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Rapid cortisol signaling in response to acute stress involves changes in plasma membrane order in rainbow trout liver

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Dindia L, Faught E, Leonenko Z, Thomas R, Vijayan MM. Rapid cortisol signaling in response to acute stress involves changes in plasma membrane order in rainbow trout liver. *Am J Physiol Endocrinol Metab* 304: E1157–E1166, 2013. First published March 26, 2013; doi:10.1152/ajpendo.00500.2012.—The activation of genomic signaling in response to stressor-mediated cortisol elevation has been studied extensively in teleosts. However, very little is known about the rapid signaling events elicited by this steroid. We tested the hypothesis that cortisol modulates key stress-related signaling pathways in response to an acute stressor in fish liver. To this end, we investigated the effect of an acute stressor on biophysical properties of plasma membrane and on stressor-related protein phosphorylation in rainbow trout (*Oncorhynchus mykiss*) liver. A role for cortisol in modulating the acute cellular stress response was ascertained by blocking the stressor-induced elevation of this steroid by metyrapone. The acute stressor exposure increased plasma cortisol levels and liver membrane fluidity (measured by anisotropy of 1,6-diphenyl-1,3,5-hexatriene), but these responses were abolished by metyrapone. Atomic force microscopy further confirmed biophysical alterations in liver plasma membrane in response to stress, including changes in membrane domain topography. The changes in membrane order did not correspond to any changes in membrane fatty acid components after stress, suggesting that changes in membrane structure may be associated with cortisol incorporation into the lipid bilayer. Plasma cortisol elevation poststress correlated positively with activation of intracellular stress signaling pathways, including increased phosphorylation of extracellular signal-related kinases as well as several putative PKA and PKC but not Akt substrate proteins. Together, our results indicate that stressor-induced elevation of plasma cortisol level is associated with alterations in plasma membrane fluidity and rapid activation of stress-related signaling pathways in trout liver.

fish; *oncorhynchus mykiss*; salmonid; glucocorticoid; stress response; membrane fluidity; cell signaling

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 (37). The principal stress hormones, epinephrine and glucocorticoids, have critical functions in the stress adaptation process (9). The fight-or-flight response involves the activation of the sympathetic nervous system, leading to the rapid release of epinephrine from chromaffin cells (9). 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 the expression of genes involved in intermediary metabolic regulation (2, 30). Although several studies have examined the genomic effects associated with stressor-induced plasma cortisol elevation (2, 30, 46), few have investigated the rapid, nongenomic effects of this steroid on peripheral tissues (4).

The plasma membrane is likely pivotal during the response to stress, as the cell membrane is tightly linked to cellular physiology (48). Subtle perturbations in the plasma membrane structure and/or composition can have important consequences to cellular physiology, including membrane transport (26), endocytosis (32), enzyme activity (14), permeability (18), and apoptosis (33). Indeed, changes to membrane biophysical properties have been shown to modulate intracellular stress-activated signaling pathways in response to temperature (28), oxidative (11, 38), and mechanical stressors (47). Despite evidence that changes in plasma membrane properties rapidly affect the cellular stress response (48), very little is known about the effect of acute physical stressors on membrane properties and associated signaling events in whole organisms.

Recently, we reported that in vitro exposure to cortisol rapidly alters fluidity, surface topography, and elasticity of hepatic plasma membranes in rainbow trout (*Oncorhynchus mykiss*) (12). In addition to modulating plasma membrane properties, stress levels of cortisol rapidly phosphorylated putative substrate proteins 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 (12). 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 stressors. To test this hypothesis, we carried out in vivo acute stress experiments in either the presence or absence of metyrapone, an 11β-hydroxylase inhibitor that blocks endogenous cortisol biosynthesis (25). Liver plasma membrane fluidity, topography, and composition along with phosphorylation of putative PKC, PKA, and Akt substrate proteins and extracellular signal-related kinases (ERK1/2) were measured after a 30-min physical stressor in rainbow trout. We report for the first time that an acute physical stressor fluidizes plasma membrane and underscores a role for cortisol in rapidly modulating the membrane biophysical properties and activating stress-related signaling pathways in trout liver.

MATERIALS AND METHODS

Animals. Juvenile rainbow trout (100–300 g), purchased from Alma Aquaculture Research Station (Alma, ON, Canada), were main-

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tained at the University of Waterloo aquatic facility exactly as described before (35). The tanks were supplied with a constant flow of aerated well water (12 ± 2°C) and were maintained under a 12:12-h light-dark photoperiod. Trout were acclimated for ≥2 wk prior to experiments and were fed commercial trout feed (Martin Mills, Elmira, ON, Canada) to satiety once daily, 5 days/wk. The animals were maintained according to Canadian Council on Animal Care guidelines and approved by the Animal Care Committee at the University of Waterloo.

Experimental protocol. There were four experimental tanks (100 liters) with groups of 12–13 trout each maintained exactly as mentioned above. Fish in two tanks were injected with 1 mg/kg metyrapone (Sigma, St. Louis, MO), whereas 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 1 h, 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 (1:1,000) and were sampled either prior to (0 min) or after 30 min of stressor exposure. Sampling consisted of quickly netting six 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 6,000 *g*) 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.

Plasma cortisol, glucose, and lactate analysis. Plasma cortisol levels were measured using a ^{3}H-labeled cortisol radioimmunoassay, as described previously (1). Plasma glucose (Trinder method; Sigma) and lactate (Trinity Biotech, St. Louis, MO) levels were measured using commercially available colorimetric kits.

Liver plasma membrane. Liver plasma membranes were isolated using sucrose gradient, as described previously (42). The membrane pellet was resuspended in TCD buffer (300 mM sucrose, 10 mM Tris·HCl, 1 mM dithiothreitol, 0.5 mM $CaCl_2$, and 1× 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 (24), 5′-nucleotidase (12), and lactate dehydrogenase (14). The fivefold Na^+/K^+-ATPase [homogenate (H): 2.8 ± 0.31 vs. membrane (M): 10.4 ± 2.3; $n = 23$] and 12-fold higher 5′-nucleotidase (H: 3.6 ± 0.56 vs. M: 41.7 ± 9.2; $n = 12$) activities (U/g protein) in the membrane fraction, compared with the initial tissue homogenate, confirm membrane enrichment. The ~97% drop in lactate dehydrogenase activity (H: 5,334.7 ± 386.2 vs. M: 163.7 ± 113.7; $n = 23$) in the membrane fraction compared with the homogenate further confirms enriched plasma membranes with negligible cytosolic contamination.

1,6-Diphenyl-1,3,5-hexatriene 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, Burlington, ON, Canada) exactly as described previously (12). Membrane samples were added to 96-well opaque plates (100 μl with approximate protein concentration of 0.3 mg/ml; Corning) and incubated with DPH (1:100 of 4.7 mM stock dissolved in tetrahydrafuran) in the dark for 30 min. Readings were taken at various temperatures starting at 2°C, followed by 12, 24, and 37 ± 1°C. The required temperature (reached within ~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.

Atomic force microscopy. 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 (27). 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, height: 10–15 μm). 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 ~15 min. Quantitative analysis of topography and phase images was conducted using Gwyddion (http://gwyddion.net/). Difference in membrane height (membrane roughness) and viscoelastic properties between different membrane regions were calculated by taking the average difference in height or phase between lower and higher membrane regions, respectively.

Lipid extraction. Membrane samples were isolated as described above and extracted as described previously (10) and stored at −20°C for gas chromatography-flame ionization detection and thin-layer chromatography-flame ionization detection analysis, as described before (43). Thin-layer chromatography was used to separate the individual phospholipids present in the sample and was performed exactly as described previously (10). The dried samples (separated phospholipids) were derivatized for gas chromatography analysis, as explained previously (10).

Immunoblotting. Liver tissue was homogenized and protein concentration measured using the bicinchoninic acid method, with bovine serum albumin used as the standard. All samples were diluted in Laemmli's sample buffer (60 mM Tris·HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, and 0.1% bromophenol blue), and the immunodetection was carried out exactly as described in Ref. 12. Briefly, total protein (40 μg) was separated on 10% SDS-PAGE and transferred to nitrocellulose membrane and blocked with a 5% solution of nonfat dry milk in 1× TTBS (2 mM Tris, 30 mM NaCl, and 0.01% Tween, pH 7.5) for 1 h at room temperature. This was followed with an overnight incubation (1:1,000 dilution) with either phospho-ERK1/2 (Thr^{202}/Tyr^{204}) or total ERK1/2 monoclonal rabbit antibodies or phospho-(Ser) PKC substrate, phospho-Akt substrate, or phospho-PKA substrate polyclonal rabbit antibodies (Cell Signaling Technology, Beverly, MA). Blots were incubated for 1 h at room temperature with anti-rabbit horseradish peroxidase-labeled secondary antibody (1:3,300 dilutions in 5% skim milk; Bio-Rad). Protein bands were detected with ECL Plus chemiluminescence (GE Health Care, Baie d'Urfe, QC, Canada) 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), and values were normalized to a standard trout liver sample run on each blot for interblot comparisons. Each immunoblot was subsequently probed with Cy3-conjugated monoclonal mouse β-actin antibody (Cy3-conjugated monoclonal mouse, 1:1,000; Sigma) for 1 h at room temperature. Densitometric values were then normalized to β-actin to control for protein-loading differences between samples.

Statistical analysis. A two-way analysis of variance was used to compare time and treatment effects. A least significant differences 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 parameter 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 ERK1/2 (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, San Jose, CA).

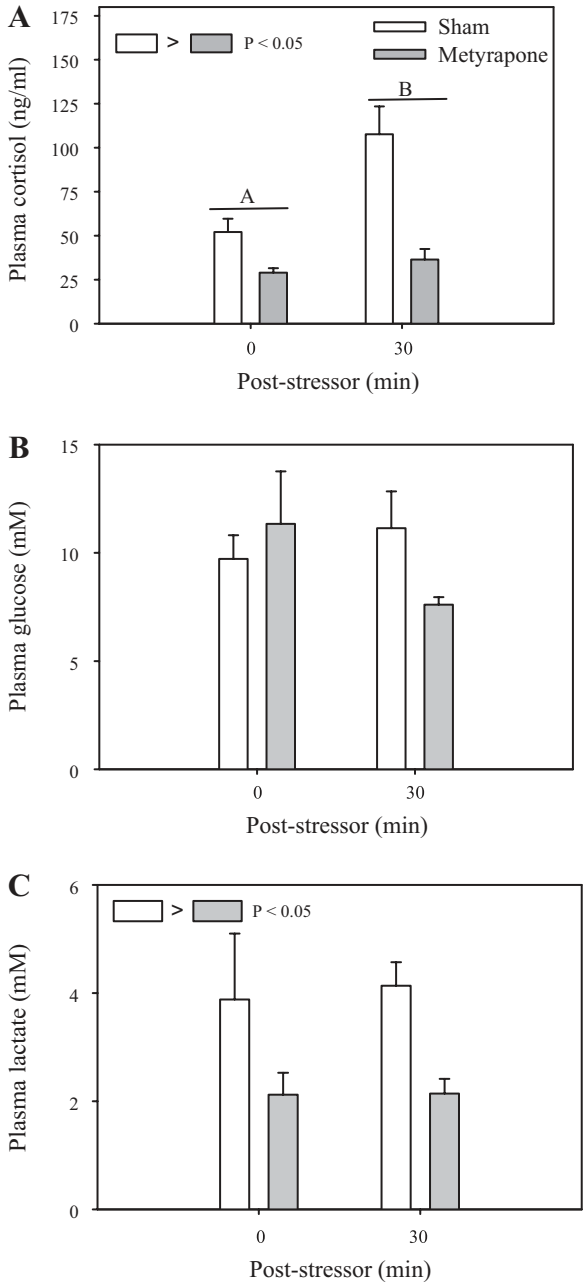

Fig. 1. Effect of acute stressor exposure in either the presence or absence of metyrapone on plasma cortisol (*A*), glucose (*B*), and lactate levels (*C*) in rainbow trout. Data represent means ± SE ($n = 4–6$). Different uppercase letters indicate significant time effects, and significant treatment effects are indicated at *top left* (2-way ANOVA, $P < 0.05$).

RESULTS

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

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. 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 with the prestressor group. Metyrapone injection did not significantly affect plasma membrane fluidity in response to stress compared with sham-treated trout. The stress-induced fluidization in the sham group was significant at 2, 11, and 24°C (Fig. 2).

Stress alters membrane biophysical properties. AFM 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*A*, *a* and *b*) and phase (Fig. 3*B*, *a* and *b*) images, there are two plasma regions (light vs. dark regions, which likely represent different plasma membrane domains). These two plasma membrane domains differ in height (Fig. 3*A*, *c* and *d*), with the solid arrow pointing to the lower domain, whereas the dotted arrow denotes a higher domain (Fig. 3*A*, *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*B*, *a* and *b*). In the phase image (Fig. 3*B*, *b*), the solid arrow indicates a harder domain, whereas the dotted arrow indicates a softer domain.

The acute stressor significantly altered membrane topography and domain organization (Fig. 3*A*) such that membrane roughness (difference in height between lower and higher domains) increased significantly following acute stress (Fig. 3*A*, *e*). In addition, as is evident in the corresponding cross-section plots, the width of lower microdomains increased after stressor exposure (Fig. 3*A*, *d*) compared with prestressor membranes (Fig. 3*A*, *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*B*, *e*).

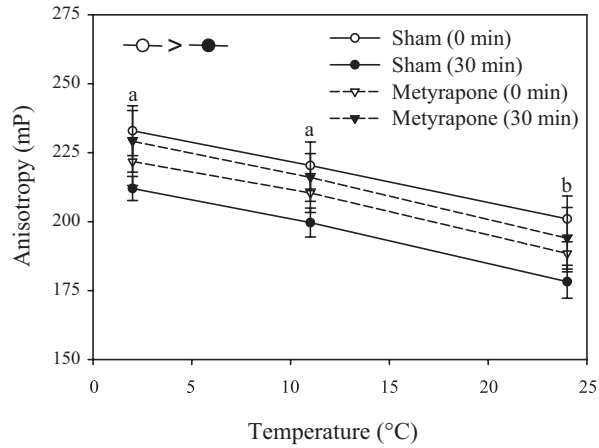

Fig. 2. Effect of acute stress on 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence anisotropy of enriched hepatic plasma membranes isolated from sham or metyrapone-treated trout before and 30 min after a stressor exposure. Data represent means ± SE ($n = 4–6$). Different lowercase letters indicate significant time effect, and treatment effect is indicated at *top left* (2-way ANOVA, $P < 0.05$; ○ > ●).

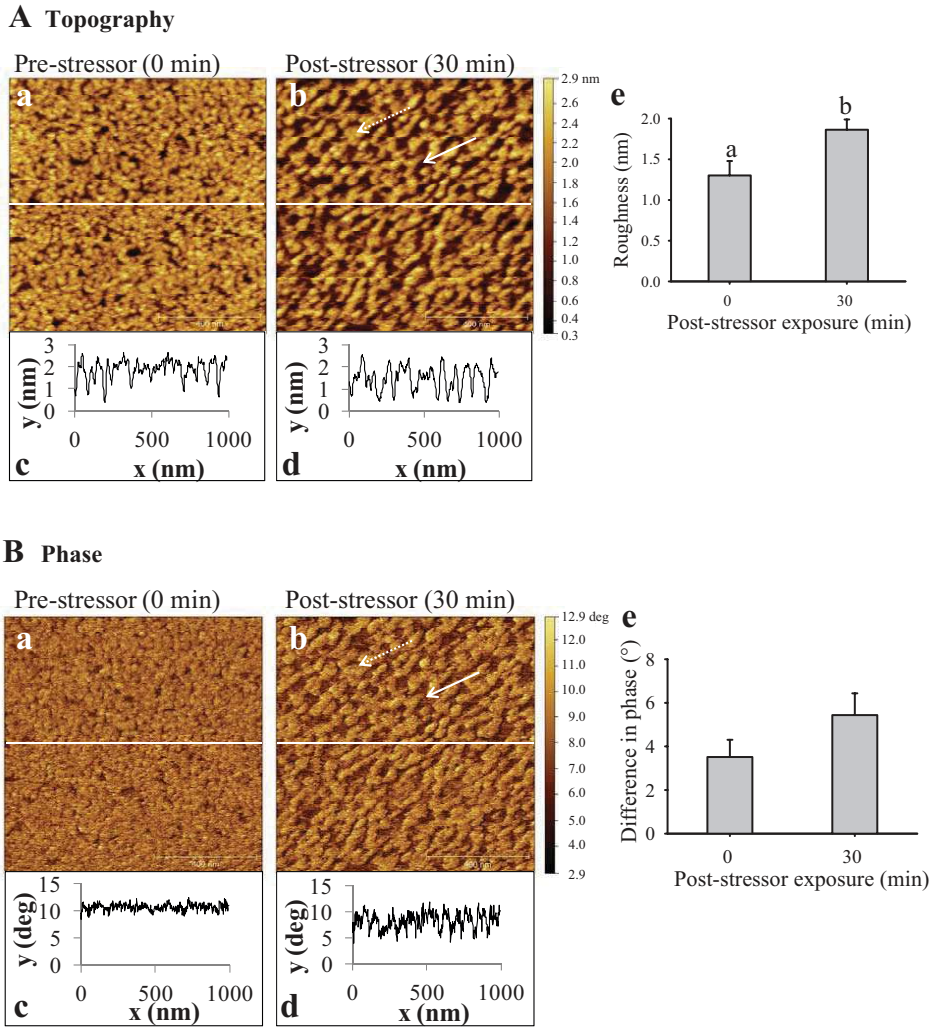

Fig. 3. Effect of acute stress on liver plasma membrane topography and surface adhesion. *A*: representative atomic force microscopy (AFM) topography images of supported hepatic plasma membrane before (*a*) and 30 min after stressor exposure (*b*). Two distinct domains, which differ in height, are visible in both unstressed and stressed plasma membranes. A representative higher domain is indicated by the dotted arrow, whereas 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 2 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, whereas 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*).

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 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 with all other groups and lower levels of FFA compared with sham trout. Within sham trout, there was no change in the total levels of saturates, monounsaturates, polyunsaturates, unsaturates, and plasmalogens after stress compared with values prior to stress (Table 1). Levels of saturated fatty acids (FA) decreased significantly 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 trout but not sham trout. Although there was no difference in overall saturation level within the sham, the levels of saturated and saturated/polunsaturated FA within PC were significantly higher 30 min poststressor compared with prestressor values (Table 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 plasmogens were significantly lower, whereas the levels of polyunsaturates were significantly higher in the metyrapone group compared with sham trout. Within PE, there were no significant changes in fatty acid composition in response to acute stress or metyrapone treatment.

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. 4) and PKA (Fig. 5) but not Akt (Fig. 6) substrate proteins with plasma cortisol levels. In addition to these protein kinase pathways, the ratio of phosphorylated to total ERK1/2 was also significantly correlated with plasma cortisol levels (Fig. 7).

DISCUSSION

The novel finding from the present study is that stressor-induced plasma cortisol elevation alters the biophysical prop-

Table 1. *Lipid components of liver plasma membrane*

Components	Sham		Metyrapone	
	Prestressor 0 min	Poststressor 30 min	Prestressor 0 min	Poststressor 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[a,b]	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[a,b]	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 (%by weight) represent means ± SE. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; C, cholesterol; FFA, free fatty acids; PL, phospholipids. Unsaturates represent mono- and polyunsaturates. Means in the same row accompanied by different superscripted letters are significantly different between treatments at least significant difference = 0.05; $n = 5$ for all components, except for PC and PE fatty acid components analysis, where $n = 3$.

erties of plasma membrane 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 and activates stress-related signaling pathways in trout hepatocytes (12). Most studies have focused on the genomic actions of cortisol in stress adaptation (2), whereas this study highlights a novel rapid action of cortisol that may be nongenomic and involves membrane structure alterations and modulation of stress-related signaling pathways in trout liver.

The plasma membrane is highly sensitive to the surrounding physical and chemical environment, leading to the proposal that the plasma membrane is the primary detector of stress stimuli and activator of the cellular stress response (48). 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 (34), temperature (44), and oxidative stress (3). This study provides additional evidence for the membrane-sensing 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. Although it is commonly stated that steroid-induced alterations to membrane fluidity occur only at supraphysiological levels, studies using mammalian cell lines have reported fluidity changes with glucocorticoids in the physiological range (7, 13). Further confirmation arises from our recent finding demonstrating that stress level of cortisol in vitro increases liver plasma membrane fluidity in rainbow trout (12). Although studies have shown that longer-term glucocorticoid exposure has a membrane-fluidizing effect in vivo (17), 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 that differ in their lipid phase primarily because of their lipid and protein composition (21). These microdomains are commonly referred to as lipid rafts, which generally have a high percentage of sphingolipid, cholesterol, and membrane proteins (21). This stressor-induced microdomain reorganization likely has important functional consequences, as lipid rafts play a critical role in influencing receptor activity and intracellular signaling (21, 28, 39). The increase in plasma membrane roughness detected

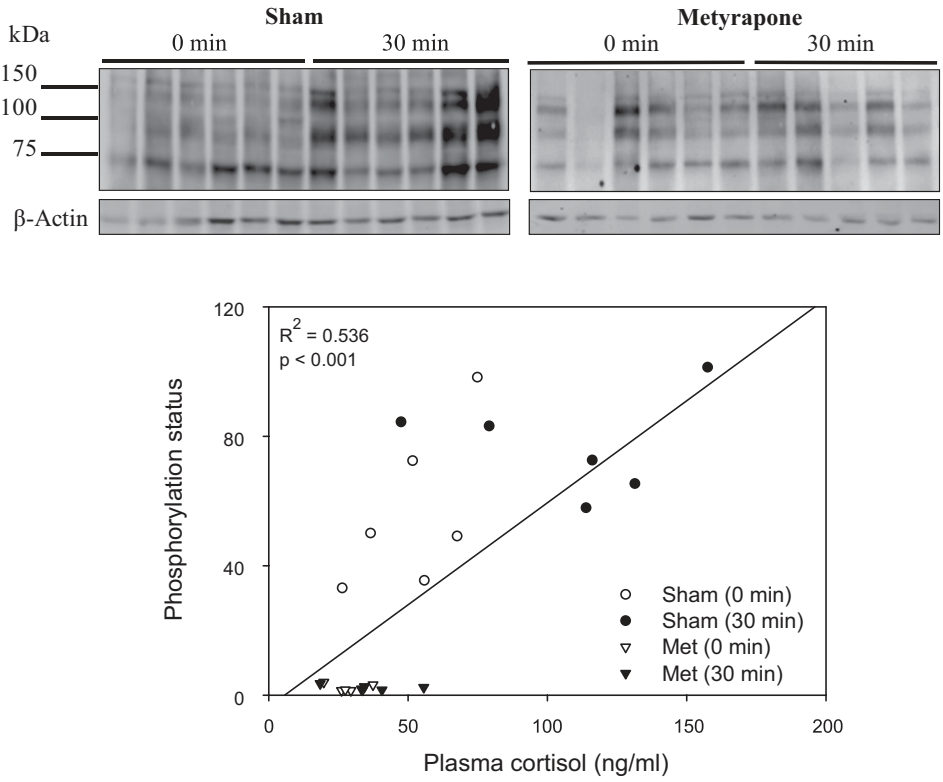

Fig. 4. Effect of acute stress on hepatic protein phosphorylation of protein kinase C (PKC) substrate proteins. Rainbow trout liver homogenates (40 μg of protein) from sham and meytrapone (Met)-treated trout before and after acute stress were probed with phospho (Ser)-PKC substrate polyclonal rabbit antibody (Cell Signaling Technology, Beverly, MA), which detects proteins that are phosphorylated within the phosphorylation motif for PKC. Protein loading was normalized 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). 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 ($r^2 = 0.607$, $P < 0.001$, $n = 23$).

in response to stressor exposure, which is associated with microdomain reorganization, may play a role in activating the liver cellular response in trout. Because cortisol treatment in vitro also increases plasma membrane roughness (12), our results suggest that stressor-mediated elevation of plasma steroid hormone levels facilitates domain reorganization in rainbow trout.

Membrane order changes may aid in the hepatic response to acute stress by regulating cellular processes that are important for stress adaptation. For instance, studies have

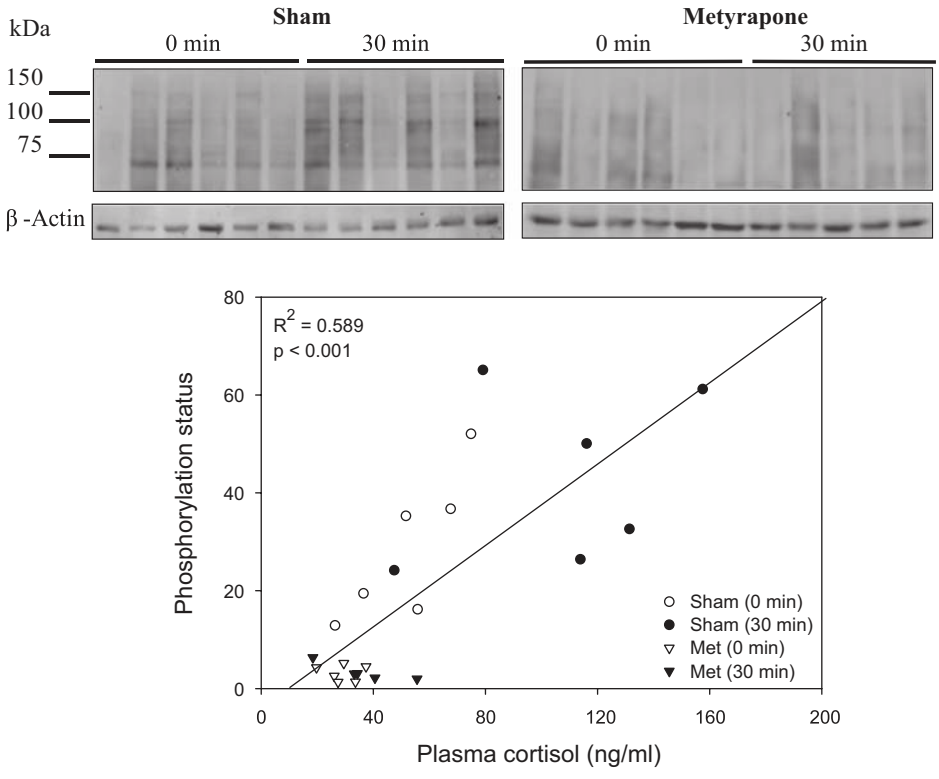

Fig. 5. Effect of acute stress on hepatic protein phosphorylation of protein kinase A (PKA) substrate proteins. Rainbow trout liver homogenates (40 μg of protein) from sham and Met-treated trout before and after acute stress were probed with phospho-PKA substrate plolyclonal rabbit antibody (Cell Signaling Technology), which detects proteins that are phosphorylated within the phosphorylation motif for PKA. Protein loading was normalized with β-actin (monoclonal mouse antibody; Sigma). 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 ($r^2 = 0.615$, $P < 0.001$, $n = 23$).

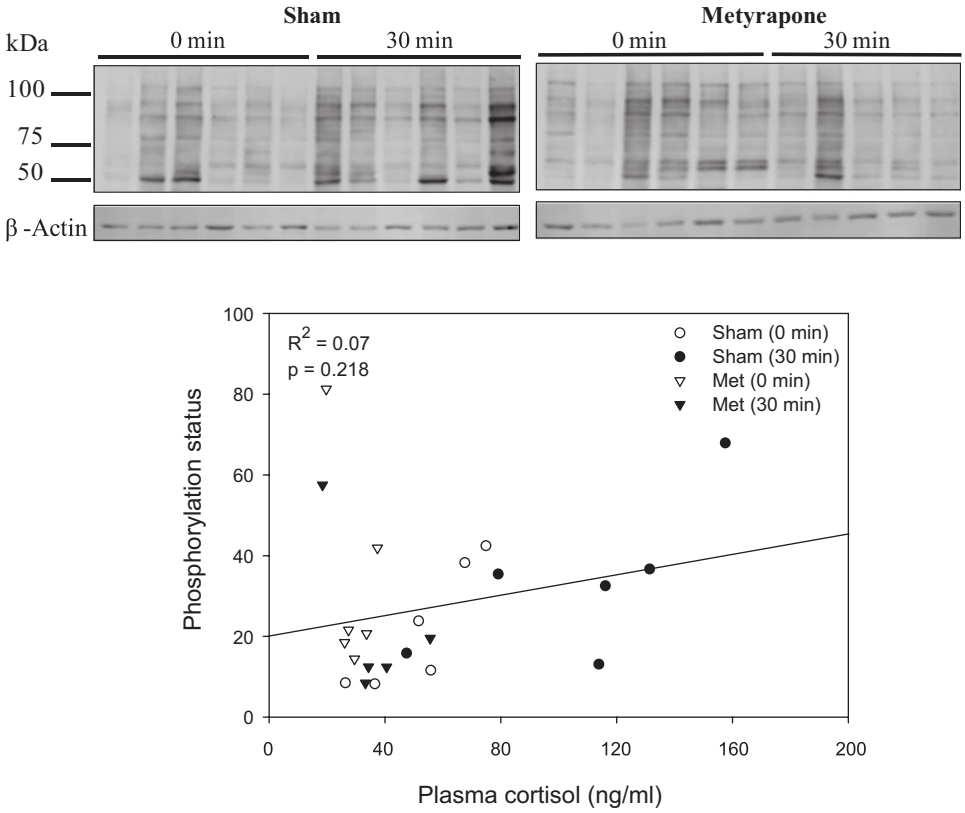

Fig. 6. Effect of acute stress on hepatic protein phosphorylation of Akt substrate proteins. Rainbow trout liver homogenates (40 μg of protein) from sham and Met-treated trout before and after acute stress were probed with phospho-Akt substrate ploly-clonal rabbit antibody (Cell Signaling Technology), which detects proteins that are phosphorylated within the Akt phosphorylation motif. Protein loading was normalized with β-actin (monoclonal mouse antibody; Sigma). 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 ($P > 0.05$, $n = 23$).

shown that glucose transport systems are sensitive to the surrounding lipid environment (26) and are affected markedly by temperature-induced changes in membrane fluidity (29). In addition to metabolite transport, alterations to the plasma membrane can directly modulate several stress-signaling pathways (19, 31, 40), thereby affecting the cellular stress response. Also, stress-mediated alterations in membrane biophysical properties may have important permissive or suppressive effects on other hormonal cell signaling pathways (23). Altogether, our results suggest that stressor exposure and the associated elevation of plasma cortisol level rapidly alter membrane order and may be playing a role in modulating the liver stress response.

Plasma membrane properties such as fluidity and domain structure are highly dependent on membrane composition (45). However, there were no stress-induced changes in the

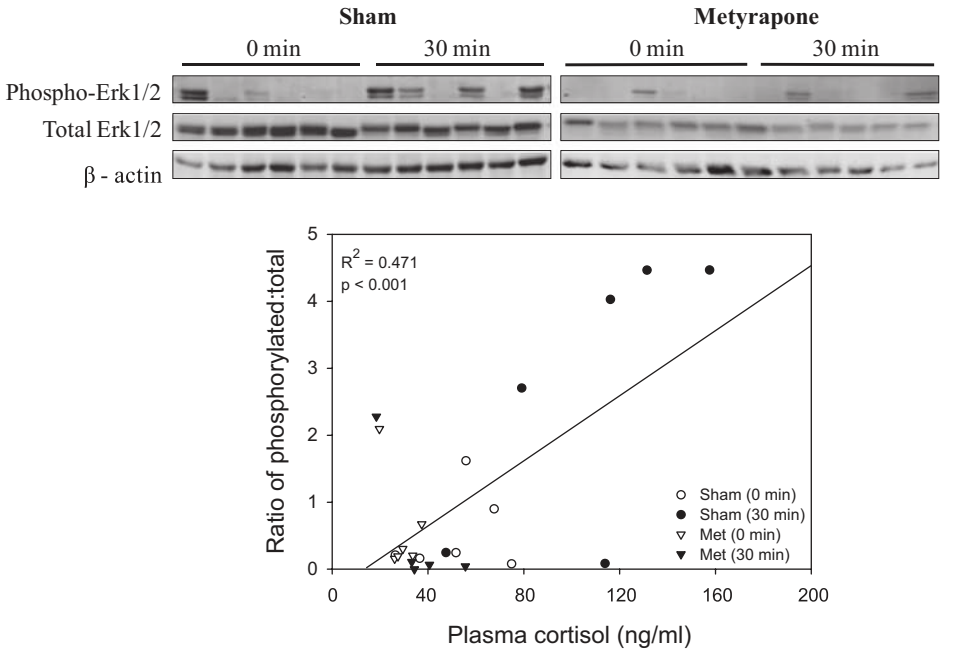

Fig. 7. Activation of extracellular signal-related kinases (ERK1/2) in response to acute stress. Rainbow trout liver homogenates (40 μg of protein) from sham and Met-treated trout before and after acute stress were probed with total or phosphospecific ERK1/2 monoclonal rabbit antibody (Cell Signaling Technology). Protein loading was normalized with β-actin (monoclonal mouse antibody; Sigma). A scatter plot of the plasma cortisol values plotted against the ratio of phosphorylated ERK1/2 to total ERK1/2, 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 ERK1/2 (ratio of densitometry values of phosphorylated to total ERK1/2; $r^2 = 0.338$, $P < 0.01$, $n = 23$).

proportion of each lipid component, except for the FFA composition within PC phospholipids. Although significant, the increase in saturation and the decrease in monounsaturates were minor (5 and 8%, respectively) and occurred only within the 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, it 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 instead related to alterations in biophysical properties. Recently, we demonstrated that stress level 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 (12). Although 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 changes in membrane order 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 ERK1/2 (ratio of phosphorylated to total) was also positively correlated with elevated plasma cortisol levels. This supports our recent finding in vitro showing that stress level 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 ERK1/2 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 ERK1/2 signaling (8, 20, 36). Furthermore, benzyl alcohol, which mediates its effects at the level of the plasma membrane (28), also activated these signaling pathways in trout hepatocytes (12), 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 glucocorticoid receptor (GR) or other membrane receptors. For instance, PKC and mitogen-activated protein kinases are thought to play a role in the central nongenomic GR-dependent inhibition of ACTH secretion (41). Also, dexamethasone (a synthetic glucocorticoid) modulated the T cell phosphorylation profile in a GR-dependent manner (5), 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 downregulation of several insulin-related kinases (22). However, glucocorticoid-mediated PKC, PKA, and ERK1/2 phosphorylation have also been reported in the absence of GR activation in the hippocampus of mammalian species (reviewed in Ref. 16), suggesting multiple modes of nongenomic signaling by this stress steroid. Although rapid nongenomic signaling by glucocorticoid has been reported in teleosts (6), a role for nongenomic signaling by GR has not been established (2). Together, the association of protein kinase activation with elevated plasma cortisol levels suggests a novel nongenomic role for cortisol in the rapid liver stress response, whereas the mechanisms involved remain to be elucidated. We hypothesize that modulation of membrane biophysical properties by stress levels of cortisol in trout may be a novel mechanism leading to rapid 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 acute stress-induced membrane perturbations are likely mediated by cortisol, underscoring a role for this steroid in rapid modulation of liver stress response. Plasma cortisol elevation was also correlated with activation of stress-associated protein kinases, further supporting a role for rapid cortisol signaling in cellular stress adaptation. However, whether cortisol mediates these changes by altering membrane order alone or in combination with activation of GR or other membrane receptors remains to be determined. We hypothesize that the alteration in plasma membrane fluidity and rapid activation of stress-related signaling pathways may play an important role in acute stress adaptation in animals.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.D. and M.M.V. contributed to the conception and design of the research; L.D., E.F., and R.T. performed the experiments; L.D., E.F., and R.T. analyzed the data; L.D., E.F., Z.L., R.T., and M.M.V. interpreted the results of the experiments; L.D. and E.F. prepared the figures; L.D. drafted the manuscript; L.D., E.F., Z.L., R.T., and M.M.V. edited and revised the manuscript; L.D., E.F., Z.L., R.T., and M.M.V. approved the final version of the manuscript.

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