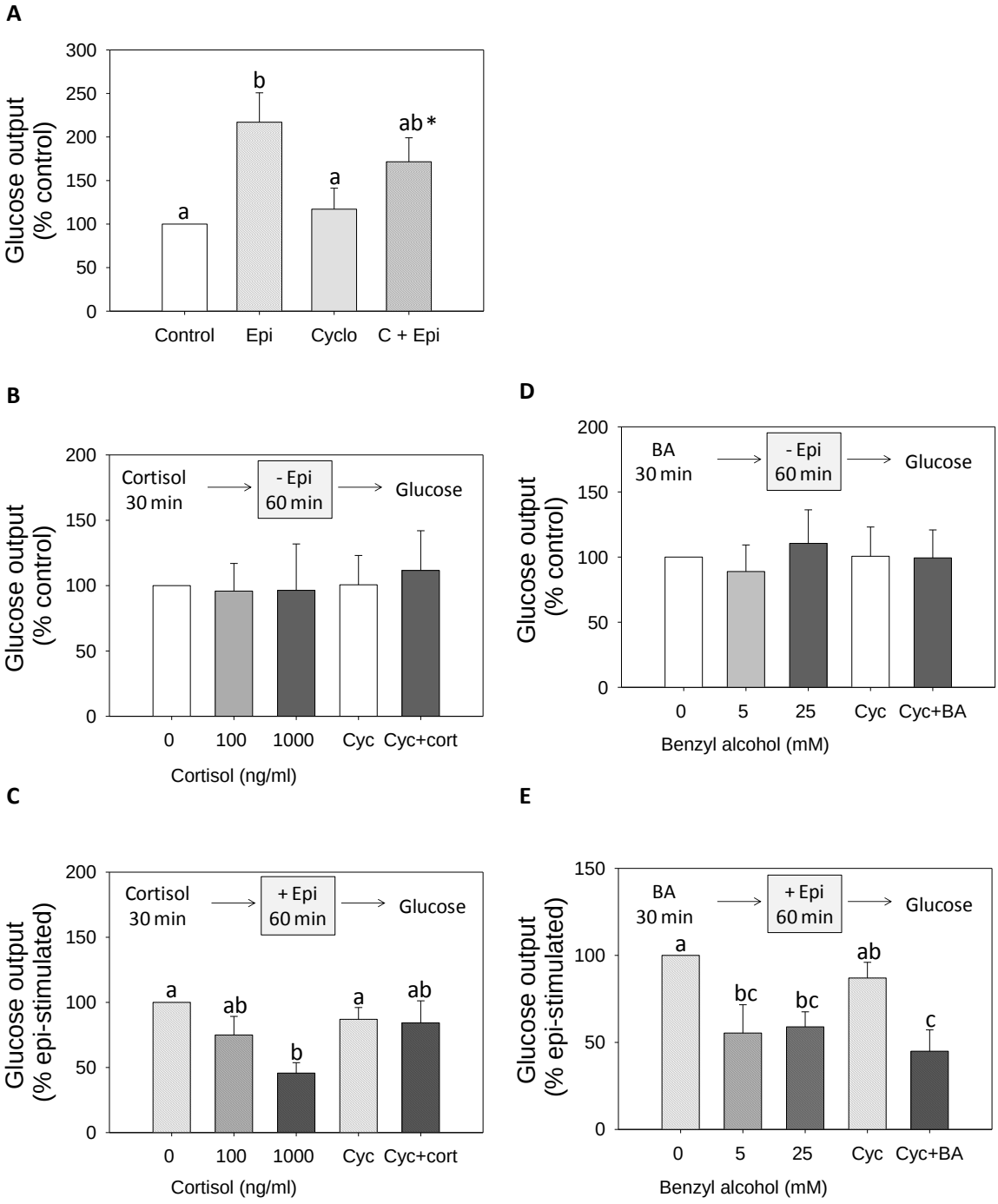
6.3 Results

6.3.1 Hepatic glucose output

To examine the possible interaction of acute stress cortisol elevation on epinephrine signaling, the effect of acute cortisol treatment on epinephrine-stimulated glucose output was determined (Fig. 6-1). As expected, epinephrine significantly increased glucose release from trout hepatocytes over a 60 min period (Fig. 6-1A). This response was partially suppressed (10 %) in the presence of cycloheximide (a translation inhibitor) (Fig. 6-1A). Cortisol treatment alone had no significant effect on hepatic glucose production (Fig. 6-1B). However, cortisol pre-treatment significantly reduced (50%) epinephrine-stimulated glucose production (Fig. 6-1C). Co-treatment with cycloheximide significantly negated the suppressive effect of cortisol on epinephrine-stimulated glucose secretion (Fig. 6-1C). Benzyl alcohol was used to test whether membrane fluidization impacts adrenergic response of trout liver. Benzyl alcohol had a similar effect to that of cortisol, such that there was no change in glucose production in response to benzyl alcohol alone (Fig. 6-1D), however pre-treatment with benzyl alcohol at low (5 mM) and high doses (25 mM) significantly reduced stimulated glucose output (Fig. 6-1D). In contrast to cortisol, the suppressive effect of benzyl alcohol on epinephrine-induced glucose output was insensitive to cycloheximide, as benzyl alcohol (25 mM) significantly reduced epinephrine-induced glucose output in the presence of cycloheximide (Fig. 6-1E).

Figure 6-1. The effect of short-term (30 min) cortisol or benzyl alcohol treatment on glucose production in response to epinephrine stimulation

(A) Media glucose levels following epinephrine treatment (10^{-5} M) for 1 hour compared to untreated liver slices, with and without cycloheximide (10 µg/ml) treatment 1.5 hour before epinephrine treatment. (B) Media glucose levels following treatment with cortisol (10, 100 or 1000 ng/ml) for 30 min prior to treatment with control for 60 min, with and without cycloheximide (Cyclo; 10 µg/ml) treatment one hour before cortisol treatment. (C) Media glucose levels following treatment with cortisol (10, 100 or 1000 ng/ml) for 30 min prior to treatment with epinephrine (Epi; 10^{-5} M) for 60 min, with and without cycloheximide (Cyclo; 10 µg/ml) treatment one hour before cortisol treatment. (D) Media glucose levels following treatment with benzyl alcohol (5 and 25 mM) for 30 min prior to treatment with control media for 60 min, in the presence and absence of cycloheximide (Cyclo; 10 µg/ml) treatment one hour before benzyl alcohol treatment. (E) Media glucose levels following treatment with benzyl alcohol (5 or 25 mM) for 30 min prior to treatment with epinephrine (Epi; 10^{-5} M) for 60 min, with and without cycloheximide (Cyclo; 10 µg/ml) treatment one hour before cortisol treatment. All media glucose levels were normalized to tissue weight (mg). Values are plotted as % cortisol and show mean ± S.E.M of the percent control values (n = 6 independent fish); treatments with different letters are significantly different from each other (repeated measures ANOVA on ranks, $P < 0.05$). Bars with an * indicates that treatment is significantly different than control (repeated measures ANOVA, $P < 0.060$).


A
Glucose output (% control)
300
250
200
150
100
50
0
a
b
a
ab*
Control
Epi
Cyclo
C + Epi
B
Cortisol 30 min
- Epi 60 min
Glucose
Glucose output (% control)
200
150
100
50
0
0
100
1000
Cyc
Cyc+cort
Cortisol (ng/ml)
D
BA 30 min
- Epi 60 min
Glucose
Glucose output (% control)
200
150
100
50
0
0
5
25
Cyc
Cyc+BA
Benzyl alcohol (mM)
C
Cortisol 30 min
+ Epi 60 min
Glucose
Glucose output (% epi-stimulated)
200
150
100
50
0
a
ab
b
a
ab
0
100
1000
Cyc
Cyc+cort
Cortisol (ng/ml)
E
BA 30 min
+ Epi 60 min
Glucose
Glucose output (% epi-stimulated)
150
100
50
0
a
bc
bc
ab
c
0
5
25
Cyc
Cyc+BA
Benzyl alcohol (mM)

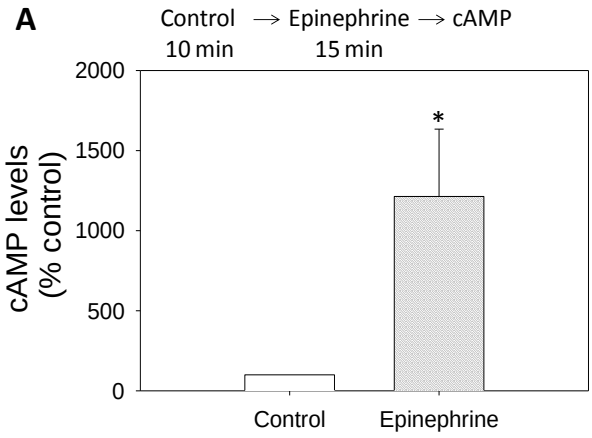
6.3.2 Second messenger - cAMP levels

Cortisol treatment significantly reduced epinephrine-stimulated cAMP levels, compared to epinephrine alone (Fig. 6-2A), when treated 10 min before epinephrine exposure (Fig. 6-2B). The effect of cortisol on epinephrine-stimulated cAMP accumulation is rapid and transient, as cortisol treatment 30 min before epinephrine treatment (Fig. 6-3C) did not significantly suppress epinephrine-stimulated cAMP accumulation (Fig. 6-3A), although cortisol alone reduced cAMP levels at this time point (40 min total cortisol incubation, Fig. 6-3B). Similarly, treatment with benzyl alcohol 30 min prior to epinephrine did not significantly modify basal or epinephrine-stimulated cAMP levels (Fig. 6-3C and D).

In the context of the hormonal response to acute stress, plasma epinephrine levels typically increase prior to an elevation in plasma cortisol levels. Therefore the ability of cortisol to rapidly modulate epinephrine-stimulated cAMP accumulation following epinephrine treatment (post-cortisol treatment) was also investigated (Fig. 6-4). In order to mimic an endogenous hormonal stress response, hepatocytes were initially treated with epinephrine for 10 min followed by brief cortisol exposure (15 min). Epinephrine significantly stimulated cAMP accumulation within 10 min (Fig. 6-4A), which was rapidly (15 min) suppressed by post-cortisol treatment (100 and 1000 ng/ml; Fig. 6-4B). In contrast, post-treatment with benzyl alcohol significantly amplified epinephrine-stimulated cAMP accumulation, by more than 2-fold (Fig. 6-4C). Cortisol exposure (10 or 30 min) after initial epinephrine exposure (15 min) increased epinephrine-stimulated CREB activation (ratio of phospho-CREB to total CREB) at 10 min post-cortisol treatment (Fig. 6-5). This effect was transient, as cortisol significantly suppressed epinephrine-stimulated CREB phosphorylation 30 min post-cortisol treatment (Fig. 6-5). This rapid and transient effect on epinephrine-stimulated CREB activation was only evident at high cortisol concentrations (1000 ng/ml).

Figure 6-2. Rapid effect of cortisol treatment on epinephrine-stimulated cAMP accumulation in trout hepatocytes.

(A) cAMP levels in response epinephrine treatment (10^{-5} M) for 15 min compared to untreated hepatocytes. (B) Hepatocytes were treated with cortisol (1000 ng/ml) either 5 or 10 min prior to treatment with epinephrine (10^{-5} M). Values are plotted as % cortisol and show mean ± S.E.M of the percent control values (n = 4 independent fish); treatments with an * indicate treatment is significantly different compared to the control (Paired t-test, $P < 0.05$), whereas treatments with different letters are significantly different from each other (Repeated measures ANVA, $P < 0.05$).


A
Control → Epinephrine → cAMP
10 min 15 min
cAMP levels (% control)
2000
1500
1000
500
0
*
Control
Epinephrine

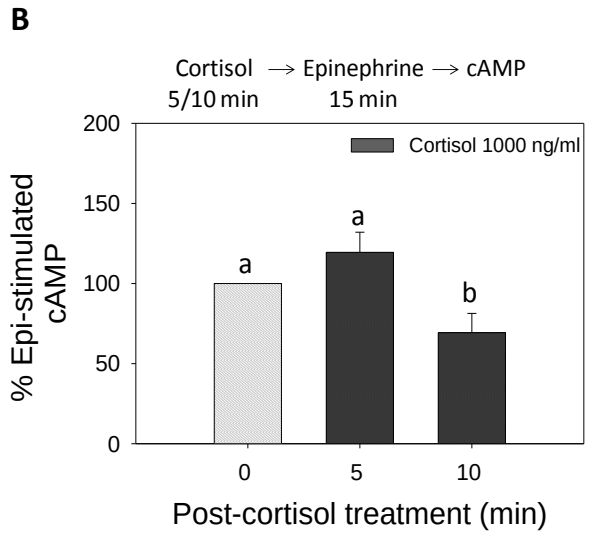

B
Cortisol → Epinephrine → cAMP
5/10 min 15 min
% Epi-stimulated cAMP
200
150
100
50
0
Cortisol 1000 ng/ml
a
a
b
0
5
10
Post-cortisol treatment (min)

Figure 6-3. The effect of short-term (30 min) cortisol or benzyl alcohol treatment on epinephrine-stimulated cAMP levels in trout hepatocytes.

(A) cAMP levels following epinephrine treatment (10^{-5} M) for 10 min compared to untreated hepatocytes. Hepatocytes were treated with cortisol ((100 or 1000 ng/ml) for 30 min prior to treatment with (B) control media or (C) epinephrine (10^{-5} M) or with benzyl alcohol (25 mM) for 30 min prior to treatment with (D) control media or (E) epinephrine (10^{-5} M). Values are plotted as % cortisol and show mean ± S.E.M of the percent control values (n = 6 independent fish); bars with an * indicate treatment is significantly different compared to the control (Paired t-test, $P < 0.05$), whereas treatments with different letters are significantly different from each other (repeated measures ANOVA, $P < 0.05$).

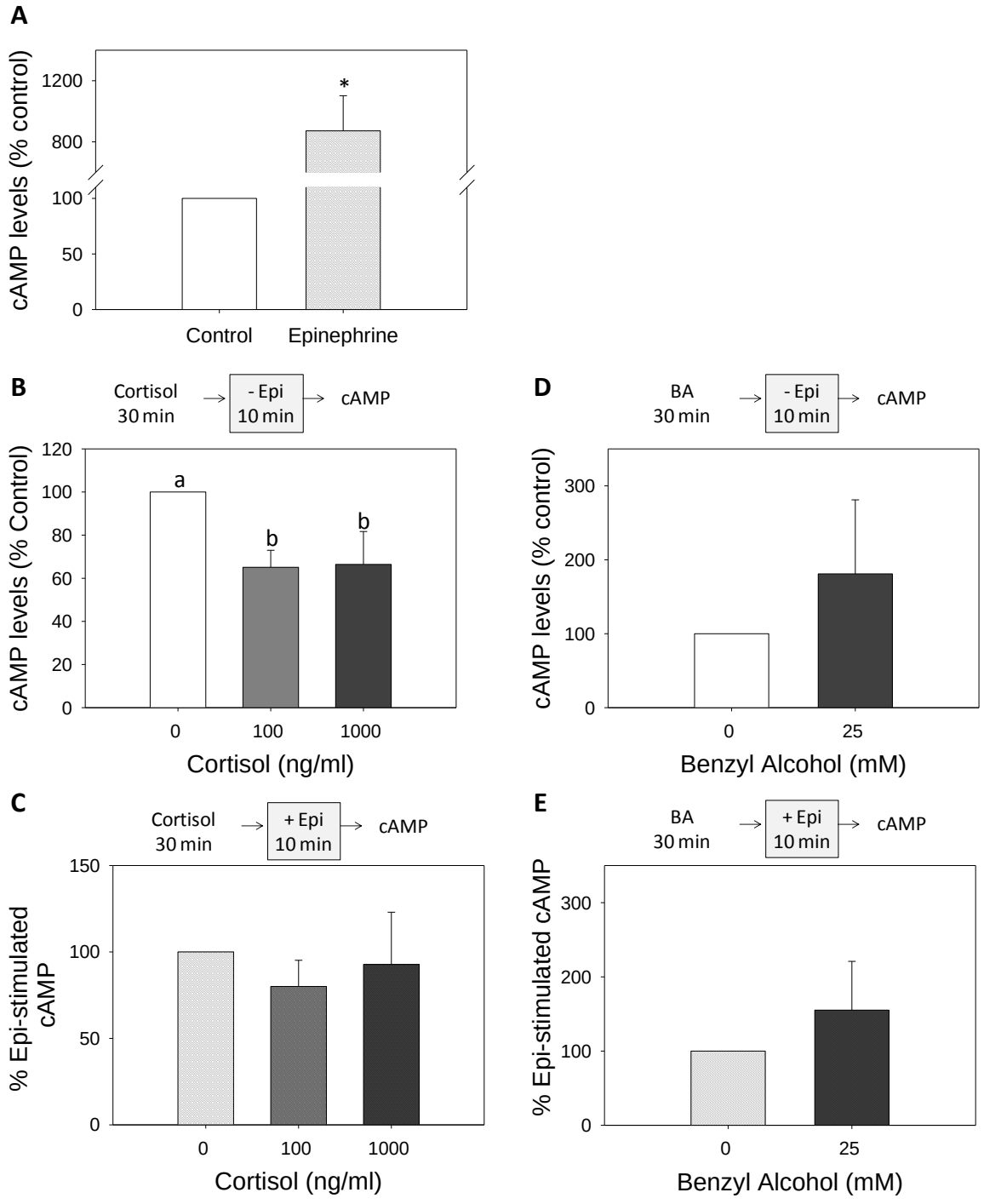
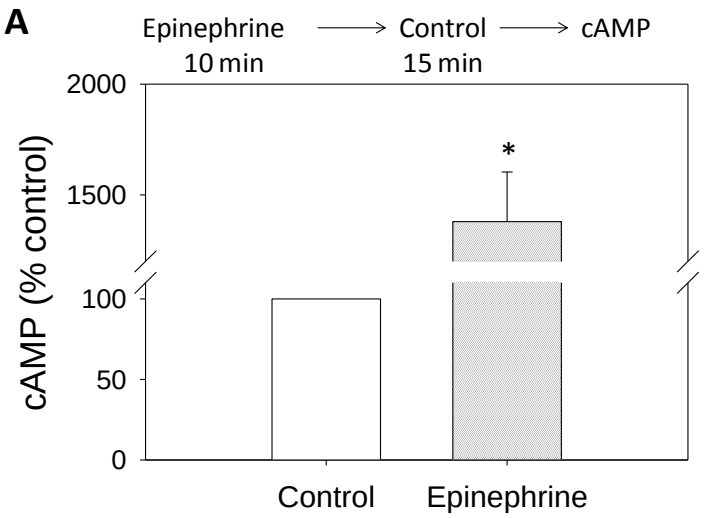
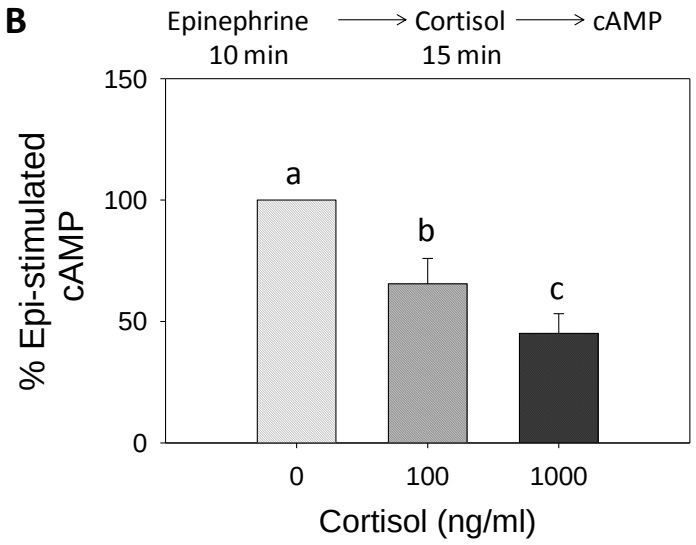
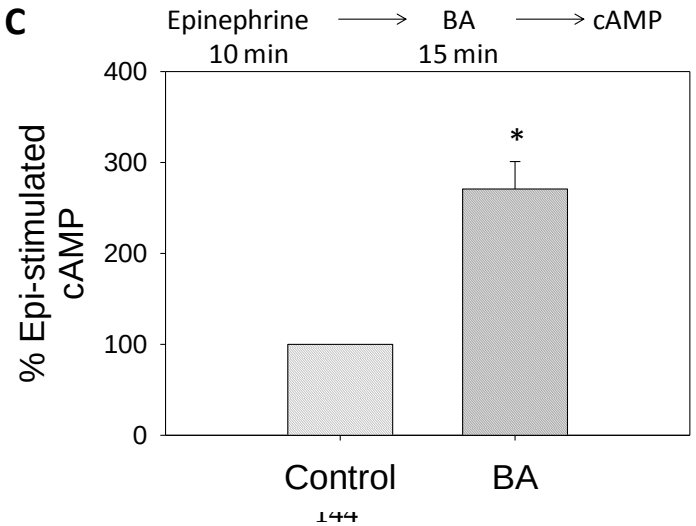

A
cAMP levels (% control)
1200
800
100
50
0
*
Control
Epinephrine
B
Cortisol
30 min
- Epi
10 min
cAMP
cAMP levels (% Control)
120
100
80
60
40
20
0
a
b
b
0
100
1000
Cortisol (ng/ml)
C
Cortisol
30 min
+ Epi
10 min
cAMP
% Epi-stimulated cAMP
150
100
50
0
0
100
1000
Cortisol (ng/ml)
D
BA
30 min
- Epi
10 min
cAMP
cAMP levels (% control)
300
200
100
0
0
25
Benzyl Alcohol (mM)
E
BA
30 min
+ Epi
10 min
cAMP
% Epi-stimulated cAMP
300
200
100
0
0
25
Benzyl Alcohol (mM)

Figure 6-4. Rapid effect of cortisol on epinephrine-stimulated cAMP accumulation in trout hepatocytes.

(A) Epinephrine (10^{-5} M) stimulated cAMP accumulation compared to control treated hepatocytes (total treatment time was 25 min). (B) The rapid (15 min) effect of cortisol treatment (100 and 1000 ng/ml) following epinephrine treatment (10 min) on cAMP levels. (C) The effect of short-term (15 min) benzyl alcohol treatment following epinephrine treatment (10 min) on cAMP levels. Values are plotted as % cortisol and show mean ± S.E.M of the percent control values (n = 4 independent fish); bars with an * indicate treatment is significantly different compared to the control (Paired t-test, $P < 0.05$), whereas treatments with different letters are significantly different from each other (repeated measures ANOVA, $P < 0.05$).


A
Epinephrine
10 min
Control
15 min
cAMP
2000
1500
100
50
0
cAMP (% control)
*
Control
Epinephrine


B
Epinephrine
10 min
Cortisol
15 min
cAMP
150
100
50
0
% Epi-stimulated cAMP
a
b
c
0
100
1000
Cortisol (ng/ml)


C
Epinephrine
10 min
BA
15 min
cAMP
400
300
200
100
0
% Epi-stimulated cAMP
*
Control
BA

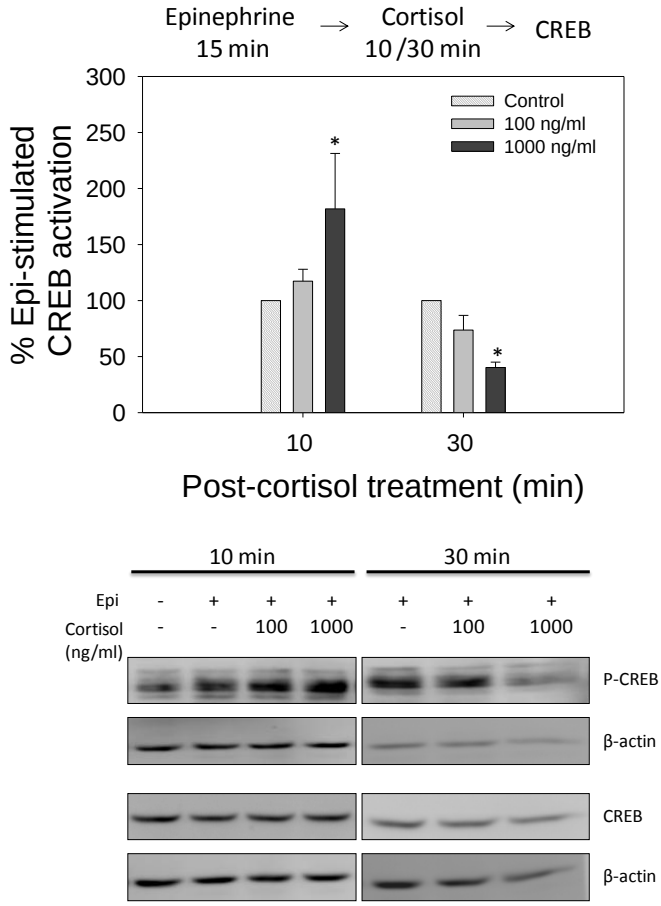


Figure 6-5. The effect of cortisol on epinephrine-induced CREB activation.

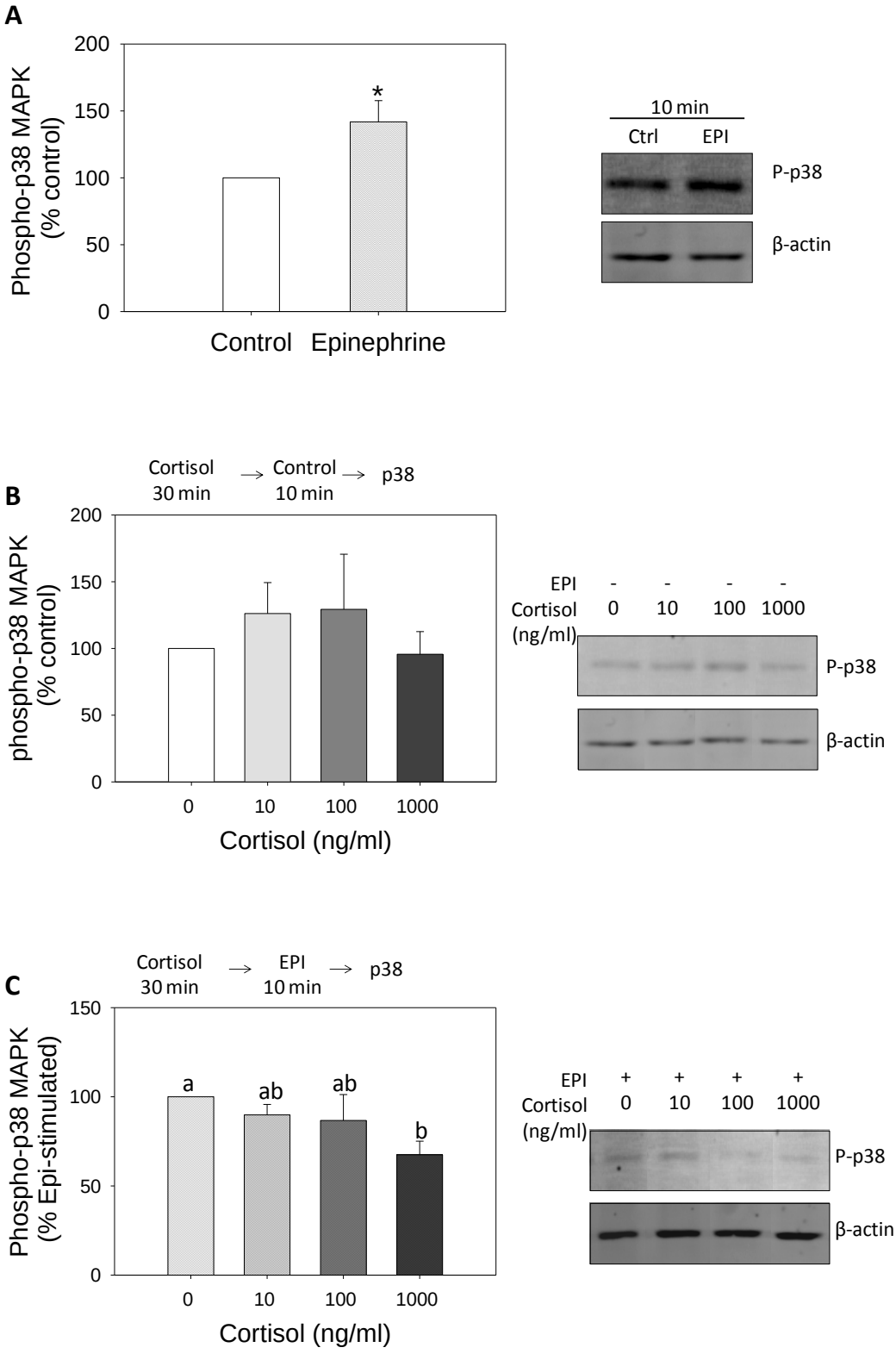
(A) Histogram and corresponding representative immunoblot of the time and dose response of the effect of cortisol (100 and 1000 ng/ml) on epinephrine-stimulated CREB activation in rainbow trout primary hepatocytes. Plated hepatocytes were initially treated with epinephrine (10^{-5} M) for 15 min, followed by cortisol treatment (0, 100 or 1000 ng/ml) for 10 or 30 min. Cell lysates (40 µg of protein) were probed with phospho-CREB (ser133) or total CREB monoclonal rabbit antibody (Cell Signaling Technology, Beverly, MA). Values plotted are the ratio of the phosphorylated to total CREB densitometric values, which were normalized to β-actin values for each protein band. Values are plotted as % control and show mean ± S.E.M of the % control values (n = 3 independent fish); An * indicates a significant interaction of the treatment within each time point (repeated measures ANOVA, $p < 0.05$).

6.3.3 MAPK phosphorylation

Accumulation of second messengers results in the activation of various protein effectors; therefore the effect of cortisol on epinephrine-stimulated p38 MAPK phosphorylation (Fig. 6-6A) was examined. Cortisol treatment on its own had no significant effect on p38 phosphorylation levels (40 min total incubation with cortisol; Fig. 6-6B), however high cortisol levels (1000 ng/ml) significantly suppressed epinephrine-stimulated p38 MAPK phosphorylation (Fig. 6-6C). Alone, benzyl alcohol, at the highest dose (25 mM), significantly increased phospho-p38 levels, whereas lower doses (5 and 10 mM) tended ($P < 0.06$) to increase phosphorylation levels (Fig. 6-7A). In comparison, benzyl alcohol had no effect on epinephrine-stimulated phospho-p38 MAPK levels (Fig. 6-7B).

Figure 6-6. The effect of short-term (30 min) pre-cortisol treatment on epinephrine-stimulated phospho-p38 mitogen-activated protein kinase (MAPK) levels.

(A) Histogram and corresponding representative immunoblot of phospho-p38 levels in liver slices treated with epinephrine (10^{-5} M) for 10 min. Histogram and corresponding representative immunoblot of phospho-p38 levels in liver slices treated with cortisol (10, 100, or 1000 ng/ml) for 30 min and were then treated with (B) control media or (C) epinephrine (10^{-5} M) for 10 min. Liver slice homogenates (40 μg protein) were probed with phospho-specific p38 MAPK monoclonal rabbit antibody (Cell Signaling Technology, Beverly, MA). Phospho-p38 MAPK densitometric values were normalized to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO) and are plotted as % control and show mean ± S.E.M of percent control values (n = 5 independent fish). Treatments with an * are significantly different compared to the control (repeated measures ANOVA, $p < 0.05$).


A
Phospho-p38 MAPK
(% control)
200
150
100
50
0
*
Control
Epinephrine
10 min
Ctrl
EPI
P-p38
β-actin
B
Cortisol
30 min
Control
10 min
p38
phospho-p38 MAPK
(% control)
200
150
100
50
0
0
10
100
1000
Cortisol (ng/ml)
EPI
Cortisol
(ng/ml)
-
-
-
-
0
10
100
1000
P-p38
β-actin
C
Cortisol
30 min
EPI
10 min
p38
Phospho-p38 MAPK
(% Epi-stimulated)
150
100
50
0
a
ab
ab
b
0
10
100
1000
Cortisol (ng/ml)
EPI
Cortisol
(ng/ml)
+
+
+
+
0
10
100
1000
P-p38
β-actin

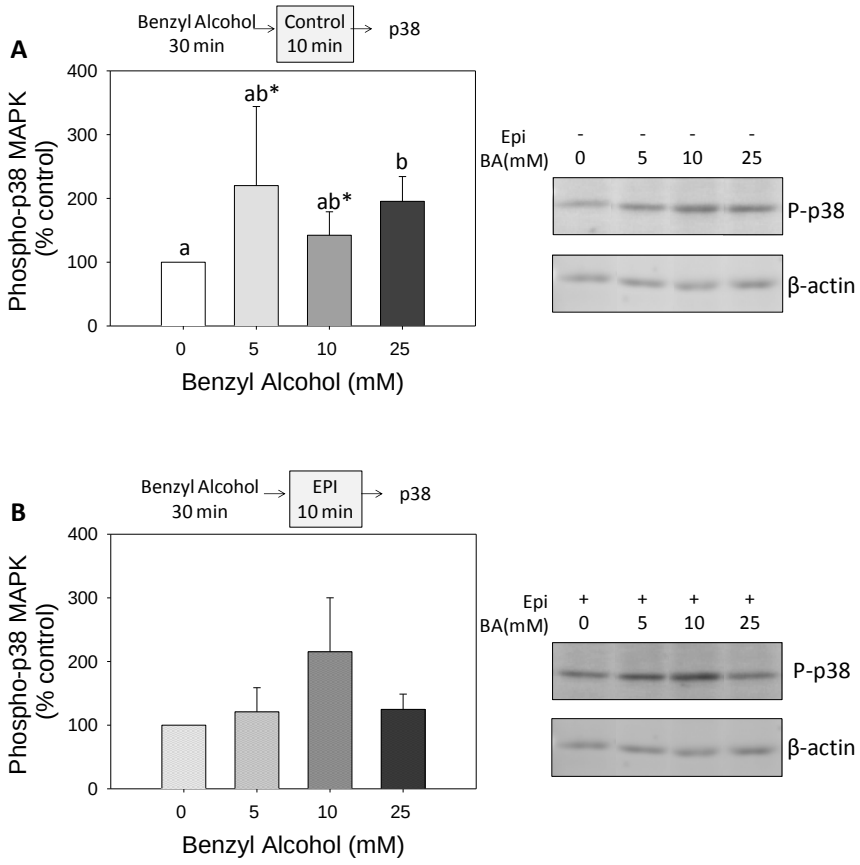


Figure 6-7. The effect of short-term pre-treatment with benzyl alcohol on epinephrine-stimulated phospho-p38 mitogen-activated protein kinase (MAPK) levels in trout liver slices.

(A) Histogram and corresponding representative immunoblot of phospho-p38 levels in liver slices treated with epinephrine (10^{-5} M) for 10 min. Histogram and corresponding representative immunoblot of phospho-p38 levels in liver slices treated with benzyl alcohol (5, 10 or 25 mM) for 30 min and were then treated with (B) control media or (C) epinephrine (10^{-5} M) for 10 min. Liver slice homogenates (40 µg protein) were probed with phospho-specific p38 MAPK monoclonal rabbit antibody (Cell Signaling Technology, Beverly, MA). Phospho-p38 MAPK densitometric values were normalized to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO) and are plotted as % control and show mean ± S.E.M of percent control values (n = 5 independent fish). Treatments with different letters are significantly different compared to the control (repeated measures ANOVA, $p < 0.05$). Bars with an * indicates that treatment is significantly different than control (repeated measures ANOVA, $P < 0.06$).

6.4 Discussion

This is the first study to characterize the rapid effects of stressor-induced cortisol levels on adrenergic responses in the liver. Importantly, this study demonstrates that cortisol rapidly suppresses epinephrine-induced glucose production in trout liver and this corresponded with alteration in signaling pathways, including reduced cAMP accumulation and modulation of CREB and p38 MAPK phosphorylation. The cortisol-mediated suppression of epinephrine-stimulated hepatic glucose production may involve genomic changes as cycloheximide negated some of these effects.

This is the first study to report rapid effect of cortisol on epinephrine-stimulated pathways in liver of any animal model. Notably, short-term cortisol treatment significantly reduced epinephrine-stimulated glucose production. This is in contrast to chronic cortisol elevations (i.e. several days), which in teleost hepatocytes enhances epinephrine-stimulated glucose production (Dugan and Moon, 1998; Reid et al., 1992; Takahashi and Iizuka, 1991) and epinephrine-simulated glycogen breakdown (Vijayan et al., 1993). Therefore, during the initial phase of the acute stress response, cortisol appears to suppress epinephrine-mediate glucose output, whereas chronic cortisol elevations enhance the hepatic adrenergic response (Vijayan et al., 1993). The initial attenuation of epinephrine-induced glucose production may prevent excessive glucose secretion and instead favour internal glucose utilization to support enhanced liver energy demand to cope with stress. In fact, cortisol acutely (2 h) depletes intracellular liver glucose levels without enhancing glucose secretion into the media in trout hepatocytes, supporting enhanced glucose metabolism within the liver (Chapter 4).

Suppression of epinephrine-induced glucose production might be the result of altered cAMP-CREB signaling, as cortisol rapidly inhibited cAMP accumulation and CREB phosphorylation in response to epinephrine in trout liver. Moreover, cortisol treatment rapidly reduced cAMP accumulation both prior to and following epinephrine treatment. There was a stronger inhibition when cortisol exposure occurred after epinephrine treatment (50 % reduction) compared to pre-treatment (10 min) with cortisol (30% reduction). A possible explanation is that cortisol-inhibition of epinephrine-induced cAMP signaling is rapid and transient, but the mechanism is currently unknown. It remains to be seen if β - adrenergic receptor down regulation and/or desensitization is involved in this rapid cortisol effect on epinephrine signaling.

In addition to altering hormone-stimulated glucose production, suppression of the epinephrine-activated cAMP accumulation could have a myriad of consequences as this is a key signaling pathway regulating metabolic processes within the liver (Exton, 2009). Particularly,

accumulation of cAMP results in the activation of PKA, which regulates numerous downstream targets including CREB, which is a well characterized transcription factor (Jiang and Zhang, 2003). Since CREB is a key mediator of epinephrine-mediated genomic regulation (Terazono et al., 2003; Viguerie et al., 2004), cortisol modulation of CREB activation likely also has longer-term genomic consequences in the liver. Moreover, CREB is activated in response to numerous stimuli besides hormones, including cytokines (Wen et al., 2010), elevated glucose levels (Jansson et al., 2008), as well as hypoxia (Leonard et al., 2008; Lin et al., 2009). Therefore, it will be interesting to investigate whether cortisol acutely modulates CREB activation in response to other stress stimuli, thereby fine tuning the cellular stress response.

Although not as well characterized as epinephrine-mediated CREB signaling, MAPK proteins, such as p38, are also activated in response to catecholamine treatment and have been implicated in glucose metabolism (Kanda and Watanabe, 2009). Consistent with this, the current study demonstrated that epinephrine treatment induces p38 MAPK phosphorylation in trout hepatocytes, and pre-treatment with cortisol attenuated this MAPK response. Although the role of p38 MAPK in adrenergic effects have not been well characterized, p38 MAPK is an important transcription factor for several metabolic genes (Cao et al., 2005; Collins et al., 2006; Xiong et al., 2007). Cortisol suppression of epinephrine-stimulated p38 MAPK phosphorylation might be mediated by MAPK phosphatase-1, which has been implicated in GC induced p38 dephosphorylation (Kassel et al., 2001), but this remains to be investigated. Nonetheless, cortisol suppression of MAPK activation by epinephrine further supports an overall attenuation of epinephrine signaling shortly after stressor exposure, likely via a nongenomic signaling mechanism.

Cortisol treatment alone has previously been shown to rapidly suppress cAMP levels in teleost species (see Chapter 3, (Borski et al., 2002). In the tilapia pituitary, cortisol rapidly suppressed prolactin release by reducing cAMP levels (Borski et al., 2002). Similar suppressive effects on epinephrine-stimulated cAMP levels have also been reported for other steroid hormones, such as estradiol, which reduces basal and epinephrine-stimulated cAMP accumulation in rabbit aortic tissues (Kishi and Numano, 1982). In contrast, aldosterone potentiates the effect of vasopressin on cAMP formation in the mammalian inner medullary collecting duct (Sheader et al., 2002). In rat hypothalamic tissue, progesterone also reduces catecholamine induction of cAMP by inhibiting the α-1-adrenergic receptor enhancement of β-adrenergic-activated cAMP production (Petitti and Etgen, 1992).

The mechanism behind cortisol-mediated inhibition of epinephrine-stimulated cAMP remains to be determined in rainbow trout liver, but may involve alterations to the plasma membrane given our recent finding (Dindia et al., 2012) and also because membrane order changes affect β – adrenergic receptor activity (Escribá et al., 2007). To this end, we also examined the effect of the chemical fluidizer, benzyl alcohol, on hepatic epinephrine responses. Interestingly, benzyl alcohol also potently suppressed glucose output following epinephrine treatment and modified cAMP signaling in response to epinephrine, suggesting that adrenergic signaling in rainbow trout liver is sensitive to membrane order perturbations. In support of this, benzyl alcohol has previously been shown to modulate epinephrine-stimulated cAMP production in rainbow trout (McKinley and Hazel, 2000) and several studies in mammal models have reported correlations between plasma membrane fluidity and β-adrenergic receptor activity (Hanski et al., 1979; Hirata et al., 1979; Lurie et al., 1985; Ma et al., 1994). Moreover, nanoscale lateral reorganization of lipid rafts induced by alterations in cholesterol levels profoundly regulates β-adrenergic receptor function in HEK293 cells (Pontier et al., 2008). Therefore, cortisol-mediated fluidization and microdomain organization (Chapter 2 and 3) may also be playing a role in the acute inhibition of adrenergic signaling.

In conclusion, the results from this study suggest that cortisol acutely modulates hepatic adrenergic signaling by altering epinephrine-stimulated glucose production. The acute effect of cortisol on epinephrine glucose production was accompanied by altered epinephrine-stimulated cAMP, CREB and p38 MAPK activity, supporting a nongenomic mediated interaction between these two stress hormones. In light of the findings presented in previous chapters, cortisol not only independently regulates hepatic signaling cascades, but also rapidly alters transduction in response to epinephrine, an important co-regulator of the stress response. The rapid effect of cortisol on adrenergic signaling further suggests an adaptive role for rapid cortisol action in regulating the integrated hepatic hormonal response during the initial phase of the stress response. We hypothesize a role for cortisol-mediated membrane perturbations as a mechanism that may be partly responsible for the observed attenuation of epinephrine responsiveness in trout hepatocytes.

6.5 Acknowledgements

This study was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada discovery grant and Discovery Accelerator Supplement to MMV. LD was the recipient of the NSERC post-graduate scholarship. Thank you to Salah Sanjar for assisting with the liver tissue experiments shown in Figure 1., Figure 6., and Figure 7.

Chapter 7**: General Conclusions**

7.1 General Conclusion

The overall goal of this thesis was to establish the involvement of rapid, nongenomic cortisol action and the mechanism(s) involved in mediating the physiological responses to this steroid hormone in the liver using rainbow trout (*Oncorhynchus mykiss*) as a model species. As a whole, the data presented provide novel insight into cortisol action in the liver by establishing, for the first time, that cortisol does stimulate rapid responses that are nongenomic in the liver *in vitro*, and that these effects likely contribute to stress adaptation, as similar effects were demonstrated *in vivo*. Particularly, the studies provide evidence for an intracellular glucocorticoid receptor-independent cortisol signaling pathway by establishing for the first time that:

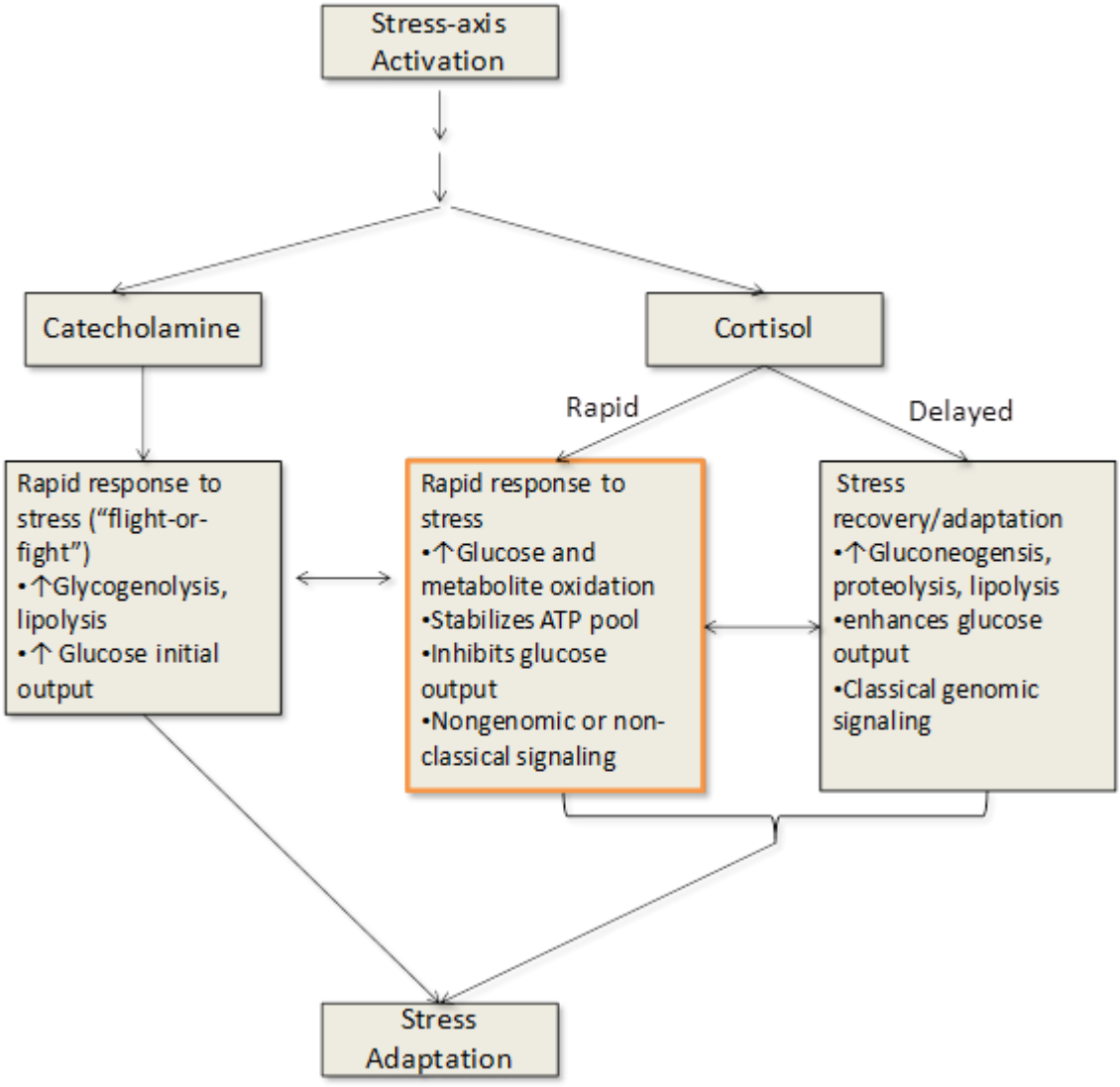
1. Cortisol rapidly alters functional properties of hepatic plasma membranes by reducing lipid order (increases lipid fluidity) and induces structural and physical microdomain changes, and modulates signaling pathways (Chapter 2).
2. Stressor-induced cortisol elevation *in vivo* results in liver plasma membrane fluidization and increases membrane roughness, supporting a role for cortisol-mediated plasma membrane perturbation as a key mechanism in acute stress adaptation (Chapter 3).
3. Rapid modulation of stress signaling pathways, including PKA, PKC, Akt and MAPKs, both *in vitro* (Chapter 2 and 4) and *in vivo* (Chapter 3) are involved in the cortisol-mediated acute effects on liver stress responses.
4. Nongenomic cortisol signaling modulates oxidative substrates, including glucose and amino acid levels, and perturbs intermediary metabolite levels without altering the activity of key enzymes involved in intermediary metabolism in trout liver (Chapter 5).
5. Rapid cortisol action alters adrenergic responses in the liver suggesting that acute stressor-induced cortisol not only has direct genomic and nongenomic effects, but also modulates liver responsiveness to other hormonal stimuli (Chapter 6).

Previously, the overriding hypothesis was that cortisol, via genomic signaling, facilitates the recovery from stress by enhancing glucose production through gluconeogenic pathways. While nongenomic cortisol signaling has been described in some detail for select mammalian systems (for instance, central nervous system, immune function, cardiovascular system), studies examining the contribution of nongenomic steroid signaling in liver metabolic adjustments to stress are for the most part unclear. The work presented in this thesis, for the first time, underscores a novel role for cortisol in mediating the immediate metabolic responses, along with catecholamine responses (Fig. 7-1). The

results suggest that cortisol acutely stimulates energy production through aerobic pathways, as cortisol significantly depleted liver glucose levels, without any changes to lactate levels, and this was also reflected in the tight regulation of adenylate energy charge ratio. In addition to direct effects, cortisol also rapidly enhances endogenous energy utilization indirectly by attenuating epinephrine-stimulated glucose output. Taken together, cortisol has distinct acute metabolic effects in the liver, which functions to maintain intracellular energy (ATP) levels. This is in contrast to genomic (delayed) cortisol action, which primarily supports stress adaptation by enhancing glucose production, through ATP consuming pathways.

Figure 7-1. Schematic diagram depicting the nongenomic cortisol action in the liver.

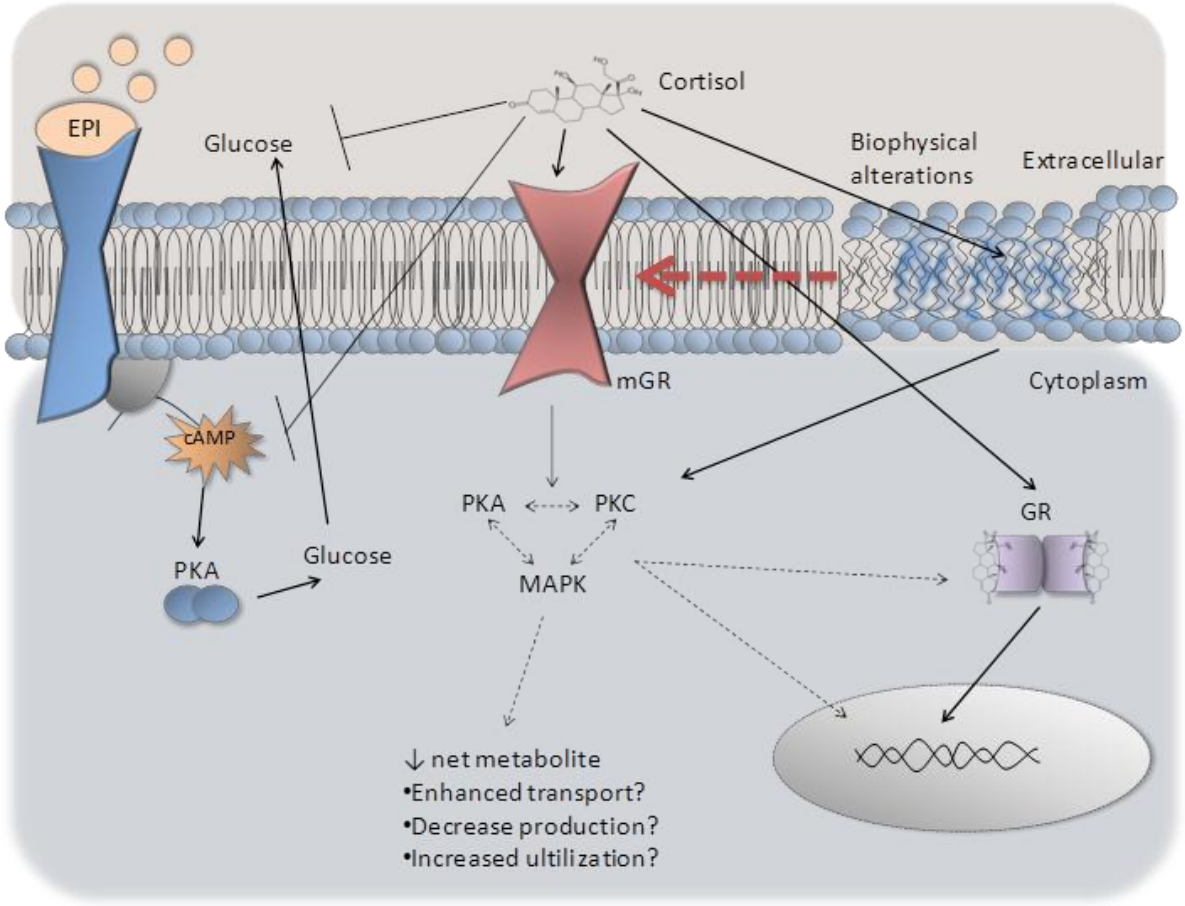
Solid arrows indicate confirmed cortisol effects; whereas dotted arrows indicate possible nongenomic cortisol effects that still need to be established. Overall, acute cortisol exposure results in altered liver metabolism, principally a decrease in liver glucose and lactate levels, possibly indicating enhanced aerobic ATP production to help cope with initial rise in energetic demands. In addition the rapid action of cortisol also regulates adrenergic responses, by attenuating intracellular signaling and glucose output in response to epinephrine (EPI). These acute metabolic effects are potentially mediated through rapid signaling events, as cortisol nongenomically alters protein kinase A (PKA), protein kinase C (PKC), AKT and mitogen-activated protein kinase (MAPK) pathways in trout liver. Although the precise mechanism involved still remains to be determined, the distinct alterations to the plasma membrane in response to cortisol elevation may facilitate this rapid response. In addition, rapid cortisol action may also be initiated through a novel membrane glucocorticoid receptor (mGR), or through the classical GR, however this awaits further experimentation.


Stress-axis Activation
Catecholamine
Cortisol
Rapid
Delayed
Rapid response to stress ("flight-or-fight")
•↑Glycogenolysis, lipolysis
•↑ Glucose initial output
Rapid response to stress
•↑Glucose and metabolite oxidation
•Stabilizes ATP pool
•Inhibits glucose output
•Nongenomic or non-classical signaling
Stress recovery/adaptation
•↑Gluconeogensis, proteolysis, lipolysis
•enhances glucose output
•Classical genomic signaling
Stress Adaptation

Although the precise mechanisms remain to be established, the studies provide evidence for nongenomic signaling through activation of the cAMP-PKA-CREB, PKC, and MAPK pathways as cortisol rapidly modulates these signaling pathways in liver tissue both *in vivo* and *in vitro.* Rapid activation of these signaling pathways may be mediated through membrane perturbations in response to cortisol-lipid interactions as hepatic signaling is responsive to chemical fluidization induced by benzyl alcohol. In addition to mechanotransduction through altered membrane properties, the classical GR signaling might play a role, as some nongenomic responses (e.g. amino acid depletion) were sensitive to mifepristone, a GR-antagonist. In addition to the intracellular GR, rapid cortisol effects might be mediated through a novel membrane-bound glucocorticoid receptor (mGR) as there is a 65-70 kDa plasma membrane protein which specifically binds cortisol in trout liver (Appendix A). The molecular weight of the cortisol-binding protein in trout liver plasma membrane corresponds to the mass of a putative glucocorticoid receptor partially purified in newt neuronal membranes. Combined, the results indicate an integrative role of cortisol in mediating the global response to acute stress within the liver, which involves direct enhancement of endogenous energy pools as well as regulating the "flight-or-fight" metabolic responses to adrenergic stimulation (Fig.7-2). Although this response may involve glucocorticoid receptors (either membrane or intracellular) the results also support a novel membrane-mediated stress adaptation response through direct perturbations to the plasma membrane in response to cortisol plasma elevation.

Figure 7-2. Schematic diagram representing acute cortisol action in rainbow trout liver.

The proposed liver response to acute cortisol involving rapid and delayed cortisol effects, which mediate both direct (e.g. altered metabolite turnover) and indirect (e.g. alter adrenergic sensitivity) physiological responses that facilitate stress adaptation. Catecholamines are thought to be principally responsible for the physiological responses immediately following stressor exposure, whereas cortisol is assumed to aid in recovery through genomic effects. The results from the studies presented in this thesis (indicated by orange outline), indicate a new role for cortisol in the immediate response by enhancing intracellular energy supplies through increased glucose oxidation and reduced epinephrine-stimulated glucose output.


Cortisol
EPI
Glucose
Biophysical
alterations
Extracellular
mGR
Cytoplasm
cAMP
PKA
PKC
GR
PKA
Glucose
MAPK
↓ net metabolite
•Enhanced transport?
•Decrease production?
•Increased ultilization?

Appendix A
Partial purification of a putative plasma membrane cortisol-binding protein

Introduction

The transcription-dependent effects of cortisol are mediated by either the mineralocorticoid (MR) or the glucocorticoid (GR) receptor, both of which belong to the nuclear receptor superfamily. The pathway leading to the activation these ligand-dependent transcription factor has been under intense investigation and is relatively well characterized. In contrast, the mechanisms leading to nongenomic actions of cortisol still remain elusive and poorly characterized. There are three main hypothesis for the initiation of rapid cortisol effects. First, cortisol is a lipophilic molecule which is able to interact with the phospholipids of cellular membranes. The incorporation of cortisol into the plasma membrane is known to alter the biophysical properties and structure of hepatic plasma membranes, which could activate cellular signaling pathways via mechanotransduction. In addition to mechanotransduction mediated steroid effects, several studies have provided evidence that rapid actions of cortisol are receptor-mediated. Recent reports indicate that in addition to regulating transcriptional events, the intracellular MR and GR are capable of eliciting rapid signaling events, particularly in the brain and in the immune system. Although not yet identified, there is also evidence for a non-classical plasma membrane glucocorticoid receptor.

In the liver, several studies have shown cortisol-specific binding sites. The high affinity binding sites have different binding characteristics compared to the intracellular GR, therefore suggesting a non-classical plasma membrane receptor. An attempt to identify a cortisol plasma membrane receptor was made using a sepharose-linked affinity chromatography column. Plasma membranes were detergent-solubilised and passed through the affinity column. A 63-70 kDa protein was specifically eluted with free cortisol from the affinity column. This is in accordance with a cortisol-binding protein identified in the newt neuronal membrane. The identity and sequence of this putative membrane protein remains to be determined.

Materials and Methods

Plasma membrane isolation

Trout were sampled by quickly netting and anesthetizing with 2-phenoxyethanol (1:1000). Liver tissue was flash frozen in liquid nitrogen and stored at -70°C for membrane preparation later. Liver plasma membranes were isolated using sucrose gradient as described previously {{212 Sulakhe, S.J. 1987}}. The membrane pellet was resuspended in TCD buffer (300 mM sucrose, 10 mM Tris-HCl, 1 mM dithiothreitol (DDT), 0.5 mM $CaCl_2$, 1X protease inhibitor cocktail, pH 7.5; Sigma, St. Louis, MO) and frozen at -70°C. All steps, including centrifugation, were carried out at 4°C.

Plasma membrane solubilisation

Membrane fraction obtained above was thawed immediately before and centrifuged at 20 000 x g for 10 min to pellet plasma membranes. Pellet was resuspended to (4-5 mg protein/ml) in solubilisation buffer (20 mM HEPES, 1mM EGTA, 5 mM MgCl2, Roche Proteinase inhibitor cocktail, pH 7.4) containing either triton-x 100 (1 or 4%) or digitonin. (1 or 4%) and gently vortexed followed by brief sonication for 20 seconds. Membranes were place at 4°C, with gentle shaking (end-over-end) overnight. Following overnight incubation, membrane fraction was centrifuged at 110 000 x g for 60 min at 4°C. Supernatant and pellet (resuspended in solubilization buffer + detergent) were collected and subjected to SDS-PAGE.

SDS-PAGE

Samples were diluted in Laemmli's sample buffer (1M tris-HCl, pH 6.8, 60 mM, glycerol 25%, SDS 2%, β-mercaptoethanol 14.4 mM, bromophenol blue 0.1%) and were separated on 10% SDS-PAGE as described by Laemmli using Bio-Rad's (Bio-Rad, Mississauga, ON) 1D electrophoresis system. Gels were stained with Coomasie blue stain (1% Coomassie, 10% acetic acid, 40% methanol) or SYPRO Ruby (Bio-Rad, Mississauga, ON) stain as per manufactures' instructions.

Cortisol-agarose affinity column

The free carboxyl group on the cortisol analogue, cortisol-21-hemisuccinate, was coupled to an amino-terminal cross-linked agarose gel (Affi-gel 102, Bio-Rad, Mississauga, On), using a carbodiimide coupling reagent as per manufactures' instructions. Briefly, 50 µM of 21-cortisol hemisuccinate was directly added to the affi-gel 102 mixed with an equal volume of dH_2O. Immediately after, 10 mg of the carbodiimide coupling reagent, 1-ethyl-3-(3-dimethylaminopropyl)

carbodiimide hydrochloride (EDC) was added. Throughout the coupling processing pH was continually readjusted to 4.7. Coupling proceeded overnight at 4°C, with gently shaking. After overnight incubation, the derivatized resin was added to a 5 ml flow-through column and was washed with 100 ml of 50 mM NaOAc (pH 7.4). dH_2O. Prior to loading the membrane fraction, the column was equilibrated with 25 ml of binding buffer (A; same composition as solubilisation buffer). Detergent soluble and insoluble fractions (4 mg/ml; 1.5 ml total) were loaded on separate gels and were rocked overnight at 4°C. Flow through fraction was collected and frozen at -80°C. The unbound proteins were washed with 40 ml of binding buffer (protein was non-detectable after 25 ml of buffer A). Cortisol-bound proteins were specifically eluted from the column in 1 ml aliquots by adding Buffer (A) containing hydrocortisone (0.5 mg/ml) and were immediately frozen at -80°C.

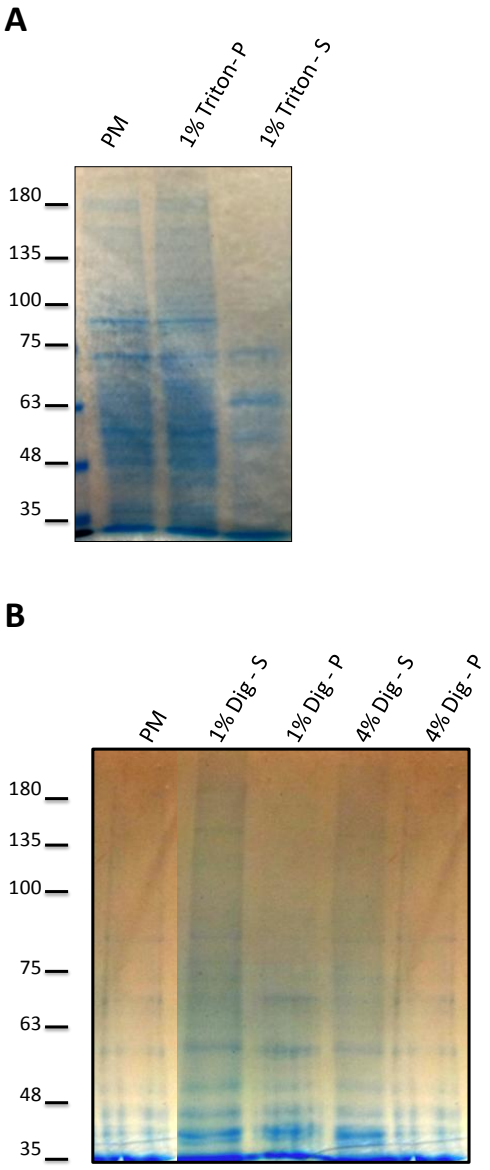
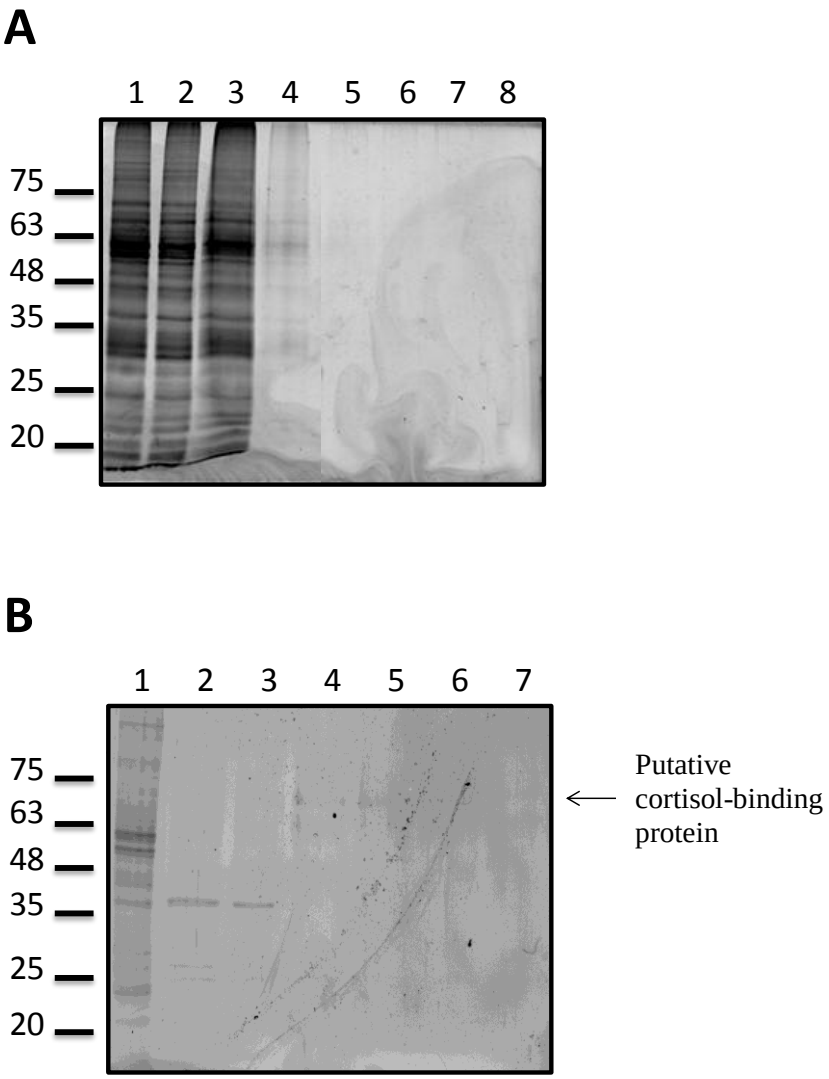


Figure A1. Coomassie stained SDS-PAGE of detergent-solubilized and insoluble plasma membrane proteins.

(A) Isolated trout plasma membranes were initially solubilised with triton-X-100 (Triton; 1%) and the solubilised (supernatant; S) and insoluble (pelleted fraction; P) were run on an SDS-PAGE (20 µg of protein). (B) For comparison, plasma membrane were solubilised with digitonin (Dig; 1 or 4%) the solubilised (supernatant; S) and insoluble (pelleted fraction; P) were run on an 10 % SDS-PAGE (20 µg of protein).

Figure A2. Sypro Ruby stained SDS-PAGE of affinity column fractions.

Sypro Ruby (Bio-Rad) stained SDS-PAGE of detergent-insoluble (A) and soluble (B) plasma membranes passed through a cortisol-linked agarose affinity column. (A) Affinity chromatography of detergent-insoluble plasma membrane proteins run on an 12% SDS-PAGE (1; plasma membrane fraction, 2; insoluble fraction, 3; initial wash; 4; fourth wash, 5-8;Eluted proteins with Buffer B in 1 ml aliquots, numbers corresponding to increasing elution aliquots). (B) Affinity chromatography of detergent-soluble plasma membrane proteins run on an 12% SDS-PAGE (1; soluble fraction, 2; initial wash with Buffer A; 3; fourth wash with Buffer A, 4-7;Eluted proteins with 1 ml aliquots of Buffer B, elutions 4-7). Arrow indicates a cortisol-specific binding protein between 63 and 75 kDa, detected in elutions 4-6.


A
1 2 3 4 5 6 7 8
75
63
48
35
25
20
B
1 2 3 4 5 6 7
75
63
48
35
25
20
Putative
cortisol-binding
protein

Appendix B

Signaling pathway activation by the peptide conjugate in rainbow trout liver: Supplemental information for Chapter 3

Materials and methods

Peptide Synthesis

Conjugation of cortisol to form a derivative was carried out as reported by Erlanger et al. (Erlanger et al., 1957). Cortisol-carboxymethyl oxime (Cortisol-CMO (4-pregnen-11b,17,21-triol-3,20-dione3-O-carboxymethyloxime, catalog number Q3888-000) was purchased from Steraloids Inc. (Newport, RI).) The peptide conjugated to the CMO is a 15 amino acid sequence of the steroidogenic acute regulatory protein (N-terminus-SGGEVVVDQPMERLY-C-terminus; Proteomics Core Facility, Washington State University, Pullman, WA). The PEP is conjugated via the serine to the CMO using a mixed anhydride technique (Erlanger et al., 1957) using *N*,*N*-dimethylformamide (DMF) as solvent, tri-*N*-butylamine, and isobutyl chloroformate. This conjugation procedure produces a product of 1:1 stoichiometry of a cortisol molecule to a single PEP sequence. The reaction is added to LH-20 Sephadex column to separate the cortisol-PEP, free cortisol, and free PEP. Based on 280 nm absorbance three peaks are derived from the separation on the column with the first peak as cortisol-PEP. This method of obtaining just the hormone conjugate has been confirmed for E2-PEP [20] using Waters QTOF-micro electrospray mass 89 spectometer with the sample introduced by direct infusion (Macromolecular Resources, Colorado State University, Fort Collins, CO; Arreguin-Arevalo and Nett). For methods on cell and tissue treatments please refer to Chapter 3.

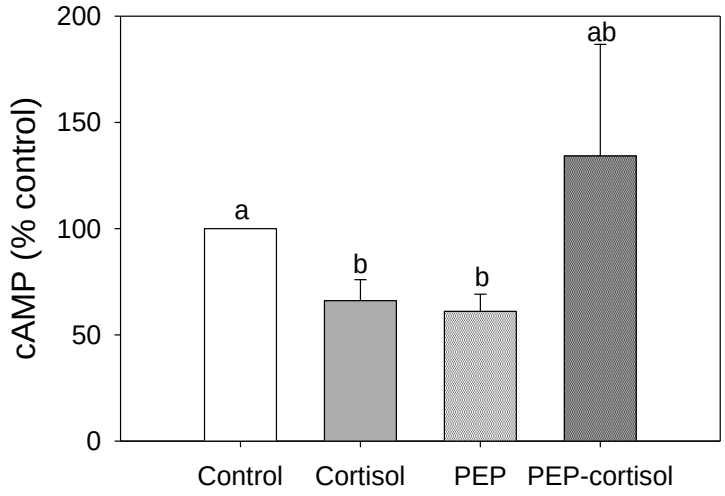


Figure B1. Rapid effect of cortisol and PEP-cortisol on cAMP in trout hepatocytes.

cAMP levels in response to brief (10 min) treatment with cortisol (1000 ng/ml), membrane impermeable cortisol-peptide conjugate (equal molarity to 1000 ng/ml of cortisol) and peptide moiety (equal molarity to 1000 ng/ml of cortisol). Values are plotted as % cortisol and show mean ± S.E.M (n = 6 independent fish); bars with different letters are significantly different (repeated measures ANOVA, $p < 0.05$).

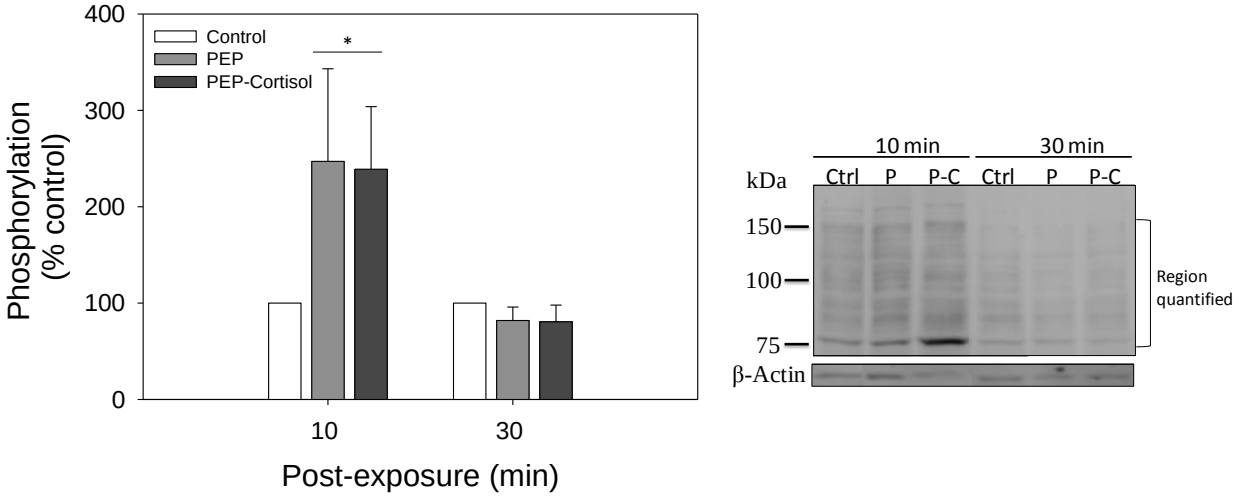


Figure B2. Phosphorylation status of protein kinase A (PKA) substrate proteins.

Histogram and corresponding representative immunoblot of the time and dose response of PKA substrate protein phosphorylation in rainbow trout liver slices following treatment with peptide moiety and cortisol-peptide conjugate (equal molarity to 1000 ng/ml cortisol). Liver samples (40 µg of protein) were probed with phospho-PKA Substrate (RRXS/T) polyclonal rabbit antibody (Cell Signaling Technology, Beverly, MA), which detects proteins that are phosphorylated within the phosphorylation motif for PKA. Equal loading was confirmed with β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometry values were obtained by quantifying the total phosphorylation intensity of each lane (region shown). Values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); bars with different symbols are significantly different within the time point (repeated measures ANOVA, $p < 0.05$).

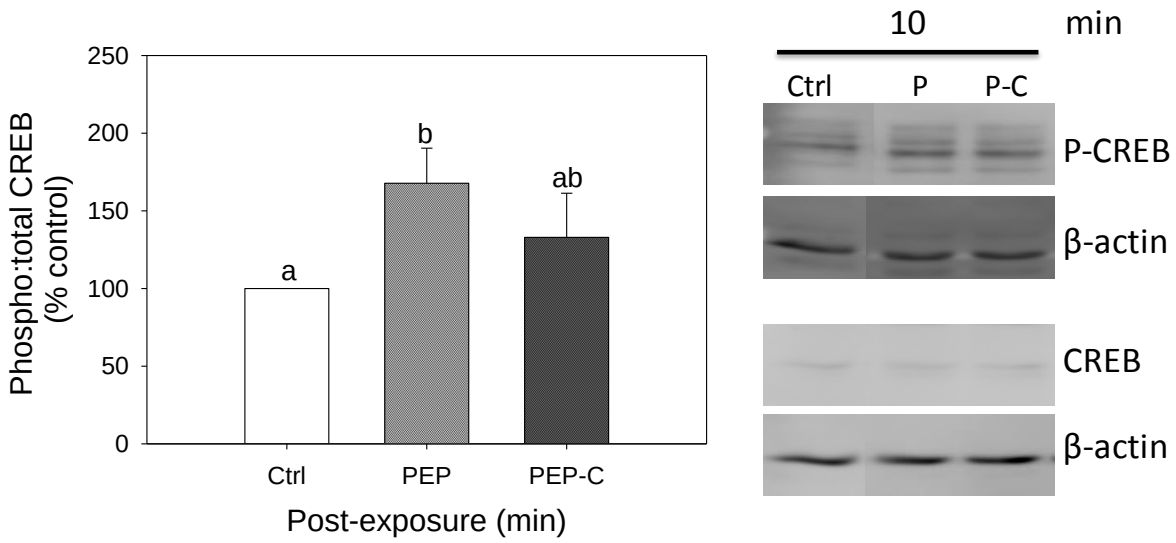


Figure B3. Rapid activation of CREB.

Histogram and corresponding representative immunoblot of CREB activation in rainbow trout primary hepatocytes following membrane impermeable peptide moiety or impermeable cortisol-peptide conjugate (equal molarity to 1000 ng/ml cortisol). Cell lysates (40 µg of protein) were probed with phospho-CREB (ser133) or total CREB monoclonal rabbit antibody (Cell Signaling Technology, Beverly, MA). Values plotted are the ratio of the phosphorylated to total CREB densitometric values. Values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); Inset indicates a significant treatment effect and bars with different letters are significantly different (repeated measures ANOVA, $p < 0.05$).

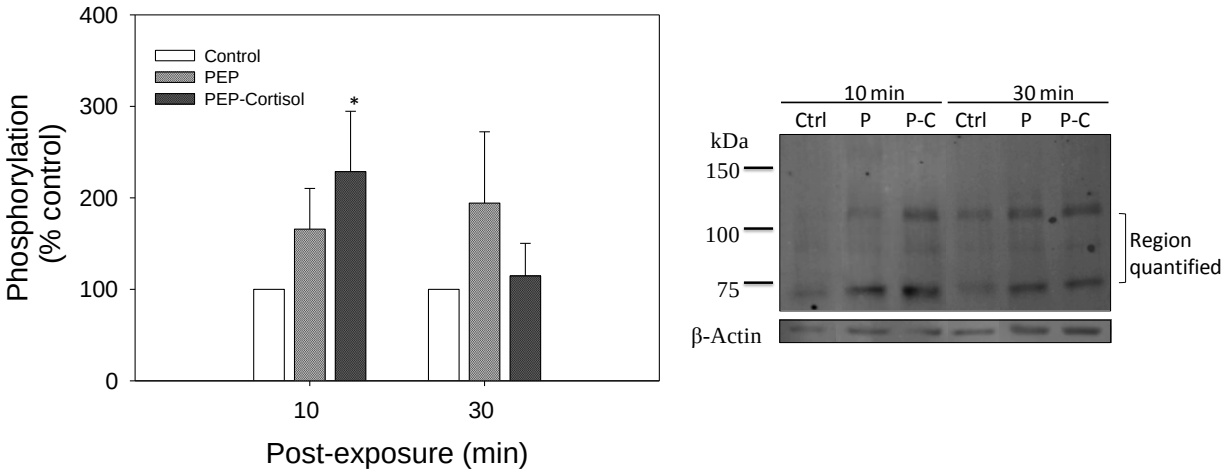


Figure B4. Phosphorylation status of protein kinase C (PKC) substrate proteins.

Histogram and corresponding representative immunoblot of the time and dose response of PKC substrate protein phosphorylation in rainbow trout liver slices following treatment with peptide moiety and cortisol-peptide conjugate (equal molarity to 1000 ng/ml cortisol). Liver samples were probed with phospho-(Ser) PKC substrate polyclonal rabbit antibody (Cell Signaling Technology, Beverly, MA), which detects proteins which are phosphorylated within the phosphorylation motif for PKC. Equal loading was confirmed with β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometry values were obtained by quantifying the total phosphorylation intensity of each lane (region shown). Values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); bars with different symbols are significantly different within the time point (repeated measures ANOVA, $p < 0.05$).

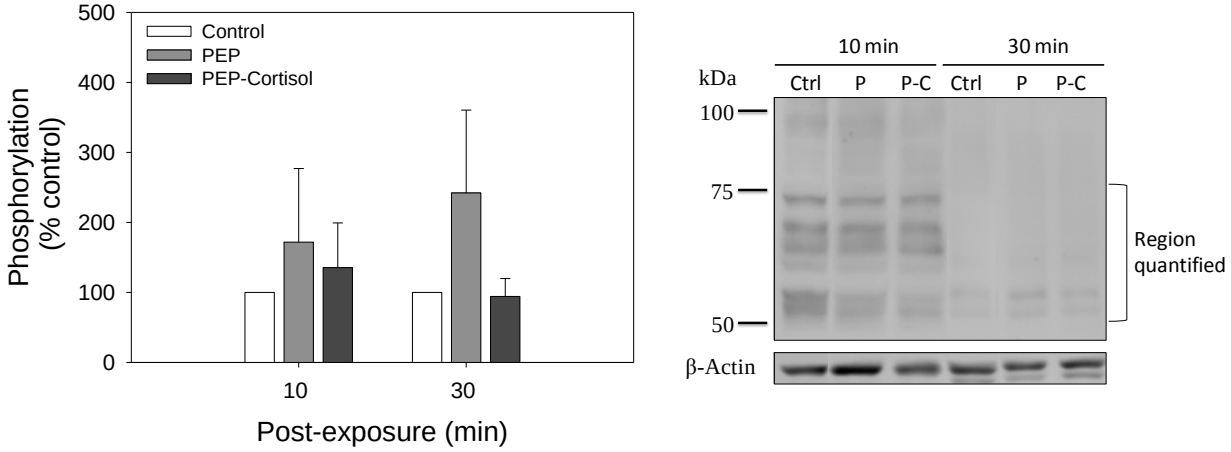


Figure B5. Phosphorylation status of AKT substrate proteins.

Histogram and corresponding representative immunoblot of the time and dose response of AKT substrate protein phosphorylation in rainbow trout liver slices following treatment with peptide moiety and cortisol-peptide conjugate (equal molarity to 1000 ng/ml cortisol). Liver samples were probed with phospho-Akt substrate (RXXS/T) polyclonal rabbit antibody (Cell Signaling Technology, Beverly, MA), which detects proteins that are phosphorylated within the AKT phosphorylation motif. Equal loading was confirmed with β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometry values were obtained by quantifying the total phosphorylation intensity of each lane (region shown). Values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); bars with different symbols are significantly different within the time point (repeated measures ANOVA, $p < 0.05$).

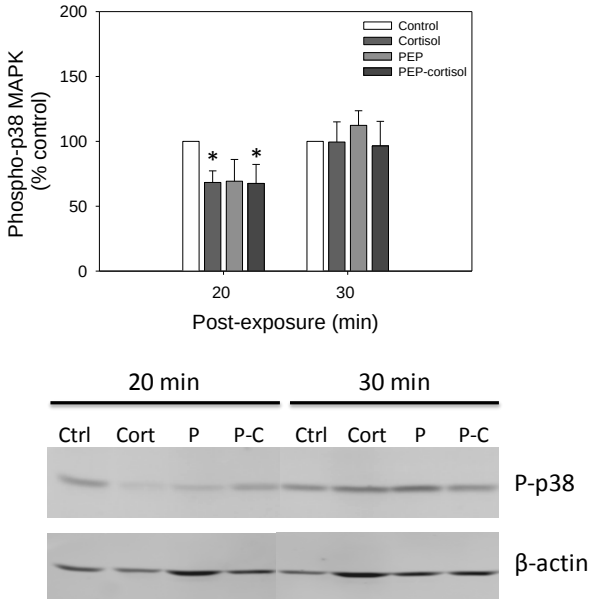


Figure B6. Regulation of p38 mitogen-activated protein kinase (MAPK) phosphorylation in trout liver slices.

Histogram and corresponding representative immunoblot of the time and dose response of p38 MAPK phosphorylation following treatment membrane impermeable peptide moiety or cortisol-peptide conjugate (equal molarity to 1000 ng/ml cortisol). Liver slices were probed with phospho-specific p38 MAPK monoclonal rabbit antibody (Cell Signaling Technology, Beverly, MA). Equal loading was confirmed with β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometric values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); bars with different symbols are significantly different within the time point (repeated measures ANOVA, $p < 0.05$).

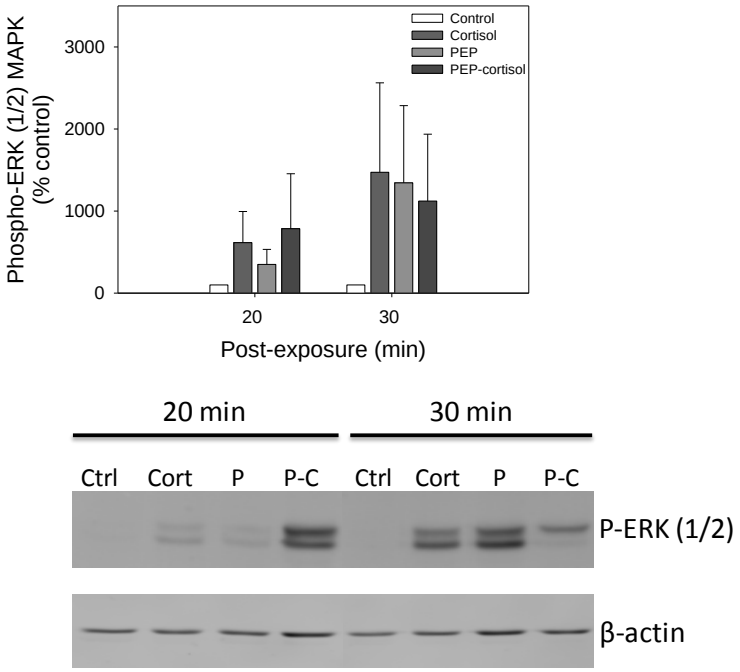


Figure B7. ERK (1/2) mitogen-activated protein kinase (MAPK) phosphorylation in trout liver slices.

Histogram and corresponding representative immunoblot of the time and dose response of ERK (1/2) MAPK phosphorylation following treatment membrane impermeable peptide moiety or cortisol-peptide conjugate (equal molarity to 1000 ng/ml cortisol). rainbow trout liver slices were probed with phospho-specific ERK(1/2) MAPK monoclonal rabbit antibody (Cell Signaling Technology, Beverly, MA). Equal loading was confirmed with β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Densitometric values are plotted as % control and show mean ± S.E.M (n = 5 independent fish); bars with different symbols are significantly different within the time point (repeated measures ANOVA, $p < 0.05$).

Appendix C

Supplemental Information for Chapter 5

Table 1C. Liver free amino acid levels (μM) in response to cortisol alone and in combination with cycloheximide (cyclo) or mifepristone (RU486).

Amino Acids	Control	Cortisol 100 ng/ml	Cortisol 1000 ng/ml	Cyclo	Cyclo + Cortisol	RU486	RU486 + Cortisol
Ala	28.12 ± 4.90	17.43 ± 3.15	16.92 ± 3.85	21.47 ± 4.58	16.84 ± 3.13	17.56 ± 3.90	18.35 ± 4.71
Asn	1.30 ± 0.24	1.14 ± 0.29	0.83 ± 0.13	1.10 ± 0.10	0.92 ± 0.13	0.96 ± 0.11	0.92 ± 0.11
2-AB	0.17 ± 0.04	0.12 ± 0.03	0.08 ± 0.02	0.13 ± 0.01	0.10 ± 0.02	0.11 ± 0.02	0.11 ± 0.02
Asp	1.63 ± 0.27	1.28 ± 0.20	0.96 ± 0.13	1.38 ± 0.17	1.12 ± 0.11	1.14 ± 0.23	1.10 ± 0.23
Glu	6.10 ± 1.24	4.33 ± 0.54	3.76 ± 0.53	5.00 ± 0.74	3.37 ± 0.35	3.50 ± 0.67	3.35 ± 0.67
Gln	0.38 ± 0.14	0.28 ± 0.08	0.16 ± 0.10	0.34 ± 0.10	0.18 ± 0.10	0.22 ± 0.09	0.19 ± 0.08
Gly	5.06 ± 1.03	3.21 ± 0.84	2.85 ± 0.70	4.14 ± 1.01	2.76 ± 0.80	3.18 ± 0.95	3.13 ± 0.94
Ile	1.35 ± 0.25	0.94 ± 0.24	0.74 ± 0.15	1.10 ± 0.23	0.88 ± 0.18	0.86 ± 0.20	0.77 ± 0.17
Leu	1.54 ± 0.32	1.01 ± 0.27	0.82 ± 0.18	1.31 ± 0.29	0.96 ± 0.20	0.94 ± 0.22	0.87 ± 0.20
Met	0.67 ± 0.14	0.50 ± 0.15	0.38 ± 0.09	0.53 ± 0.12	0.41 ± 0.12	0.39 ± 0.10	0.48 ± 0.17
Phe	0.72 ± 0.10	0.60 ± 0.09	0.57 ± 0.08	0.81 ± 0.20	0.62 ± 0.20	0.70 ± 0.20	0.65 ± 0.12

Table 1. Continued

Amino Acids	Control	Cortisol 100 ng/ml	Cortisol 1000 ng/ml	Cyclo	Cyclo + Cortisol	RU486	RU486 + Cortisol
Thr	3.42 ± 0.86	2.66 ± 0.80	1.75 ± 0.45	2.14 ± 0.45	1.38 ± 0.46	1.40 ± 0.42	1.33 ± 0.46
Tyr	1.51 ± 0.25	1.35 ± 0.33	0.98 ± 0.18	1.38 ± 0.33	1.00 ± 0.23	1.15 ± 0.28	1.03 ± 0.28
Val	1.32 ± 0.25	0.91 ± 0.25	0.67 ± 0.15	1.13 ± 0.24	0.85 ± 0.20	0.80 ± 0.17	0.74 ± 0.15

Values represent mean ± S.E.M (n = 6).Alanine (Ala), Asparagine (Asn), aminobutyrate (2-AB), Aspartate (Asp), glutamate (Glu), glutamine (Gln), Glutathione (GSH), glycine (Gly), isoleucine (Ile), leucine (Leu), methionine (Met), phenylalanine (Phe), threonine (Thr), tyrosine (Tyr), valine (Val)

Table 2C. Liver metabolite levels (μM) in response to cortisol alone and in combination with cycloheximide (cyclo) or mifepristone (RU486).

Metabolite	Control	Cortisol 100 ng/ml	Cortisol 1000 ng/ml	Cyclo	Cyclo + Cortisol	RU486	RU486 + Cortisol
ATP	0.25 ± 0.03	0.22 ± 0.04	0.19 ± 0.03	0.28± 0.06	0.24 ± 0.03	0.27 ± 0.06	0.22 ± 0.05
ADP	0.20 ± 0.04	0.13 ± 0.02	0.13 ± 0.02	0.18 ± 0.03	0.14 ± 0.02	0.15 ± 0.03	0.12 ± 0.02
AMP	0.39 ± 0.08	0.26 ± 0.06	0.31 ± 0.06	0.3 ± 0.06	0.25 ± 0.04	0.24 ± 0.05	0.21 ± 0.04
Acetate	1.50 ± 0.35	0.40 ± 0.08	0.98 ± 0.22	1.48 ± 0.40	0.90 ± 0.21	1.10 ± 0.31	1.14 ± 0.31
Acetone	0.84 ± 0.10	0.70 ± 0.07	0.61 ± 0.08	0.70 ±0.09	0.06 ± 0.09	0.65 ± 0.05	0.56 ± 0.06
Choline	0.70 ±	0.33 ± 0.03	0.45 ± 0.11	0.50 ± 0.09	0.38 ± 0.09	0.37 ±0.06	0.36 ± 0.09
Citrate	0.91 ± 0.21	0.47 ± 0.06	0.46 ± 0.08	0.75 ± 0.17	0.51 ± 0.10	0.53 ± 0.08	0.53 ± 0.08
Creatine	0.29 ± 0.07	0.15 ± 0.04	0.11 ± 0.03	0.21 ± 0.05	0.13 ± 0.03	0.15 ± 0.05	0.15 ± 0.04
Creatinine	0.24 ± 0.04	0.17 ± 0.02	0.13 ± 0.03	0.16 ± 0.01	0.13 ± 0.02	0.15 ± 0.02	0.13 ± 0.03
Formate	3.82 ± 0.45	3.83 ± 0.44	3.06 ± 0.28	4.20 ± 3.23	3.23 ± 0.42	3.19 ± 0.36	3.10 ± 0.49
Fumarate	0.20 ± 0.03	0.17 ± 0.04	0.13 ± 0.03	0.16 ± 0.03	0.16 ± 0.03	0.14 ± 0.02	0.13 ± 0.02
Glucose	16.41 ± 3.56	8.62 ± 1.17	9.22 ± 2.98	12.45 ± 3.0	9.24 ± 2.69	8.40 ±1.50	8.27 ± 2.20
GSH	1.06 ± 0.18	0.86 ± 0.09	0.89 ± 0.15	1.00 ± 0.19	0.80 ± 0.17	0.71 ± 0.07	0.73 ± 0.10

Table 2C. Continued.

Metabolite	Control	Cortisol 100 ng/ml	Cortisol 1000 ng/ml	Cyclo	Cyclo + Cortisol	RU486	RU486 + Cortisol
GPC	0.44 ± 0.10	0.28 ± 0.07	0.24 ± 0.07	0.36 ± 0.08	0.24 ± 0.07	0.26 ± 0.07	0.21 ± 0.04
Lactate	15.29 ± 3.70	6.09 ± 0.85	7.70 ± 2.27	11.79 ± 3.90	6.64 ± 2.75	8.28 ± 3.32	8.36 ± 2.75
Malonate	1.56 ± 0.25	0.97 ± 0.22	0.89 ± 0.17	0.97 ± 0.12	1.1 ± 0.1	1.0 ± 0.15	1.0 ± 0.2
PC	0.60 ± 0.09	0.37 ± 0.07	0.34 ± 0.09	0.57 ± 0.22	0.40 ± 0.12	0.41 ± 0.14	0.34 ± 0.09
Sarcosine	0.12 ± 0.02	0.07 ± 0.01	0.07 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	0.08 ± 0.02	0.09 ± 0.3
Succinate	1.21 ± 0.23	0.78 ± 0.14	0.60 ± 0.13	0.80 ± 0.23	0.49 ± 0.18	0.75 ± 0.23	0.76 ± 0.24
Taurine	29.50 ± 6.66	14.95 ± 3.13	14.70 ± 3.65	20.71 ± 4.59	11.22 ± 2.67	14.80 ± 4.35	13.91 ± 4.18
TMAO	0.34 ± 0.06	0.22 ± 0.05	0.20 ± 0.05	0.36 ± 0.06	0.15 ± 0.03	0.26 ± 0.09	0.27 ± 0.08
Xanthine	3.18 ± 0.56	2.39 ± 0.41	2.01 ± 0.26	2.67 ± 0.60	1.98 ± 0.22	1.59 ± 0.28	1.06 ± 0.34

Values represent the mean ± S.E.M. (n = 6). Sn-glycero-3-phosphocholine (GPC), 0-phosphocholine (PC), trimethylamine N-oxide (TMAO).

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Chapter 1

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Chapter 2

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