Research paper

In-vivo regulation of Krüppel-like factor 9 by corticosteroids and their receptors across tissues in tadpoles of Xenopus tropicalis

Leena H. Shewade, Katelin A. Schneider, Audrey C. Brown, Daniel R. Buchholz *

Department of Biological Sciences, University of Cincinnati, 312 Clifton Court, Cincinnati, OH 45221, USA

A R T I C L E   I N F O

Article info
Received 19 November 2016
Accepted 17 February 2017
Available online 20 February 2017

Keywords:
Amphibian metamorphosis
Corticosterone
Aldosterone
Glucocorticoid receptor
Mineralocorticoid receptor
RU486
Spironolactone

A B S T R A C T

Corticosteroids are critical for normal development and for mediating effects of stress during development in all vertebrates. Even though gene knockout studies in mouse and zebrafish have identified a number of developmental roles of corticosteroids and their receptors, the numerous pleiotropic actions of these hormones affecting various aspects of development are understudied. For the most part, neither the endogenous hormone(s) nor their receptor(s) regulating developmental processes during natural development have been determined. Here, we address this issue by elucidating the endogenous regulation of the transcription factor Krüppel-like factor 9 (klf9) across tissues during development by corticosteroid hormones (aldosterone and corticosterone) and their nuclear receptors (type-I and type-II receptors). First, we measured the developmental expression profiles of klf9 and type-I and type-II corticosteroid receptors in key target tissues, brain, lungs, and tail, during larval and metamorphic stages in Xenopus tropicalis. We also studied the corticosteroid regulation of klf9 in these tissues in-vivo using exogenous hormone treatments and receptor antagonists. klf9 and the corticosteroid receptors were expressed in each tissue and significantly increased in expression reaching a peak at metamorphic climax, except for the type-II receptor in brain and tail whose expression did not change significantly across stages. Both corticosteroid hormones induced klf9 in each tissue, although aldosterone required a five times higher dose than corticosterone to cause a significant induction. The upregulation of klf9 by both corticosteroids was completely blocked by the use of the type-II receptor antagonist RU486 and not the type-I receptor antagonist spironolactone. These results are consistent with previous in-vitro studies and indicate for the first time in-vivo that corticosteroid regulation of klf9 occurs exclusively via corticosterone and type-II receptor interaction across tissues.

© 2017 Elsevier Inc. All rights reserved.

1. Introduction

Corticosteroids are primary vertebrate stress hormones that play vital roles during development. They are responsible for organ maturation in preparation for life history transitions, such as birth and metamorphosis, and for mediating the effect of the environment through altered timing of organ maturation (Fowden and Forhead, 2015; Fowden et al., 1998; Khulan and Drake, 2012; Wada, 2008). The two corticosteroids, aldosterone and corticosterone (or cortisol in humans), act via two nuclear receptor proteins, type-I receptor (also known as the mineralocorticoid receptor, MR) and type-II receptor (also known as the glucocorticoid receptor, GR), to regulate gene expression underlying developmental responses to corticosteroids. While the type-I receptor binds with a very high affinity to both ALDO and CORT, the type-II receptor shows a comparatively lower affinity to CORT and does not bind ALDO at physiological concentrations (Funder, 1997; Roubos et al., 2009).

Gene knockout studies have begun to sort out which corticosteroid hormone and which receptor are responsible for specific corticosteroid-dependent developmental events. Mice lacking the type-II receptor die at birth due to lung atelectasis, but these mice also show severely impaired adrenal glands and decreased gluconeogenic activity (Cole et al., 1995). Brain-specific type-II receptor mutants only show behavioral abnormalities characteristic of anxiety and learning phenotypes (Tronche et al., 1999). Type-I receptor knockout mice show increased hypothalamic hormone activity suggestive of its role in corticosteroid feedback regulation as well as reduced neurogenesis (Berger et al., 1998, 2000, 2001). A zebrafish type-II receptor knockout model shows only a moderate stress behavior phenotype suggesting a possible compensatory mechanism (Griffiths et al., 2012). The complexity and pleiotropy of the developmental actions by
corticosteroids (Fowden and Forhead, 2015) leaves the specific hormone or receptor responsible for many stress and corticosteroid actions difficult to determine. Type-I receptor knockdowns in mice cause increased type-II receptor activity compounding the difficulty in identifying specific developmental roles for the two corticosteroid hormones and their receptors (Berger et al., 2006).

The dramatic effects of hormones on amphibian metamorphosis provide a valuable model to examine the role of stress hormones and their receptors in development (Buchholz, 2015). However, few if any corticosteroid-dependent events during metamorphosis have been attributable to a specific hormone/ligand interaction. The amphibian stress hormone, corticosterone (CORT), is believed to be the hormonal mediator of the environmental effects of stress because an increase in CORT in response to stress has been measured in a number of tadpole species (Bonett et al., 2010; Hoopfer et al., 2002; Krain and Denver, 2004; Middlemis Maher et al., 2013; Roubos et al., 2009; Yao et al., 2008). Also, in-vivo and in-vitro studies have shown that exogenous CORT can synergize with exogenous thyroid hormone to accelerate metamorphic events, while type-II receptor antagonists and CORT synthesis blockers reduce the effects of stress on metamorphosis (Bagamasbad et al., 2012; Bonett et al., 2009; Kulkarni and Buchholz, 2012). However, exogenous treatment with ALDO similarly affects thyroid hormone-dependent metamorphic development (Jolivet Jaudet and Leloup Hatey, 1984; Kikuyama et al., 1983, 1986; Ul et al., 1983; Niki et al., 1981). Also, plasma levels of both CORT and ALDO rise to a peak at metamorphic climax (Jolivet Jaudet and Leloup Hatey, 1984). Importantly, the pituitary hormone ACTH, which is responsible for CORT production, also induces ALDO production when injected into tadpoles (Krug et al., 1983; Macchi and Phillips, 1966), as in mammals (Funder, 2016). So far, no study has conducted measurements of ALDO after stress treatments in tadpoles. Thus, ALDO and/or CORT could be responsible for corticosteroid-dependent developmental effects.

In an effort to identify specific roles for corticosteroids in mediating developmental effects during metamorphosis, we here examine the regulation via corticosteroids and their receptors of a known CORT-response gene, kruppel-like factor 9 (klf9) in tadpoles undergoing metamorphosis. No other CORT-response gene is well-described in tadpoles. klf9 is a transcription factor induced by both thyroid hormone and CORT individually and synergistically (Bagamasbad et al., 2012; Bonett et al., 2009; Hoopfer et al., 2002). klf9 is known for its developmental role in response to stress in the brain of X. laevis tadpoles as well as mice, where it can alter neuronal structure and differentiation (Bagamasbad et al., 2012; Bonett et al., 2009). The developmental expression of klf9 has been shown for several tadpole tissues, including brain, tail, and intestine, and treatment with CORT induces klf9 expression in all tadpole tissues previously examined (Hoopfer et al., 2002). The induction of klf9 in-vivo by ALDO has not been tested.

Both nuclear receptors for corticosteroids are expressed in all tadpole tissues tested, namely tail and pituitary for type-I receptor and tail, brain, and intestine for type-II receptor (Bonett et al., 2010; Krain and Denver, 2004; Roubos et al., 2009; Thurman et al., 1986). An enhancer element upstream of klf9 has been identified in frogs and mammals as a CORT and thyroid hormone synergy module containing hormone response elements for thyroid hormone and corticosteroids (Bagamasbad et al., 2012). Because hormone response elements for type-I and type-II receptors in corticosteroid-regulated genes have the same sequence motif (Funder, 1997; Pearce and Yamamoto, 1993), it is not clear which corticosteroid/receptor interaction(s) regulate klf9 expression in-vivo.

Studies using type-I- and type-II-specific agonists and antagonists have provided insight into the corticosteroid regulation of klf9 (Bonett et al., 2009). In juvenile Xenopus (tadpoles were not tested), stress-induced induction of klf9 in the brain was completely or almost completely blocked using RU486 (a type-II receptor specific antagonist) pointing to the use of the type-II receptor, at least in post-metamorphic stages (Bonett et al., 2009). Using cell lines derived from Xenopus and mouse, CORT-induced klf9 expression was almost completely suppressed by RU486 but mostly not by spironolactone (a type-I receptor specific antagonist) (Bagamasbad et al., 2012; Bonett et al., 2009). Further, dexamethasone (a type-II receptor-specific agonist) can induce klf9 and be blocked by RU486 in cell lines and mouse primary cerebrocortical
cells (Bagamasbad et al., 2012; Gil-Ibáñez et al., 2014). In a mouse cell line overexpressing type-I receptor, ALDO can induce klf9, including at concentrations likely too low to activate type-II receptors though not always significantly (Bagamasbad et al., 2012). Also, in a different mouse cell line overexpressing type-I receptor and transfected with a klf9 enhancer reporter construct, ALDO-induced reporter expression was partially blocked by RU26752 (a type-I receptor-specific antagonist) (Bagamasbad et al., 2012). These data, from juvenile frogs and in-vitro brain-derived cell culture studies, suggest that the CORT-type II receptor interaction is the main if not only contributor to the induction of klf9 with equivocal evidence for a possible role of ALDO and type-I receptor, but in any case the in-vitro situation during development has not been determined.

Here, to gain insight into the role of corticosteroids during metamorphosis, we examined the developmental expression profiles of type-I and type-II receptors as well as klf9 in key CORT target tissues (brain, lungs, and tail) in tadpoles across developmental stages through metamorphosis. We then tested the effect of corticosteroid hormone treatments and receptor antagonists on klf9 expression to examine the possible in-vivo action of corticosteroids and their receptors on the regulation of klf9 expression among tissues.

2. Materials and methods

2.1. Animal husbandry

Lab-reared male and female adult Xenopus tropicalis were mated by priming with 20 U of human chorionic gonadotropin (Sigma) in the evening and boosting with 200 U the next morning. Resulting tadpoles were reared at 26°C and fed Sera Micron twice daily with daily water changes. The use of animals in experiments was in accordance with the guidelines of University of Cincinnati.

Fig. 2. Developmental profiles of type-I and type-II corticosteroid receptor expression in the brain, lungs, and tail during Xenopus metamorphosis. Expression of both receptors was detectable in each tissue at each stage and increased significantly during metamorphic climax in most cases. Total RNA was extracted from tadpole tissues at the indicated developmental stages to measure receptor mRNA expression by quantitative PCR. Bars show the mean mRNA levels relative to the reference gene rpl8. Error bars indicate SEM. The letters above the error bars indicate significance groups among stages based on Tukey’s honest significant difference test (p < 0.05, n = 5 for brain and tail and n = 3 for lungs per stage). No letters above bars indicates a lack of significant difference among stages within the tissue.
Institutional Animal Care and Use Committee (IACUC protocol #06-10-03-01).

2.2. Hormone treatments and gene expression analysis

For the developmental expression profiles of klf9 and type-I and type-II receptors, brain, lungs, and tail were harvested from tricaine-anaesthetized tadpoles at developmental stages throughout the larval period and metamorphosis, staged according to Nieuwkoop and Faber (NF) with n = 5–10 per tissue per stage (Nieuwkoop and Faber, 1956). For the dose response experiment, tadpoles at the end of premetamorphosis (NF54), were treated for 24 h with 100 nM or 500 nM of CORT or ALDO (Acros Organics) or EtOH vehicle control by addition to aquarium water before harvesting tails (n = 10 per experimental condition). For the receptor antagonist experiments with CORT treatments, brain, lungs, and tail were harvested from premetamorphic tadpoles (NF 54) treated for 24 h with i) EtOH control, ii) 100 nM CORT, iii) 100 nM CORT + 150 nM RU486 (Apexbio Technologies LLC), iv) 100 nM CORT + 150 nM spironolactone (Alfa Aesar), v) 100 nM CORT + 150 nM RU486 + 150 nM spironolactone, vi) 150 nM RU486, and vii) 150 nM spironolactone (n = 10 per experimental group). The antagonist doses were set to 50% more than the hormone to block the receptor binding (Rollins-Smith et al., 1997). A parallel receptor antagonist experiment was repeated for ALDO, except 500 nM ALDO vehicle control was used, only tails were harvested, and the antagonist-only treatments were not repeated. Tissues were snap frozen immediately following harvest on dry ice and stored at −80 °C. For brain and lungs, two and three individuals, respectively, were pooled together to obtain enough RNA. Total RNA extraction was performed using TriReagent (Molecular Research Centre Inc.) following manufacturer’s instructions. One µg of RNA was used per sample to synthesize cDNA (Biotool Inc) using manufacturer’s protocol. One µl of cDNA was used in 20 µl reactions for quantitative PCR with FAM labeled primer-probe sets and Taqman Universal master mix (Life technologies) on 7300 Real time PCR system, using PCR conditions and primer/probe sequences as previously described (Choi et al., 2014). The relative quantification method was used to measure the expression levels of klf9 normalized to the reference gene rpl8 (Livak and Schmittgen, 2001).

2.3. Statistical analysis

Data analysis was performed using JMP Pro 12 statistical analysis software. Analysis of Variance (ANOVA) was performed to identify significant differences in klf9 expression among developmental stages and among hormone and antagonist treatments. A p-value less than 0.05 was considered to be statistically significant. Pair-wise comparisons were done using the Tukey-Kramer post-hoc test. All error bars indicate standard error of the mean. All hormone treatment experiments show results from a single experiment, which were independently repeated 2–4 times using different clutches of offspring with similar results.

3. Results

3.1. Developmental expression profile of klf9

To confirm the expected expression of klf9 among tadpole tissues, we measured klf9 mRNA levels in brain, lungs, and tail of X. tropicalis tadpoles through metamorphosis from premetamorphosis (NF54) through metamorphic climax (NF62) to tail resorption (NF66) including intervening stages (Fig. 1). The levels of klf9 peaked at metamorphic stages in the brain (Fig. 1A), mimicking the profiles of thyroid hormone and CORT content in the blood, which reach peak amounts at NF62 and decline towards NF66 (Krain and Denver, 2004). In the lungs and tail, the expression remained low until early metamorphosis but significantly elevated during climax and remained high until the end (Fig. 1B, C).
It is not known if klf9 levels remain elevated indefinitely in lungs in juveniles and adults.

3.2. Developmental expression profile of type-I and type-II receptors

The expression of type-I and/or type-II receptor is likely a prerequisite for tissues to express klf9 in response to corticosteroids. We thus compared the developmental expression of both corticosteroid receptors in the brain, lungs, and tail of X. tropicalis tadpoles from premetamorphosis to the end of climax (Fig. 2). For the type-I receptor, the expression levels in the brain, lungs, and tail all increased significantly reaching a peak at the climax of metamorphosis. As seen for the type-I receptor, the type-II mRNA in the lungs showed significant increases and remained elevated at NF66. However, type-II receptor expression in brain and tail, though present, did not change significantly across metamorphosis.

3.3. Dose response for CORT and ALDO on klf9 expression

Because both type-I and type-II receptors are expressed in each tissue throughout metamorphosis, we wanted to further examine the potential role of these receptors in klf9 expression. Dose response and time course experiments were carried out first to establish suitable treatments for our receptor antagonist experiments in the next section. In the dose response experiments (Fig. 3), CORT-induced klf9 expression occurred at 100 nM but was not significant at 500 nM consistent with an inverted U-shaped dose response or indicative of more rapid transient expression kinetics at 500 nM. However, for ALDO from 100 nM to 500 nM, klf9 mRNA levels increased in a dose-dependent manner (Fig. 3). For the time course experiment (Fig. 4), klf9 expression increased significantly in each tissue reaching highest levels at 4 h in brain and lungs and at 24 h in tail. Thus, we used 100 nM CORT and 500 nM ALDO at a 4-h time point for the brain and lungs and a 24-h time point for the tail in the following experiments.

3.4. Effect of receptor antagonists on corticosteroid-induced klf9 expression

To examine the in-vivo receptor(s) used by corticosteroids, we studied the effect of RU486 (type-II antagonist) and spironolactone (SL, type-I antagonist) on klf9 expression induced by CORT (Fig. 5) and ALDO (Fig. 6). Addition of receptor antagonists alone had no impact on klf9 regulation in-vivo, but RU486 blocked the induction of klf9 by CORT completely in all three tissues, while addition of SL had no effect on CORT-induced klf9 induction (Fig. 5). Similarly, ALDO-induced klf9 expression was completely blocked by RU486 while SL had no effect (Fig. 6).

4. Discussion

Stress has numerous pleiotropic actions on organ maturation and development as mediated by corticosteroid hormones (Fowden and Forhead, 2015). While corticosteroid signaling is vital for maturation of major organ systems at the end of the gestational period, the endogenous roles of these hormones, their receptor preferences and subsequent gene targets are yet to be established. Here, we chose to address this issue by examining the endogenous regulation of the only known CORT-response gene in tadpoles, klf9 by CORT, ALDO, and their receptors in three corticosteroid-responsive tissues in-vivo in tadpoles.

We found that expression of klf9 starts low and by metamorphic climax achieves a 10-, 20-, and 100-fold increase in brain, lungs, and tail, respectively. These data concur with previous studies in brain and tail and add lung as another tissue with dynamic klf9 expression (Hoopfer et al., 2002). Similarly, our measurements for both corticosteroid hormone receptors showing expression in brain, lungs, and tail and increases in expression across development in the case of the type-I receptor for each tissue and only in the lung for the type-II receptor are in line with previous studies in brain for type-II receptor (Krain and Denver, 2004) and are novel for the lung and for the type-I receptor in brain, tail, and lung. For tail type-II expression, we found no significant differences among stages, though there was a 50% increase from NF54 to NF64, compared to a threefold increase in type-II receptor in the tail from a previous study using Northern blot analysis (Krain and Denver,
Thus, both corticosteroid hormone receptors are present and available to regulate klf9. In addition, previous studies showing significant increases in CORT and ALDO levels (Carstensen et al., 1961; Jolivet Jaudet and Leloup Hatey, 1984) and our current results and results from others on dynamic type-I and type-II receptor expression during development (Fig. 2) (Funder, 1997; Langlois and Martyniuk, 2013; Roubos et al., 2009) strongly suggest developmental roles each corticosteroid hormone and receptor.

To identify potential functional roles of corticosteroid signaling in vivo, we examined which corticosteroid(s) and receptor(s) are able to regulate klf9 in different tissues. Our dose response study using premetamorphic tadpoles confirmed klf9 induction in tails by CORT (Bonett et al., 2009) and showed ALDO was also capable of inducing klf9 but at a higher concentration (500 nM). Receptor antagonist studies showed that RU486 (type-II receptor-specific antagonist) was able to completely block CORT induction of klf9.

Fig. 5. Effect of receptor antagonists on CORT-induced klf9 expression in brain, lungs, and tail. CORT-induced klf9 expression was completely suppressed by type-II receptor antagonist but not by type-I receptor antagonist in each tissue. Tadpoles were raised to NF54 and treated with 100 nM CORT, 150 nM RU486, 150 nM spironolactone (SL), and EtOH control as indicated before tissue harvest at 4 h (brain and lungs) and 24 h (tails). Total RNA was isolated from brain, lungs, and tail, and klf9 mRNA levels were measured using quantitative PCR and normalized to the reference gene rpL8. Error bars indicate SEM. The letters above the error bars indicate significance groups among treatments based on Tukey’s honest significant difference test (p < 0.05, n = 5 for brain and tails and n = 3 for lungs per treatment group).
If the type-I receptor played a role, one would expect RU486 to only partially block klf9 induction. Our result using RU486 is consistent with the result from spironolactone (type-I receptor-specific antagonist), which did not block klf9 induction, indicating that even though type-I receptor is present, CORT seems to use only the type-II receptor in klf9 regulation.

Similar to the results with CORT, aldosterone is also able to induce klf9, but the higher dose required, 500 nM, represents supraphysiological levels which may have been sufficient to activate the type-II receptor. Indeed, we found that RU486 and not SL blocked ALDO induction of klf9. Thus, ALDO induction of klf9 does not involve the type-I receptor. Also, because the low affinity of ALDO for the type-I receptor is present, CORT seems to use only the type-II receptor in klf9 regulation.

Our findings expand on results from studies of postnatal mice, juvenile Xenopus, and cell culture experiments that CORT acts through type-II receptors and is the ligand/receptor pair that carries out the effects of corticosteroids on klf9 regulation (Bagamasbad et al., 2012; Bonett et al., 2010; Terrién and Prunet, 2013). Our studies focused on a single corticosteroid-response gene, klf9. Whether this result will hold for other CORT-response genes or for the effects of stress on development in general remains to be examined. Increased understanding would proceed from measuring ALDO levels in response to stress in tadpoles or using knockout hormone/receptor fish lines to assess the developmental roles of these hormones during metamorphosis. In addition, the peak in ALDO levels at metamorphic climax and the dynamic developmental expression profile of the type-I receptor in all three tissues highlights additional unanswered questions in the role of corticosteroids and their receptors during development.

Disclosure statement

The authors have nothing to disclose.

Acknowledgments

Our work was supported by Graduate Student Governance Association Research fellowship and Wieman Wendel Benedict Award granted by University of Cincinnati to LHS. This research was also partially supported by the McMicken Undergraduate STEM Experiences program through the McMicken College of Arts and Sciences, University of Cincinnati awarded to KAS. Additional support came from NSF-REU program funds awarded to ACD.

References


Carstensen, H., Burgers, A.J.C., Li, Ch., 1961. Demonstration of aldosterone and corticosterone as the principal steroids formed in incubates of adrenals of the American bullfrog (Rana catesbeiana) and stimulation of their production by mammalian adrenocorticotropin. Endocrinology 1 (1), 37–50. http://dx.doi.org/10.1210/mem.1.1.37.


Terrien, X., Pruner, P., 2013. Crossregulation of the thyroid hormone and corticosteroids in amphibians and fish: the effects of endocrine disruption. INTECH Open Access Publisher.


