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Glucocorticoid receptors are involved in the regulation of pulsatile urea excretion in toadfish

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Abstract The objectives of this study were to characterize the pattern of pulsatile urea excretion in the gulf toadfish in the wake of exogenous cortisol loading and to determine the receptors involved in the regulation of this mechanism. Toadfish were fitted with indwelling arterial catheters and were infused with isosmotic NaCl for 48 h after which fish were treated with cortisol alone, cortisol + peanut oil, cortisol + RU486 (a glucocorticoid receptor antagonist) or cortisol + spironolactone (a mineralocorticoid receptor antagonist). Upon cortisol loading, fish treated with cortisol alone, cortisol + oil or cortisol + spironolactone experienced a two- to threefold reduction in pulsatile urea excretion. This reduction was due to a decrease in urea pulse size with no effect on pulse frequency compared to values measured during the control NaCl infusion period. In addition, these fish showed an increase in plasma urea concentrations upon treatment. These apparent effects of cortisol treatment were abolished in fish treated with cortisol + RU486. In contrast, these fish showed an increase in pulsatile urea excretion mediated by a twofold increase in pulse size with no change in frequency. Likewise, fish treated with cortisol + RU486 showed a significant decrease in plasma urea concentrations over the course of the experiment. The findings of this study indicate that high levels of cortisol reduce pulsatile urea excretion by decreasing pulse size. In addition, it appears that glucocorticoid receptors and not mineralocorticoid receptors are involved in the regulation of the toadfish pulsatile urea excretion mechanism.

Keywords Cortisol · RU486 · Spironolactone · Toadfish, *Opsanus beta* · tUT · Urea

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Introduction

The gulf toadfish (*Opsanus beta*) has a fully functional ornithine-urea cycle and can shift from ammoniotelism to ureotelism when exposed to air, ammonia, crowding or confinement (Walsh et al. 1990, 1994; Walsh and Milligan 1995). What makes the toadfish unique in its facultative ureotelism is the pulsatile nature of its branchial urea elimination. As in most teleost fish, the gill of the toadfish accounts for >90% of nitrogenous waste excretion. Urea excretion across the toadfish gill is facilitated by a urea transport protein (tUT; Genbank accession #AF165893) that shows higher than 60% homology at the amino acid level to mammalian UT-A2 facilitated diffusion urea transporters (Smith et al. 1998; Walsh et al. 2000). While in most other teleosts nitrogenous wastes are excreted continuously across the gill, the branchial urea clearance in the toadfish is pulsatile, possibly due to the periodic insertion or activation of tUT. The result is the excretion of urea a few times daily in distinct pulses that last 0.5–3 h in duration (Wood et al. 1995, 1997, 1998). In the periods between pulses there is little excretion of urea.

In mammals, arginine vasopressin (AVP) and glucocorticoids are two important regulators of UT transport systems (reviewed by Sands 1999). AVP causes an increase in facilitated urea diffusion and is potentially involved in mediating two separate mechanisms: an acute, cAMP-dependent change in activity of membrane-bound transporter proteins and a gradual recruitment of transporters to the membrane from intracellular pools (Grantham and Burg 1966; Star et al. 1988; Inoue et al. 1999; reviewed by Smith and Rousselet 2001). Glucocorticoids, on the other

hand, have the opposite effect on UT-mediated urea transport (Knepper et al. 1975; Naruse et al. 1997) apparently via an inhibition of UT-A transport gene expression (Peng et al. 2002). Specifically, recent evidence illustrates that glucocorticoids suppress the activity of the promoter region responsible for the transcription of three of four major renal isoforms of the mammalian UT-A urea transporter (Peng et al. 2002).

With respect to the toadfish, arterial injection of arginine vasotocin (AVT; the teleost homologue of AVP) does cause a urea pulse event to occur, but only at supraphysiological doses (10^{-10} to 10^{-9} mol l^{-1}) and at most 10% the size of natural pulses (Perry et al. 1998; Wood et al. 2001). In addition, circulating AVT levels are low (10^{-12} to 10^{-11} mol l^{-1}) and show no relationship to the occurrence of natural urea pulses (Wood et al. 2001). In contrast, evidence suggests that circulating cortisol concentrations in toadfish may be an important regulator of pulsatile urea excretion (Hopkins et al. 1995; Wood et al. 1997, 2001). When ureotelic, toadfish maintain high plasma cortisol concentrations that are typical for chronically stressed teleosts (reviewed by Mommsen et al. 1999). However, 2–4 h preceding a natural urea pulse event, plasma cortisol levels fall steadily and then rise rapidly thereafter (Wood et al. 1997, 2001). This decline in cortisol is believed to be permissive to a pulse event rather than the direct trigger because often times plasma cortisol levels will fall without a natural pulse occurring (Wood et al. 2001).

The direct trigger of pulsatile urea excretion is now believed to be serotonin (5-hydroxytryptamine, 5-HT; Wood et al. 2003). Circulating levels of 5-HT fluctuate considerably around natural urea pulses and intravenous injection of 5-HT and α-methyl-5-HT, a 5-HT_2 receptor agonist, at physiological doses (3×10^{-6} mol l^{-1}) results in large urea pulse events (Wood et al. 2003; McDonald and Walsh 2004). Potentially relevant to the regulation of pulsatile urea excretion, there is a well-described interaction between 5-HT and cortisol; 5-HT has been shown to stimulate cortisol secretion in fish (Winberg et al. 1997; Øverli et al. 1999; Höglund et al. 2002) and at least in mammals, central 5-HT synthesis and/or release is under complex control by glucocorticoids (reviewed by Chaouloff 1993). The hypothesis to be tested in this study is that the periodic declines measured in circulating cortisol concentrations prior to a urea pulse are permissive to the pulse event. Thus, elevation of plasma cortisol levels by continuous exogenous infusion, so as to prevent these periodic declines, would inhibit pulsatile excretion of urea. To test this hypothesis, the pattern of pulsatile urea excretion in the wake of exogenous cortisol loading, by way of continuous arterial infusion, was characterized. In addition, the roles of glucocorticoid receptors and mineralocorticoid receptors in the regulation of pulsatile urea excretion were investigated by interperitoneal injection of RU486, a corticosteroid type II receptor antagonist (Bertagna et al. 1984; Gaillard et al. 1985) or injection the mineralocorticoid receptor antagonist spironolactone (Delyani 2000) in toadfish infused with cortisol.

Materials and methods

Experimental animals

Gulf toadfish [*O. beta*; 0.095 ± 0.004 kg ($n = 33$), range 0.055–0.158 kg] were caught by commercial shrimpers in Biscayne Bay, Florida, in the winter of 2000–2001 and the spring of 2002. The toadfish were held in an outdoor tank at the shrimper's holding facility with running seawater (ambient seasonal conditions) for no longer than 24 h following capture, then transferred to the laboratory. Fish were treated with a dose of malachite green (final concentration 0.05 mg l^{-1}) in formalin (15 mg l^{-1}) (AquaVet, Hayward, Calif., USA) on the day of transfer to the laboratory in order to prevent infection by the cilate, *Cryptocaryon irritans* (Stoskopf 1993). Initially the fish were kept in 50 l glass aquaria with flowing, aerated seawater. Fish were maintained in this relatively crowded environment (> 10 fish per tank) in order to initiate a switch to ureotelism (Walsh et al. 1994). In order to guarantee fish were ureotelic, crowded conditions were further maintained by transferring groups of four fish to smaller plastic tubs (6 l) served with flowing seawater at least 48 h prior to surgery. The water temperature was 24–26°C. Fish were fed weekly with squid up until the time of surgery.

Experimental protocol

As outlined by Wood et al. (1997) and McDonald et al. (2000), caudal artery catheterizations were performed on fish anaesthetized with MS-222 (0.5 g·l^{-1}; Sigma Chemicals) and wrapped with wet towels. The fish were left to recover in a 2-l flux chamber with flowing seawater for 24 h during which the patency of the arterial catheters was confirmed and an initial blood sample was taken from each fish. This and all subsequent blood samples were 200 µl followed by replacement of all red blood cells resuspended in toadfish saline (Walsh 1987). At the same time, water flow to the fish box was stopped, the water level was set to a volume mark of 1.5 l and an initial water sample was taken for the measurement of urea and ammonia concentration. Thereafter, water samples (5 ml) were taken every 2 h for the remainder of the experiment and the box was rapidly flushed with fresh seawater over a 15-min period at 24-h intervals. Vigorous aeration maintained thorough mixing and the P_{O_2} close to air saturation during times when water flow to the box was stopped.

As described by McDonald et al. (2003), following the initial blood sample, the arterial catheter was connected to one channel of a Gilson-8 channel peristaltic pump and fish from all treatment groups were infused for 48 h with an isosmotic load of NaCl (150 mmol l^{-1})

at an infusion rate of 3 ml kg^{-1} h^{-1}; the rate was checked by periodic measurements of the weight of the infusion reservoir. After the 48 h infusion with NaCl, a second blood sample was taken. Toadfish were then separated into four treatment groups. The fish treated with cortisol alone (mean weight 0.089 ± 0.005 kg; $n = 10$) were then infused with cortisol (hydrocortisone hemisuccinate salt; Sigma Chemicals) in isosmotic NaCl at a rate of 270 μg cortisol kg^{-1} h^{-1} for 48 h followed by a subsequent 48 h recovery infusion of NaCl alone. For fish in this treatment group, blood samples were taken at 1, 4, 8, 16, 24, 36 and 48 h after the switch to cortisol. Following the switch back to NaCl alone, blood samples were taken at 1, 4, 8, 16, 24, 36, and 48 h.

Fish treated with cortisol + peanut oil (mean weight 0.090 ± 0.009 kg; $n = 6$) were infused with cortisol in isosmotic NaCl at a rate of 270 μg cortisol kg^{-1} h^{-1} for 96 h during which the toadfish were injected intraperitoneally with 0.3 ml peanut oil starting immediately before the cortisol infusion. Injections were repeated every 12 h for the remainder of the experiment. Injections were done quickly and with little handling so as to minimize disturbance, using a fresh 18-gauge needle. This treatment group served as a vehicle and injection control for the next two treatment groups. Fish treated with cortisol + RU486 (mean weight 0.089 ± 0.011 kg; $n = 7$) were infused with cortisol for 96 h during which they were injected intraperitoneally with 5 mg RU486 (mifepristone, 11β-[4-dimethylamino] phenyl-17β-hydroxy-17 [1-propynyl] estra-4,9-dien-3-one; Sigma Chemicals) in 0.3 ml peanut oil as described above. Similarly, fish treated with spironolactone + cortisol (mean weight 0.107 ± 0.006 kg; $n = 10$) were infused with cortisol for 96 h and injected intraperitoneally every 12 h with 5 mg spironolactone (Sigma Chemicals) in 0.3 ml peanut oil. For fish that were injected with oil alone or spironolactone, blood samples were taken at 1, 4, 8, 16, 24, 36, 48, 60, 72, 84 and 96 h following the switch from simple saline to saline with cortisol. For fish that were injected with RU486, blood samples were taken at 1, 4, 16, 24, 36, 48, 60 and 72 h following the switch from simple saline to saline with cortisol. The intraperitoneal injection of lipophilic compounds (such as RU486 and spironolactone) in oil vehicles (i.e. peanut oil, coconut oil, corn oil) mediates the slow release of these substances into the circulation (Vijayan and Leatherland 1989; Chandrashekar et al. 1991; Christensen et al. 1999).

The concentration of cortisol infused was aimed at raising circulating cortisol levels to approximately five-fold higher than in a typical ureotelic toadfish and was determined in a range finding experiment. The amount of receptor antagonist injected was chosen such that circulating levels of antagonist were tenfold greater than circulating cortisol concentrations. Blood samples were centrifuged at 10,000 *g* for 1 min and the plasma decanted. Samples were then frozen immediately in liquid N_2 and stored at −80°C for later analysis of urea, ammonia and cortisol. Water samples were analyzed immediately for urea and ammonia.

Analytical techniques and calculations

Urea concentrations in blood and water were measured using the diacetyl monoxime method of Rahmatullah and Boyde (1980), with appropriate adjustments of reagent strength for the different urea concentration ranges in water and blood plasma. Ammonia concentrations in the water were measured by the indophenol blue method (Ivancic and Degobbis 1984) and in the plasma by a Sigma Diagnostics ammonia (L-glutamate dehydrogenase) kit. Plasma glucose concentrations were measured with a Sigma Diagnostics Infinity glucose (hexokinase) kit. Plasma cortisol concentrations were measured using a commercial ^{125}I radioimmunoassay kit (ICN) with standards diluted to the same protein range as toadfish plasma.

The majority of urea excretion is via the gills (> 90%), the kidney of toadfish contributing only a small percentage (< 10%) (McDonald et al. 2000). In addition, the permeability of the skin to urea is extremely low ($5.07 \pm 0.56 \times 10^{-7}$ cm s^{-1}, $n = 8$; Part et al. 1999). Thus, the excretion (E; μmol kg^{-1}) of any substance (X) was calculated from the increase in concentration of the substance in the water $[\Delta X]_w$ during a pulse corrected for fish body weight (wt) and calculated as:

$$E_x = \frac{[\Delta X]_w \times V_f}{wt}, \quad (1)$$

where V_f is the volume of water surrounding the fish. A pulse was identified as a sudden increase in urea appearance in the surrounding water of at least 20 μmol kg^{-1}.

The metabolic clearance rate (MCR; ml kg^{-1} h^{-1}) for cortisol was estimated by adapting an approach described by Brown et al. (1986) from the known rate of cortisol infusion (Ic) and the steady-state value of cortisol in the plasma ($_{ss}[C]_p$) and calculated as:

$$MCR = \frac{I_C}{{}_{ss}[C]_p} \quad (2)$$

Statistics

Data have been reported as mean ± 1 SEM (n = number of fish). The significance of differences between means was evaluated using Student's paired or unpaired two-tailed t-test ($P < 0.05$) as appropriate (Nemenyi et al. 1977). An ANOVA with time as the main factor was followed by a comparison of individual means using the Bonferroni correction for multiple sample comparisons.

Results

The toadfish used in this study were predominantly ureotelic, excreting $75.6 \pm 3.1\%$ ($n = 33$) urea-N

(83.3 ± 8.8 μmol-N kg^{-1} h^{-1}, $n = 33$) and $24.4 \pm 3.1\%$ ($n = 33$) ammonia-N (22.5 ± 3.0 μmol-N kg^{-1} h^{-1}, $n = 33$) when averaged over the initial NaCl infusion control period. Ammonia excretion was not significantly different amongst groups and did not change over the course of the experiment (Table 1). Over the control infusion period, $93.9 \pm 1.3\%$ ($n = 33$) of the urea excretion occurred in discrete pulses in fish of all four treatment groups, with a mean frequency of 4.7 ± 0.3 pulses per 24 h ($n = 33$) and a mean size of 280 ± 3 μmol kg^{-1} ($n = 33$). Average plasma urea concentrations during the control period were 6.12 ± 0.42 mmol l^{-1} ($n = 33$) and plasma cortisol concentrations were 235.7 ± 32.8 ng ml^{-1} ($n = 33$). Plasma ammonia and glucose concentrations were not substantially different amongst groups and were unchanged by each respective treatment (Table 1).

Fish in the cortisol-only group had cortisol concentrations and plasma urea concentrations that were relatively steady during the initial 48 h infusion with NaCl (Fig. 1a, b). Upon cortisol loading, plasma cortisol concentrations increased significantly within the first h of infusion and continued to increase until approximately 8 h into cortisol loading ($t = 56$ h) when cortisol concentrations stabilized (Fig. 1a). During this time, plasma urea concentrations showed a slight increasing trend (Fig. 1b) and the pulsatile component of urea excretion was reduced by threefold, causing significant reduction in total urea excretion (Fig. 5a). The reduction in pulsatile urea excretion appeared to be a result of a significant fivefold decrease in pulse size, whereas pulse frequency during cortisol infusion was unchanged (Fig. 6a, b). Upon removing the cortisol from the infusate, cortisol concentrations quickly decreased, returning back to resting concentrations in approximately 16 h ($t = 112$; Fig. 1a). The metabolic clearance rate of cortisol of these fish was estimated at 550.5 ± 54.8 ml kg^{-1} h^{-1} ($n = 10$). During this time, the excretion of urea recovered and the pulsatile component of excretion increased to levels not significantly different than initial control values (Fig. 1c, 5a).

Fish in the cortisol + oil treatment (i.e. control injection group) had plasma cortisol concentrations and plasma urea concentrations that did not significantly change during the initial 48 h infusion with NaCl (Fig. 2a, b). Upon cortisol and oil treatment, plasma cortisol concentrations increased significantly within the first hour of infusion ($t = 49$ h) and stabilized 3 h later ($t = 52$ h). However, at $t = 96$ h, cortisol levels began to show an increasing trend (Fig. 2a). Similar to fish treated with cortisol only, the metabolic clearance rate of cortisol was estimated at 568.1 ± 58.7 ml kg^{-1} h^{-1} ($n = 6$). At this time, the total urea excreted was significantly reduced as a result of a 2.5-fold decrease in the pulsatile component of excretion (Fig. 5b). Similar to fish infused with cortisol alone, the reduction in pulsatile excretion was a consequence of a twofold decrease in

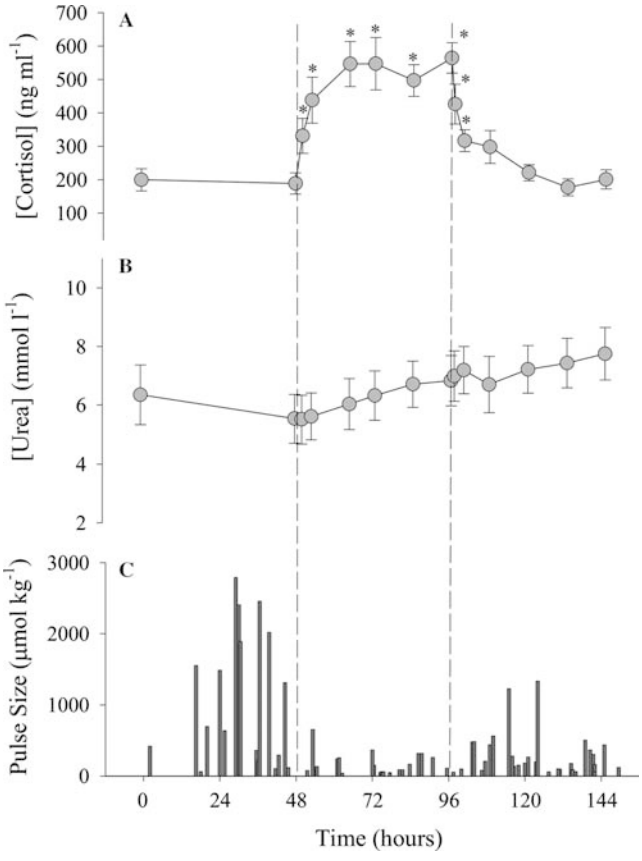

Fig. 1 **a** Plasma cortisol concentrations, **b** urea concentrations and **c** individual pulses from cortisol-treated fish during the initial NaCl infusion, cortisol infusion and subsequent recovery NaCl infusion. *Dashed lines* separate the three consecutive treatments. Values are mean ± 1 SEM ($n = 10$); $^*P < 0.05$ compared to the last sample taken during the initial NaCl infusion

Table 1 Parameters that are often affected by cortisol treatment. No significant differences between the control NaCl infusion and the respective treatments of each group were found. Values are mean ± 1 SEM (n)

	Cortisol	Cortisol + oil	Cortisol + RU486	Cortisol + Spironolactone
NaCl Infusion				
NH_3 excretion (μmol kg^{-1} h^{-1})	34.7 ± 8.6 (10)	52.0 ± 14.8 (6)	70.9 ± 17.0 (7)	32.8 ± 8.2 (10)
$[Ammonia]_{plasma}$ (μmol l^{-1})	164.1 ± 18.0 (10)	284.7 ± 63.2 (6)	125.9 ± 16.8 (7)	174.4 ± 47.3 (8)
$[Glucose]_{plasma}$(mmol l^{-1})	1.55 ± 0.15 (10)	1.41 ± 0.11 (6)	1.22 ± 0.15 (7)	1.65 ± 0.32 (10)
Treatment				
NH_3excretion (μmol kg^{-1} h^{-1})	42.2 ± 11.3 (10)	40.6 ± 8.1 (6)	67.4 ± 16.0 (7)	52.0 ± 7.6 (10)
$[Ammonia]_{plasma}$ (μmol l^{-1})	147.5 ± 15.2 (10)	258.9 ± 27.0 (6)	139.4 ± 27.9 (7)	196.0 ± 25.4 (8)
$[Glucose]_{plasma}$(mmol l^{-1})	1.72 ± 0.22 (10)	2.00 ± 0.32 (6)	1.08 ± 0.08 (7)	1.98 ± 0.38 (10)

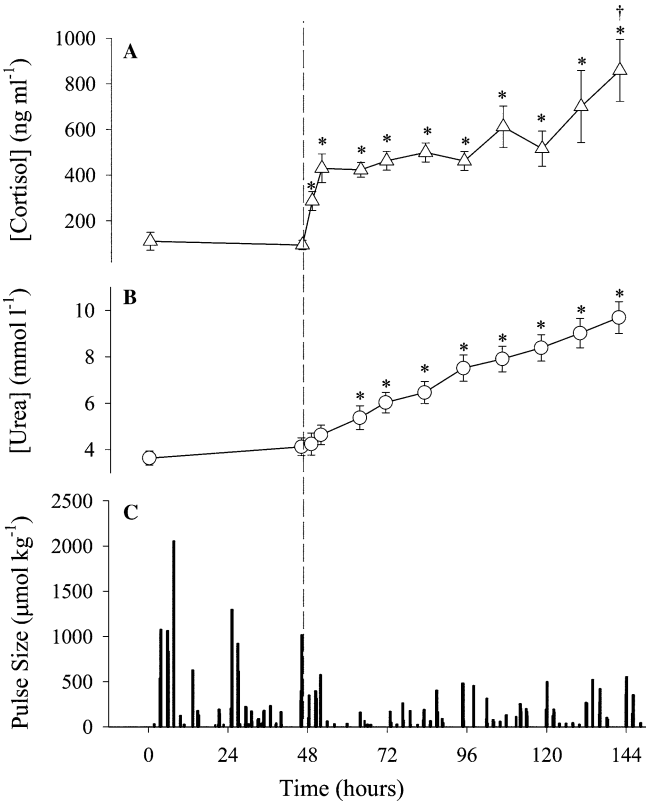

Fig. 2 **a** Plasma cortisol concentrations, **b** urea concentrations and **c** individual pulses from cortisol + oil-treated fish during the initial NaCl infusion and subsequent cortisol infusion and oil injection. *Dashed lines* separate the two consecutive treatments. Values are mean ± 1 SEM ($n = 6$); *$P < 0.05$ compared to the last sample taken during the initial NaCl infusion

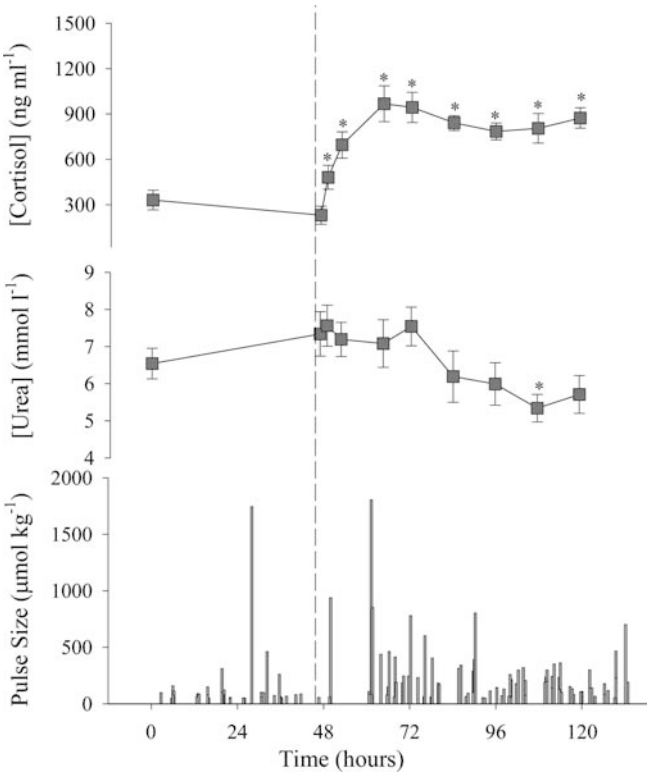

Fig. 3 **a** Plasma cortisol concentrations, **b** urea concentrations and **c** individual pulses from cortisol + RU486-treated fish during the initial NaCl infusion and subsequent cortisol infusion and RU486 injection. *Dashed lines* separate the two consecutive treatments. Values are mean ± 1 SEM ($n = 7$); *$P < 0.05$ compared to the last sample taken during the initial NaCl infusion

pulse size as pulse frequency was not affected (Fig. 6c, d). Consequently, plasma urea concentrations steadily increased and final values were almost threefold greater than plasma levels before the cortisol infusion (Fig. 2b).

Like the above treatment groups, fish in the RU486 group had cortisol concentrations and plasma urea concentrations that were relatively constant during the initial 48 h infusion with NaCl. Upon cortisol and RU486 treatment, plasma cortisol concentrations significantly increased within the first hour of infusion ($t = 49$ h) and continued to increase until $t = 56$ h when cortisol concentrations stabilized for the remainder of the experiment (Fig. 3a). The metabolic clearance rate of cortisol of these fish was significantly lower than that measured in the cortisol only and oil injected groups and was estimated at 310.2 ± 22.3 ml kg^{-1} h^{-1} ($n = 7$). In contrast to the other two groups, the total excretion of urea was significantly elevated during cortisol + RU486 treatment, due to a significant twofold increase in the pulsatile component of urea excretion (Fig. 5c). This increase was due to a twofold increase in pulse size with no corresponding change in pulse frequency (Fig. 6e, f). Most likely reflecting their mode of entry into the circulation, the protective action of RU486 against cortisol was delayed despite the treatments of cortisol (via infusion) and RU486 (via intraperitoneal injection) being initiated at the same time. Cortisol appeared to have an effect within 2 h, completely eliminating pulsatile excretion of urea until approximately 10 h post-infusion, after which the pulsatile excretion of urea returned and indeed was stimulated by RU486. In contrast to the control group, plasma urea concentrations showed a slight decreasing trend during cortisol + RU486 treatment and were significantly lower than initial concentrations at $t = 112$ h (Fig. 3b).

Fish in the spironolactone group had cortisol concentrations that were relatively constant during the initial 48 h infusion with NaCl but were slightly higher than initial concentrations measured in the other treatment groups (Fig. 4a). Initial plasma urea concentrations were also elevated in these fish compared to the fish of the other groups, but remained constant during the saline infusion (Fig. 4b). When treated with cortisol + spironolactone, plasma cortisol concentrations steadily increased reaching a final concentration that was much higher than in fish of other groups (Fig. 4a). The metabolic clearance rate of cortisol of these fish was significantly lower than that measured in the other groups and was estimated at 197.0 ± 9.6 ml kg^{-1} h^{-1} ($n = 10$). As in toadfish, in the cortisol-alone and cortisol + oil-injection groups, the pulsatile component of

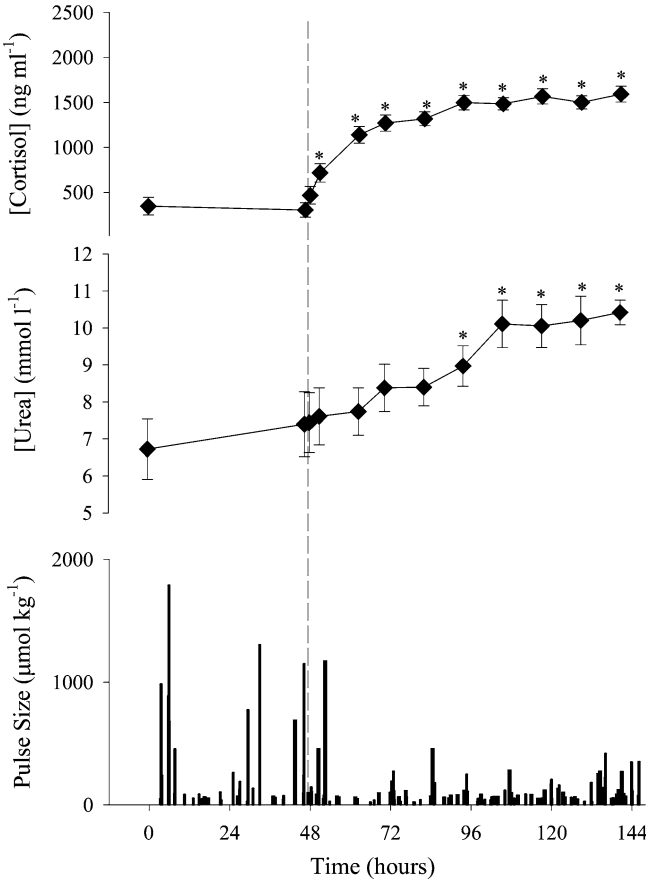

Fig. 4 **a** Plasma cortisol concentrations, **b** urea concentrations and **c** individual pulses from cortisol + spironolactone-treated fish during the initial NaCl infusion and subsequent cortisol infusion and spironolactone injection. *Dashed lines* separate the two consecutive treatments. Values are mean ± 1 SEM ($n = 10$); *$P < 0.05$ compared to the last sample taken during the initial NaCl infusion

urea excretion showed a twofold reduction upon cortisol + spironolactone treatment, due to a 2.7-fold decrease in the size of urea pulses (Figs. 5d, 6g). There was no change in pulse frequency (Fig. 6h). In addition, plasma urea concentrations increased steadily, reaching a final concentration that was significantly higher than initial values (Fig. 4b).

Discussion

To date, a drop in cortisol has been considered to play a permissive role in toadfish pulsatile urea excretion as plasma cortisol concentrations consistently decline immediately preceding a pulse event (Wood et al. 1997, 2001). The findings of the present study suggest that high levels of cortisol downregulate the activity of the urea transporter involved in pulsatile excretion, as there was a significant decrease in the size of urea pulse events accompanying cortisol infusion. Not only is this effect prevented by the glucocorticoid antagonist, RU486, suggesting the involvement of these types of receptors in

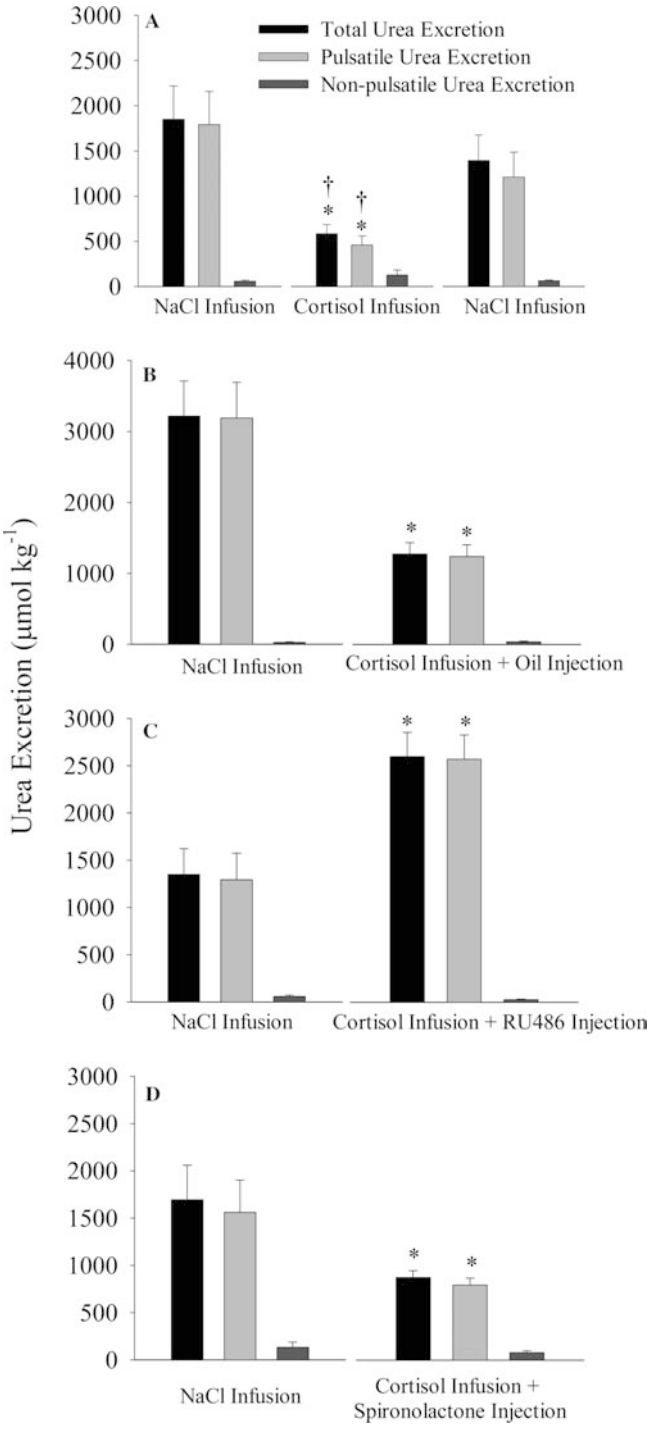

Fig. 5a–d The total urea excretion separated into pulsatile and non-pulsatile components during the initial NaCl infusion and subsequent cortisol treatments demonstrating a significant decrease in the pulsatile component when treated with cortisol alone, cortisol + oil or cortisol + spironolactone treatments. Treatment with cortisol + RU486 caused a significant increase in the pulsatile component of urea excretion. There was no change in the non-pulsatile component with any of the treatments. Values are mean ± SEM [cortisol alone ($n = 10$); + oil ($n = 6$); + RU486 ($n = 7$); + spironolactone ($n = 10$)]; *$P < 0.05$ significantly different than value measured during initial NaCl infusion; †$P < 0.05$ significantly different than during the recovery NaCl infusion

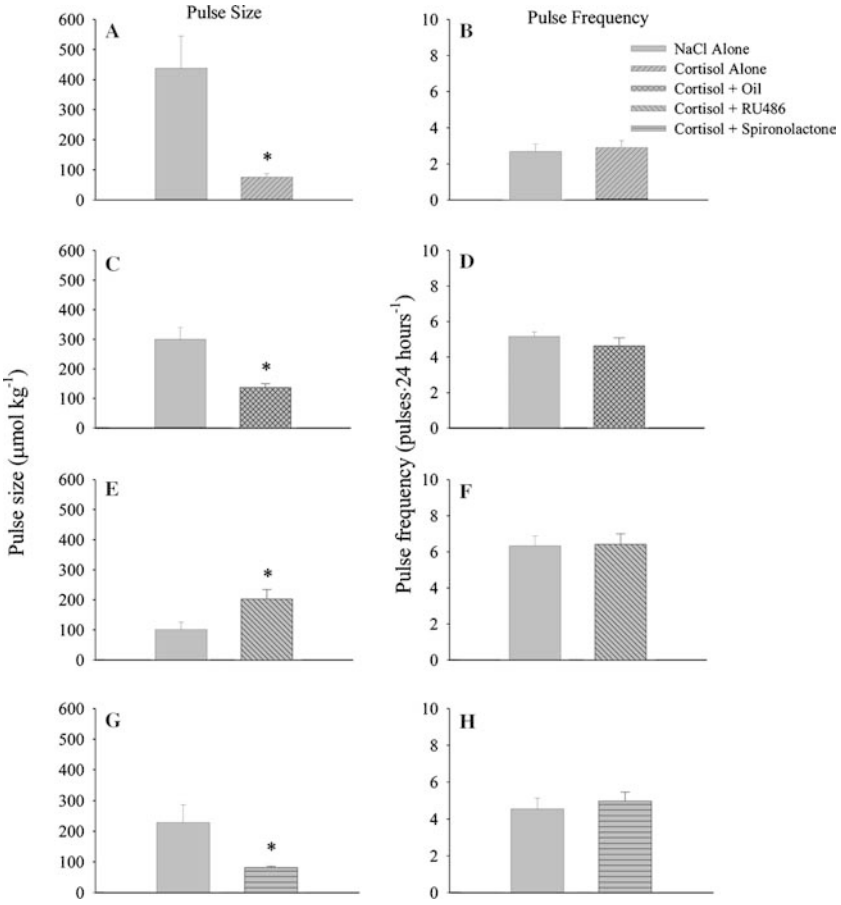

Fig. 6a–h Pulse size (*left panels*) and pulse frequency (*right panels*) from fish of all four treatment groups demonstrating a significant decrease in pulse size with cortisol alone, cortisol + oil or cortisol + spironolactone treatments. Treatment with cortisol + RU486 caused a significant increase in pulse size. There was no change in pulse frequency with any of the treatments. Values are mean ± SEM [cortisol alone ($n = 10$); + oil ($n = 6$); + RU486 ($n = 7$); + spironolactone ($n = 10$)]; *$P < 0.05$ significantly different than during initial NaCl infusion

pulsatile urea excretion, but the urea excretion mechanism is actually upregulated in the presence of RU486. However, the direct trigger eliciting pulses is unaffected, as there was no change in pulse frequency with elevated cortisol treatment or with RU486 treatment.

Plasma cortisol concentrations measured during the initial NaCl infusion period were extremely variable between groups of fish, which emphasized the necessity for a pharmacological rather than a physiological dose of cortisol in order to achieve the desired response in all the fish used in the study. Despite the high cortisol levels achieved by the cortisol infusion, plasma ammonia concentrations were not different than levels measured in a previous study (100–200 μmol l^{-1}; Wood et al. 1995). Increased gluconeogenesis, which results in a rise in circulating ammonia and glucose, is a common indicator of stress in fish (reviewed by Mommsen et al. 1999), but neither plasma ammonia nor plasma glucose increased with cortisol treatment in the present study. In addition, the excretion of ammonia was within the expected range (20–100 μmol kg^{-1} h^{-1}; Wood et al. 1995; McDonald et al. 2003) and was unaffected by cortisol treatment in all groups. These three parameters indicate that, despite levels being high, circulating cortisol concentrations achieved by infusion were not of any measurable detriment to the fish. Furthermore, in a recent study, endogenous plasma cortisol levels in subordinate, cannulated fish exceeded 400 ng ml^{-1} without any exogenous loading and without any measurable harm to the fish (K.A. Sloman et al., unpublished observations).

During the initial NaCl infusion, the cortisol-only and cortisol + oil fish had cortisol values that were not substantially different than previously measured values in cannulated and confined, ureotelic toadfish (100–200 ng ml^{-1}; Wood et al. 1997, 2001). However, RU486-treated and spironolactone-treated fish had initial circulating cortisol concentrations that were on average threefold greater than those measured in the two other groups. In reflection of this difference, both RU486-treated and spironolactone-treated fish had lower urea excretion values than fish in two other groups during the initial NaCl infusion period, lending further support of the sensitivity of the urea excretory mechanism to elevated cortisol. However, despite this initial variability, upon cortisol treatment it was only fish treated additionally with RU486 that showed an increase in urea excretion. This is not only in contrast to the other groups of the present study but also to toadfish that were infused with saline + urea for a similar duration in which branchial urea excretion remained constant over the entire 132-h infusion period (McDonald et al. 2003).

Under laboratory confinement, circulating cortisol levels in toadfish decrease 2–4 h preceding a urea pulse event, the urea pulse then occurs and cortisol levels rise immediately thereafter (Wood et al. 1997, 2001). During

the chronic cortisol elevation maintained in the present study by exogenous cortisol loading over a 48- to 96-h period, there was an inhibition of pulsatile excretion mediated by a decrease in pulse size. Theoretically, within 2 h and certainly within 48 h, cortisol could diffuse through the cell membrane and alter the transcription of the specific genes involved in pulsatile urea excretion. However, increasing evidence suggests that steroid hormones can exert rapid onset effects (within seconds to minutes) and that glucocorticoid receptors are distributed in membrane as well as intracellular compartments (Grote et al. 1993). A recent study revealed that mRNA expression of tUT around the time of a pulse (and at varying cortisol levels) was constant, suggesting a non-genomic action of cortisol (Walsh et al. 2000). Furthermore, rats treated with dexamethasone, a glucocorticoid agonist, showed a significant decrease in mRNA expression for UT-A1 (71%), UT-A3 (75%) and UT-A3b (75%) but no comparable decrease in UT-A2 (Peng et al. 2002). Since the toadfish tUT shows high homology to mammalian UT-A2 transporters, it may not be affected on the transcriptional level by elevations in circulating cortisol. In addition, a pharmacologically induced fall in plasma cortisol (via metyrapone injection) consistently results in a small pulse event within 30–90 min (Wood et al. 2001); a rapid onset and an equally fast recovery after hormone removal being characteristic of non-genomic action (Borski et al. 2002). Also relevant to the present study, the rapid actions of glucocorticoids in fish have been shown to be blocked by the corticosteroid antagonist, RU486 (Shih et al. 1990).

As plasma cortisol concentrations fluctuate dramatically within a short time period in ureotelic toadfish, the metabolic clearance rate for cortisol ($MCR_{cortisol}$) in toadfish could potentially be higher than that measured in other fish. Values for $MCR_{cortisol}$ in other fish range from 45 ml kg^{-1} h^{-1} in the American eel (*Anguilla rostrata*; Butler 1973) to 260 ml kg^{-1} h^{-1} in rainbow trout (*Oncorhynchus mykiss*; Brown et al. 1986, 1989; reviewed by Mommsen et al. 1999). In comparison, the $MCR_{cortisol}$ values measured for toadfish treated with cortisol or cortisol + oil were at least twofold and up to 12-fold greater than other fish. However, $MCR_{cortisol}$ is normally calculated using [^{3}H]-cortisol without raising circulating cortisol levels (Oppenheimer and Gurpide 1979; Brown et al. 1986, 1989) as the clearance of cortisol is highly modified by the environment and will vary considerably with stress, salinity, maturity and nutritional state (reviewed by Mommsen et al. 1999). Thus, the $MCR_{cortisol}$ values determined from toadfish treated with cortisol or cortisol + oil are likely an overestimation of what would be calculated for toadfish under resting conditions, as suggested by the reduced $MCR_{cortisol}$ values measured in fish treated with cortisol + RU486 or cortisol + spironolactone (see below). In these fish, $MCR_{cortisol}$ values fall very close to the rates measured in other fish, suggesting that toadfish do not have an elevated clearance rate of cortisol under normal resting conditions. However, it would be interesting to measure $MCR_{cortisol}$ for toadfish around the time of a urea pulse event when cortisol levels are rapidly changing.

Despite the absence of aldosterone, the major circulating mineralocorticoid in mammals, mineralocorticoid receptors (Colombe et al. 2000) are present and mediate specific actions of cortisol in teleost fish (Sloman et al. 2001). Glucocorticoid receptors appear to be involved in the regulation of the pulsatile excretion mechanism as RU486 blocked the inhibitory action of cortisol. In comparison, there was no protective effect of the mineralocorticoid receptor antagonist, spironolactone, against the cortisol-mediated inhibition of urea excretion. Interestingly, the difference between initial and maximum plasma cortisol levels in fish treated with RU486 ($\sim$600 ng ml^{-1}) or spironolactone ($\sim$1200 ng ml^{-1}) was greater than that observed in fish of the other two groups ($\sim$350 ng ml^{-1}), despite being given the same dose of cortisol. In addition, the $MCR_{cortisol}$ values measured in fish treated with cortisol + RU486 or cortisol + spironolactone were significantly lower than fish in the other two groups. While discrepancies in initial cortisol concentrations between groups of fish could have accounted for part of this difference (i.e. initial cortisol levels affecting $MCR_{cortisol}$), this observation may also suggest a direct effect of RU486 or spironolactone on $MCR_{cortisol}$ or on cortisol production. An increase in cortisol production has been observed in mammals as well as teleost fish in response to RU486 and is due to an altered negative feedback control of cortisol release from the interrenal cells (homologous to the mammalian adrenal gland; Bertagna et al. 1984; Gaillard et al. 1985; Healy et al. 1983; McDonald and Wood 2004).

In conclusion, cortisol and glucocorticoid receptors are involved in the regulation of pulsatile urea excretion in the gulf toadfish, although it is unclear if the inhibitory action of cortisol is directed towards tUT or a second urea transporter isoform. Clearly, this can be addressed in future research through molecular approaches directed at the mRNA (Northern analysis) and protein level (Western analysis). In addition to this, further investigation is necessary in order to determine whether the actions of cortisol on the pulsatile mechanism are through genomic regulation or via non-genomic pathways.

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