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The liquorice root derivative glycyrrhetinic acid can ameliorate ionoregulatory disturbance in rainbow trout (*Oncorhynchus mykiss*) abruptly exposed to ion-poor water

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ABSTRACT

To consider the idea that a dietary botanical supplement could act as an adaptogen in a teleost fish, the effect of a liquorice root derivative (18β-glycyrrhetinic acid, 18βGA) on rainbow trout following an acute ionoregulatory stressor was examined. Freshwater (FW) trout were fed a control or 18βGA supplemented diet (0, 5, or 50 μg 18βGA/g diet) for 2 weeks, then abruptly exposed to ion-poor water (IPW) for 24 h. Following IPW exposure, muscle moisture content and serum cortisol levels elevated and serum [Na^+] and/or [Cl^-] reduced in control and 50 μg/g 18βGA-fed fish. However, these endpoints were unaltered in 5 μg/g 18βGA-fed fish. Gill tissue was investigated for potential mechanisms of 18βGA action by examining mRNA abundance of genes encoding corticosteroid receptors (CRs), 11β-hydroxysteroid dehydrogenase 2 (*11β-hsd2*), and tight junction (TJ) proteins, as well as Na^+-K^+-ATPase and H^+-ATPase activity, and mitochondrion-rich cell (MRC) morphometrics. Following IPW exposure, CR and *11β-hsd2* mRNA, MRC fractional surface, Na^+-K^+-ATPase and H^+-ATPase activity were unaltered or decreased in 50 μg 18βGA fish, as was mRNA encoding select TJ proteins. In contrast, 5 μg 18βGA-fed fish exhibited elevated *11β-hsd2* and CR mRNA abundance versus 50 μg 18βGA-fed, and reduced MRC apical area as well as some differences in TJ protein mRNA abundance versus control fish. Data suggest that 18βGA, at low levels, may be adaptogenic in trout and might help to ameliorate ionoregulatory perturbation following IPW exposure. This seems to occur, in part, through 18βGA-induced alterations in the biochemistry and physiology of the gill.

1. Introduction

The root of the liquorice plant (*Glycyrrhiza* sp.) can be an immensely beneficial botanical if taken/administered correctly, and this is reflected in the deep history of its use in traditional medicine across many cultures (for review, see Asl and Hosseinzadeh, 2008). Liquorice root (LR) or liquorice root derivatives (LRDs) possess a considerable number of properties including those that are anti-inflammatory, anti-viral, and antimicrobial (for review, see Asl and Hosseinzadeh, 2008). However, it has been shown in mammals that excessive LR consumption can lead to fundamental changes in salt and water balance that manifest as hypertension resulting from pseudohyperaldosteronism (Ruszymah et al., 1995; Ferrari, 2010; Lin et al., 2012). An important cause of LR derived homeostatic perturbation is the principal LRD, glycyrrhizic acid (GL) (Asl and Hosseinzadeh, 2008). When LR is ingested, GL is converted into 18β-glycyrrhetinic acid (18βGA) by intestinal bacteria (Akao et al., 1998; Chilke, 2012), and 18βGA acts systemically as an inhibitor of 11β-hydroxysteroid dehydrogenase type-2 (11βHSD2) enzyme activity. This impacts the process that converts cortisol to its inactive cortisone analogue, and given that 11βHSD2 would normally prevent hyper-activation of the mineralocorticoid receptor (MR) (Tanahashi et al., 2002), excessive LR or LRD consumption can result in abnormal MR activation (Makino et al., 2012). Furthermore, LRDs such as GL and 18βGA have also been reported to directly interact with mammalian corticosteroid receptors (CRs) in cortisol sensitive tissues (Ulmann et al., 1975; Armanini et al., 1983). Therefore, LR and LRDs have been widely investigated for their effects on the mammalian endocrine system (Armanini et al., 1983, 1999, 2004; Latif et al., 1990; Räikkönen et al., 2010; Tamir et al., 2000; Ulmann et al., 1975; Van Uum et al., 2002).

In addition to the aforementioned observations, it has been reported that LR and LRDs have adaptogenic properties and as such, could be useful for the treatment of stress or stress hormone associated disorders (Head and Kelly, 2009). At appropriate doses, adaptogenic herbs (or adaptogens), are botanical compounds that do not disrupt systemic endpoints of homeostasis under 'normal' circumstances (Panossian et al., 1999). But in the presence of a stressor (or stressors), an adaptogen can lessen perturbation and in doing so, reduce physiological

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stress and aid in the restoration of systemic equilibrium (Brekhman and Dardymov, 1969; Panossian et al., 1999). To date, and to the best of our knowledge, the adaptogenic properties of botanicals (including LR or LRDs) have been examined exclusively in mammals. However, LRDs have been reported to alter the physiology of non-mammalian vertebrates (or tissues taken from these animals) (Ishikawa and Saito, 1980; Alderman and Vijayan, 2012; Chen et al., 2015). For example, 18βGA was demonstrated to suppress short circuit current across isolated frog skin as well as potentiate the actions of aldosterone, the latter of which enhanced active Na^+ transport (Ishikawa and Saito, 1980). In addition, Alderman and Vijayan (2012) utilized 18βGA to demonstrate a role for 11β-HSD2 in the zebrafish brain, where treatment with 18βGA in zebrafish caused an elevation in whole body cortisol levels. More recently, however, it has been demonstrated that dietary LRD-supplementation (either GL or 18βGA) can alter circulating cortisol levels in rainbow trout, as well as biochemical/molecular properties of gill tissue that relate to the stress response and transepithelial ion transport (Chen et al., 2015). But in rainbow trout these changes took place in the absence of any apparent systemic alteration in salt and water balance (Chen et al., 2015). Therefore under 'normal' FW conditions, LRDs, at the doses used by Chen et al. (2015), do not appear to perturb systemic endpoints of homeostasis in trout. Yet changes in the biochemical and molecular properties of gill tissue suggest that the capacity of LRD-treated trout to cope with ionoregulatory disturbance may have been altered. If these alterations should prove to be beneficial when an environmental stressor is presented to the fish, this would provide evidence that LRDs could, within the context of salt and water balance, function as adaptogens in rainbow trout. To consider this possibility, the current study examined what effect (if any) dietary supplementation with the LRD 18βGA will have on the ability of rainbow trout to cope with an environmental stressor, abrupt ion-poor water (IPW) exposure. In a broader sense this was examined by investigating endpoints of ionoregulatory homeostasis in rainbow trout following the introduction of this stressor (i.e. serum ion levels and muscle moisture content). However, to gain some insight into possible mechanisms of 18βGA action following the introduction of an ionoregulatory stressor, biochemical and molecular endpoints of the stress axis (i.e. mRNA encoding CR genes as well as 11β-HSD2) and epithelial ion transport (i.e. gill Na^+-K^+-ATpase and H^+-ATPase activity, mitochondrion-rich cell morphometrics and tight junction protein mRNA abundance) were examined in gill tissue. This is because the gill epithelium interfaces directly with surrounding water and plays an important role in the maintenance of salt and water balance in fishes. Furthermore, transcellular as well as paracellular mechanisms of ion transport are very sensitive to the actions of cortisol, a major stress hormone and the principal corticosteroid of fishes (e.g. Chasiotis et al., 2010; Kelly and Wood, 2001, 2002; Kelly and Chasiotis, 2011; Laurent and Perry, 1990; McCormick, 2001; McCormick and Bradshaw, 2006). Should evidence be found that adaptogenic botanicals such as LRDs manifest beneficial properties in the stress physiology fishes, these properties could prove useful in an array of settings in order to harmlessly mitigate alterations in systemic salt and water balance in the stress physiology of fishes.

2. Materials and methods

2.1. Experimental animals

Rainbow trout (40–45 g) were obtained from a local supplier (Humber Springs Trout Club and Hatchery, Orangeville, ON, Canada) and were held in 200 L opaque polyethylene aquaria supplied with flow-through, de-chlorinated city of Toronto tap water (13 ± 1 °C, pH 7.4, approximate ion composition in μM: Na^+ 590; Cl^- 920; Ca^{2+} 900; K^+ 50). Photoperiod was 12 h L:12 h D and experimental water was aerated. Following transfer from the supplier, fish were allowed to acclimate to laboratory conditions for 2 weeks. During acclimation to laboratory conditions, fish were fed *ad libitum* once daily with commercial trout pellets (Martin Profishent, Elmira, ON, Canada). Fish husbandry, animal experiments and tissue collection methods were conducted in accordance with an approved York University Animal Care Protocol that conformed to the guidelines of the Canadian Council on Animal Care.

2.2. Preparation and feeding of diets supplemented with 18βGA

The LRD 18βGA was obtained from Sigma-Aldrich (Oakville, ON, Canada). 18βGA was dissolved in absolute ethanol and sprayed onto commercial trout pellets (Martin Profishent) so as to obtain experimental diets supplemented with either 5 μg 18βGA/g diet or 50 μg 18βGA/g diet. Once sprayed onto the pellets, the ethanol was allowed to evaporate overnight at room temperature, and diets were stored at −20 °C until use. A control diet (0 μg 18βGA/g diet) was prepared using absolute ethanol alone, but otherwise the pellets were treated in the same manner as the experimental diets. Fish ($n = 24$ per tank) were fed either a control or 18βGA-supplemented diet (3% body weight once daily on a scheduled regime) for 14 days.

2.3. Abrupt exposure to IPW

Following a 14 day feeding period (last meal 24 h prior to sampling) 10 fish from each treatment were rapidly net captured, anesthetized, and sampled for blood and tissue collection as described below in Section 2.4, Tissue Sampling. The de-chlorinated city of Toronto tap water in the experimental tanks was then rapidly replaced with IPW (approximate composition in μM; [Na^+], 85; [Cl^-], 70; [Ca^{++}], 4.7; [K^+], 0.7; pH 6.5) using inlet and outlet pipes that caused minimal disturbance to the fish. Once the water had been exchanged, fish were held in flow-through IPW for 24 h under conditions identical to those described previously. Fish were not fed during this period, and after 24 h 10 fish were collected for sampling as described above. The water in the tanks was then switched back to regular de-chlorinated FW to confirm that all remaining fish survived the IPW exposure.

2.4. Tissue sampling

Fish anesthetized in tricaine methanesulfonate (MS-222; Syndel Laboratories Ltd., Canada) were rapidly processed for blood and tissue collection by multiple individuals to reduce sampling time. Blood was collected from the caudal vein using a 20-gauge sterile syringe (all fish typically completed within 4–5 min from anaesthesia). Fish were then killed by spinal transection. Blood samples were allowed to clot at room temperature for 30 min then centrifuged (4 °C, 10,600 ×*g*, 10 min) to collect serum. Serum was subsequently stored in aliquots in −30 °C until use. For biochemical/molecular analysis of gill tissue, gill arches from the left branchial basket were removed and flash frozen in liquid nitrogen. For morphometric analysis of gill tissue using scanning electron microscopy (SEM), gill arches from the right branchial basket were carefully removed and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 4 h at 4 °C. Following fixation, samples were washed (2 × 10 min) with ice-cold 0.1 M phosphate buffer (pH 7.2) and stored in the same buffer at 4 °C until further processing. Finally, a strip of epaxial white muscle was collected into a pre-weighed tube for the determination of muscle moisture content.

2.5. Serum ions and cortisol, muscle moisture content and gill enzyme activities

Using a Multiskan Spectrum Spectrophotometer (Thermo Electron Corp, Nepean, ON, Canada), serum [Cl^-] was determined photometrically according to Zall et al. (1956). Serum [Na^+] was determined by using ion-selective micro-electrode (ISME) to calculate the concentration from Na^+ activity. Serum cortisol levels were determined using a commercial EIA kit (Oxford Biomedical Research, Oakland County, Michigan) in accordance with manufacturer instructions. Muscle