Chicken 11β-hydroxysteroid dehydrogenase type 2: Partial cloning and tissue distribution

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ABSTRACT

NAD+-dependent 11β-hydroxysteroid dehydrogenase (11HSD2) converts glucocorticoids to 11-oxo derivatives and thus decreases their local concentration and prevents them from activating corticosteroid receptors. In this paper we report the partial cloning, characterization and tissue distribution of chicken 11HSD2. A cDNA of 991 bp was cloned from kidney mRNA by reverse transcription and polymerase chain reaction. At the amino acid level, the sequence of PCR product had 56–59% homology with mammalian and 46–48% with fish 11HSD2. The consensus sequences of the short-chain dehydrogenase/reductase superfamily such as the catalytic activity motif Tyr-X-X-X-Lys and cosubstrate-binding motif Gly-X-X-Gly-X-Gly, were found in the cloned cDNA. Analysis of the tissue expression of chicken 11HSD2 mRNA and NAD+-dependent 11β-oxidase activity showed a similar tissue distribution pattern in the majority of tissues. High levels of expression and activity were found in kidney, small intestine, colon and oviduct; low in ovary and almost zero in brain, liver and testis.

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1. Introduction

It is well established that the glucocorticoids, cortisol and corticosterone bind with high affinity to not only glucocorticoid but also to mineralocorticoid receptors. The circulating levels of glucocorticoids reach every organ, but the intracellular availability of these hormones is determined not only by their plasma concentration but also by the local metabolism of glucocorticoids, which is involved in the intracrinic pre-receptor regulation of steroid hormone activity [1]. The key enzyme responsible for the local metabolism of glucocorticoids is 11β-hydroxysteroid dehydrogenase (11HSD), which has been cloned and characterized in various mammalian tissues and in some fish. 11HSD exists in two types that catalyze the interconversion of biologically active C11-hydroxy corticosteroids (cortisol, corticosterone) and their inactive C11-oxo derivatives (cortisone, 11-dehydrocorticosterone) with different physiological roles and tissue distributions [2,3]. 11HSD1 is a NADP+(H)-dependent enzyme with high K_m for glucocorticoids that operates predominantly as a reductase in vivo and thus regenerates active glucocorticoids and amplifies local glucocorticoid activity. In contrast, 11HSD2 is a NAD+-dependent oxidase with low K_m for glucocorticoids that inactivates these steroids to their C11-oxo derivatives. The distribution of 11HSD2 reflects the distribution of mineralocorticoid target cells because the key role of this enzyme is to prevent the glucocorticoid activation of mineralocorticoid receptors, which are nonselective between cortisol, corticosterone and aldosterone [4].

In teleost fishes, in which the presence of aldosterone still remains doubtful and cortisol seems to play an important role in regulating salt and water balance [5,6], 11HSD2 is the final enzyme in the biosynthesis of 11-ketotestosterone, the major fish androgen [7]. Fish 11HSD2 was recently cloned...
and it was shown that the enzymes from various fish species share about 40% homology with mammalian 11HSD2 proteins [8,9]. Though the metabolism of corticosterone to 11-dehydrocorticosterone and its role in the determination of aldosterone specificity has been identified in various non-mammalian species such as amphibians [10–12] and birds [13–16] the structural and functional aspects of avian 11HSD2 have yet to be defined. Birds are susceptible to sodium deficiency due to the relatively weak concentration ability of the avian kidney plus a relatively low sodium dietary intake, which is offset in mineralocorticoid target tissues by strongly upregulated aldosterone-dependent Na⁺ absorption [17]. However, the plasma concentration of aldosterone is much lower than that of corticosterone, the dominant glucocorticoid in birds [18] and avian mineralocorticoid receptors bind aldosterone and corticosterone with nearly equal affinity [19,20].

In this paper we describe the partial cloning of chicken 11HSD2 (ch11HSD2), the distribution of its mRNA and the NAD⁺-dependent 11β-dehydrogenase (oxidase) activity in various tissues.

2. Experimental

2.1. Animals

Experiments were performed on 5–7 weeks old Brown Leghorn chickens obtained from the hatchery of the Institute of Molecular Genetics (Czech Acad. Sci., Prague) fed a commercial poultry diet. The birds were killed by decapitation and exsanguination and the tissues were quickly removed. To study the expression and activity of 11HSD2 in the oviduct, some chickens were treated daily with an s.c. injection of 2 mg diethylstilbesterol (DES) per kg in polypropylene glycol for 7 days starting from day 22. The animal protocol was approved by the Institutional Animal Care Committee.

2.2. Chicken 11HSD2 sequence identification and verification

Based on a comparison of the high sequence homology among the known sequences of 11βHSD2 in mammals (human, rabbit, rat, mouse, pig, sheep, cattle) and fishes (zebrafish, tilapia), the predicted mRNA sequence of this enzyme in chickens was constructed using the program CLUSTAL W. Briefly, the known sequences were used to search chicken EST (expressed sequence tags) in the free internet NCBI database for other overlapping fragments and thus elongate the sequence. The fragments found were used to further search the database for other overlapping fragments and thus elongate the sequence.

The generated sequence of more than 1200bp was searched for an open reading frame using the Basic Local Alignment Search Tool. Based on the sequences of putative ch11HSD2, the ORF-specific forward and reverse primers were designed by the program Lasergene (DNASTAR, Madison, WI) (Table 1) and used in the subsequent PCR. Total RNA was isolated from chicken kidney by RNA Blue (Top Bio, Prague, Czech Republic), cDNA was synthesized from 5 μg of RNA by reverse transcription with oligo(dT) primers (Sigma, St. Louis, MO) and M-MLV Reverse Transcription Reagents (Invitrogen) and the synthesized cDNA with ORF-specific primers were obtained to obtain the 11HSD2 amplicon. The PCR reaction mixture contained 2.5 units of Platinum Tag polymerase, 20 pmol of both the sense and antisense primers, 200 μM dNTPs, 50 mM KCl, 15 mM Tris–HCl, 0.5 mM MgCl₂ and 1 μl of chicken cDNA. Although we attempted to obtain the full-length cDNA coding the ch11HSD2 protein via several protocols using rapid amplification of the cDNA end (5’ RACE kit, Invitrogen), the 3’-end was not isolated and only a 170 bp shorter fragment was obtained. The isolated cDNA fragment was inserted into the pGEM Easy Vector System (JM109 competent cells; Promega); the plasmid DNA was purified with a QIAprep Spin Miniprep kit (Qiagen) and sequenced by the ABI PRISM 3100 DNA sequencer in the Academy of Sciences Sequencing Facility. The sequences obtained were compared with predicted transcripts and with the ab initio predicted transcript downloaded from www.ensembl.org (ID: ENSGALT00000003471). Matching regions were used to design real-time PCR primers (Table 1) using the program Lasergene.

2.3. Analysis of ch11HSD2 gene expression

Total RNA from various tissues was extracted by RNA Blue (Top Bio, Prague, Czech Republic), cDNA was synthesized from 5 μg RNA using M-MLV Reverse Transcriptase Reagents (Invitrogen) and oligo(dT) (Sigma). Amplification of the target cDNA was performed in a LightCycler using LightCycler FastStart DNA Master SYBR Green I (Roche) and primers designed with the program Lasergene (DNASTAR, Madison, WI) (Table 1). Relative concentrations were calculated from crossing points using the standard curve method. Expression of the target genes was normalized to β-actin and to total RNA since the usefulness of normalization against the β-actin gene in various tissues is limited [21]. The data are expressed as mean ± S.E.M.

Table 1 – ORF-specific primers and primers used for semi-quantitative real time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense (5’→3’)</th>
<th>Antisense (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF-specific primers 11HSD2</td>
<td>AACGTCCCATCCCATCCATCCC</td>
<td>TCTACGGCGGCGAGGTCAGG</td>
</tr>
<tr>
<td>Semi-quantitative PCR primers 11HSD2</td>
<td>GCCCCGGGCTACGCGTGCAG</td>
<td>GCCCGGCTACGCGTGCAGTTC</td>
</tr>
<tr>
<td>β-Actin</td>
<td>TGATATGCTGGCGTGTTGGA</td>
<td>CATGGCTGGGCGTGTTGGAAGGTCTC</td>
</tr>
</tbody>
</table>
2.4. **11HSD enzymatic assay**

The activity of 1120HSD2 was studied in tissue slices and homogenates. Tissues were homogenized (1:9 w/v) in ice-cold buffer containing 200 mM sucrose and 10 mM Tris/HCl (pH 8.5) with a Polytron homogenizer. The homogenates were centrifuged at 1000 × g for 10 min and the supernatant was assayed for protein concentration by the Bradford Coomassie blue method. The NAD⁺- and NADP⁺-dependent 11β-oxidase activities were assayed in tubes containing the buffer (100 mM KCl, 50 mM Tris/HCl, pH 8.5), cosubstrate (0.8 mM NAD⁺ or NADP⁺) and substrate (35 nM [3H]corticosterone) [22]. In some experiments 50 mM sodium pyrophosphate was used to block pyrophosphatases and eliminate the possibility that NADP⁺ would be converted to NAD⁺ [23]. The amounts of protein and incubation times were optimized to ensure linearity of the enzymatic reaction.

The tissue slices were prepared and their metabolism analyzed as described previously [22,24]. Briefly, freshly removed tissues were washed in ice-cold 150 mM NaCl, cut into slices less than 1 mm thick and placed in tubes containing preheated and oxygenized DMEM, 0.8 mM NAD⁺ and 35 nM [3H]corticosterone [22]. In some experiments 50 mM sodium pyrophosphate was used to block pyrophosphatases and eliminate the possibility that NADP⁺ would be converted to NAD⁺ [23]. The amounts of protein and incubation times were optimized to ensure linearity of the enzymatic reaction.

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The reactions were halted by cooling, unlabelled corticosterone and 11-dehydrocorticosterone was added and the steroids extracted on SepPak cartridges (Waters, Milford, MA, USA). After the evaporation of methanol under nitrogen, the steroids were separated and quantified by HPLC with on-line detection using a flow-cell detector (Radiomac 150TR, Canberra Packard, Meriden, CT, USA) as mentioned earlier [25]. 11HSD2 activity was expressed in picomoles as the sum of 11-dehydrocorticosterone and 11-dehydro-20-dihydrocorticosterone per hour and milligram of protein (homogenate) or dry weight (tissue slices). Data are expressed as mean ± S.E.M.

The corticosteroids 11-dehydrocorticosterone (4-pregnen-21-ol-3,11,20-trione), corticosterone (4-pregnen-11β,21-diol-3,20-dione), 20-dihydrocorticosterone (4-pregnen-11β,21-triol-3-one) and 11-dehydro-20-dihydrocorticosterone (4-pregnen-20β,21-diol-3,11-dione) were purchased from Steraloids (Newport, RI).

3. **Results**

3.1. **Sequence analysis**

The identification of the putative 11HSD2 was based on a search of the chicken EST database for the cDNA fragments with the highest similarity to the conserved regions of this enzyme in mammals and fish. The identified fragments were then used to further search the database for other overlapping fragments and thus elongate the sequence. Based on these sequences, primers were designed to characterize an open reading frame (ORF) of 1161 bp starting with an ATG sequence of nucleotides. This ORF encodes a protein of 386 amino acids with a predicted molecular mass of 42 kDa. Figs. 1 and 2 show the amplified 11HSD2 cDNA that was 170 pb shorter than the deduced fragment. The missing part represents the 5’-end sequences that consist of a GC-rich region (79% within the first 200 nucleotides), which suggests the possibility of a significant mRNA secondary structure. Computer analysis of the secondary structure was performed using the Mfold tool (available at http://bioweb.pasteur.fr) and revealed the formation of a tight hairpin structure in that region. The amplified PCR product was inserted into a plasmid and sequenced. The sequence obtained was searched for an ORF of 981 bp encoding a protein of 326 amino acids with a predicted molecular mass of 36 kDa (Fig. 3). Multiple alignment of the chicken protein sequence with other species revealed that ch11HSD2 displays 56–59% sequence identity to those of mammals and 46–48% to those of fish (Fig. 4). Similar to other enzymes of the short-chain dehydrogenase/reductase (SDR) family, 11HSD2 has both of the following characteristic motifs [26,27]: a conserved NAD⁺ binding site in the N-terminal part of the molecule where a common Gly-X-X-Gly-Gly can be found and the catalytically active triad consisting of tyrosine and lysine residues (Tyr-X-X-Lys) with a serine 14 residue upstream.

3.2. **Expression of 11HSD2 mRNA and 11β-oxidase activity in chicken tissues**

The tissue distribution of 11HSD2 was determined by semi-quantitative RT-PCR on a range of chicken tissues. Transcripts were clearly detectable in the kidney, intestine, ovary and ovary. On the other hand, 11HSD2 mRNA was very low or nearly absent in the liver, brain and testis (Fig. 5). The size of the amplified fragment was 600 bp and no other reaction products were found on electrophoretic analysis (not shown).

We next compared ch11HSD2 mRNA abundance with 11β-oxidase activity. A survey of 11β-oxidase activities in intact tissue slices is presented in Fig. 6. It is obvious that there is a great variability in activity. Similar to ch11HSD2 mRNA abundance, the greatest activity of tissue slices was found in the kidney, intestinal segments and oviduct, whereas the activity was low or nearly absent in the gonads, brain and liver. The NAD⁺-dependent 11β-oxidase activity in tissue homogenates had similar distribution as 11β-oxidase activity in intact tissue slices - it was high in the kidney, intestine and oviduct, low in the ovary and very low or absent in the brain, liver and testis (Table 2). The NAD⁺-dependent 11β-oxidase activity was significantly different from NAD⁺-dependent activity. The presence of pyrophosphate in the incubation buffer decreased the conversion of corticosterone to 11-dehydrocorticosterone; in intestinal homogenate by more than 50%. Corticosterone was converted not only to 11-dehydrocorticosterone but also to 11-dehydro-20-dihydrocorticosterone in both slices and homogenates. The activity of 20-hydroxysteroid dehydrogenase was highest in the kidney, intestine and oviduct (not shown).

4. **Discussion**

In this study we cloned a partial-length cDNA of avian 11HSD2 from chicken. The cloned ch11HSD2 is a protein of 327 amino
Fig. 1 – Nucleotide sequence of chicken 11HSD2 cloned by PCR with predicted nucleotide sequence and ab initio 11HSD2 downloaded from www.ensembl.org. Numbers to the right correspond to the nucleotide sequence. (*) Indicates identical nucleotides in the cloned and predicted sequences. The primers designed for semi-quantitative real-time RT-PCR are in bold.
acids, with an identity of 56–59% to the mammalian and 46–48% to the piscine enzyme. A closer examination of the amino acid sequence of ch11HSD2 shows that the residues that are characteristic for the cosubstrate binding site and catalytic activity of the SDR superfamily [26,28] are also present in ch11HSD2. These are the dinucleotide-binding loop containing the Gly-X-X-Gly-X-Gly motif and the strictly conserved Tyr-X-X-X-1ys motif in concert with a conserved Ser 14 residue upstream. The evidence that this sequence encodes ch11HSD2 was reinforced by enzymatic studies and by the ch11HSD2 mRNA tissue distribution.

11β-oxidase activity and ch11HSD2 mRNA abundance studies on various tissues showed that the kidney, intestine and oviduct express 11HSD2 mRNA and oxidize corticosterone at C11 and that the NAD+-dependent activity is noticeably different from the NADP+-dependent activity. The presence of 11HSD2 in the kidney and intestine is in keeping with the conclusion that 11HSD2 operates in chicken mineralocorticoid target tissues similarly to mammals, where 11HSD2 prevents mineralocorticoid receptors being activated by glucocorticoids [4]. In birds, the mineralocorticoid target tissues are not just the kidney and colon [17,29,30] but also the small intestine [31]. In addition, our recent report has demonstrated that ch11HSD2 protects the intestinal mineralocorticoid regulatory pathway against stimulation by corticosterone [22].

The present finding of a relatively high 11HSD2 mRNA abundance and 11β-oxidase in the oviduct suggests the possibility that this organ might also be a target for aldosterone or that 11HSD2 might protect this reproductive organ against glucocorticoids [32]. In agreement with our finding in chickens, enzymatic studies on the quail oviduct demonstrated a high activity of 11β-oxidase that was NAD+-dependent but a very low activity of 11β-oxidase that was NADP+-dependent [15], which further confirms the presence of ch11HSD2. In contrast to the kidney, intestine and oviduct, the levels of 11HSD2 mRNA abundance and activity was low in the ovary and nearly absent in the brain, liver and testis. The presence of 11HSD2 mRNA and NAD+-dependent 11β-oxidase in ovary is consistent with the findings in mammals where 11HSD2 has been located to granulosa cells of the prelutein follicle [33,34].

Although the interpretation of enzyme activity data using NAD+ and NADP+ does not allow for absolute discrimination between 11HSD1 and 11HSD2, the different utilization of NAD+ and NADP+ in chicken tissues supports the conclusion that functional 11HSD2 is expressed in birds. Whereas 11HSD2 is unable to utilize NADP+ as a cosubstrate, 11HSD1 is able to bind and use both NAD+ and NADP+ (preferential cosubstrate) [2,3] such that NAD+-dependent 11β-oxidation reflects both 11HSD1 and 11HSD2. Consequently, the higher NADP+-dependent 11β-oxidation may reflect the higher activity of 11HSD1 in avian kidney. In contrast, the lower NADP+-dependent 11β-oxidation than NAD+-dependent 11β-oxidation in intestine and oviduct indicates that 11β-oxidase reflects predominantly 11HSD2 in these tissues. The fact that the inhibition of pyrophosphatases decreases NADP+-dependent activity further supports this assumption. Our failure to demonstrate strong NADP+-dependent 11β-oxidase activity in avian liver may reflect inadequate enzyme assay for corticosterone oxidation catalyzed by 11HSD1 in the liver since recent paper of Katz et al. [35] has identified reduction of 11-dehydrocorticosterone to corticosterone in membrane fraction of chicken liver.

The mechanism by which 11HSD2 controls glucocorticoid occupancy and glucocorticoid activation of mineralocorticoid receptors is not fully elucidated. In vivo experiments in rats have shown that most mineralocorticoid receptors in epithelial cells are occupied but not activated by corticosterone [36]. However, if 11HSD2 conversion of biologically active glucocorticoids to their receptor-inactive 11-oxo derivatives is blocked or inactive, glucocorticoid-occupied mineralocorticoid receptors are activated [37,38]. The generation of NADH from NAD+ has to be decreased under these conditions and thus not only the conversion of C11-hydroxy glucocorticoids to C11-oxo glucocorticoids but also the redox state (the balance of

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**Table 2 – Cosubstrate dependence of 11HSD2 activity in tissue homogenates**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>NAD+</th>
<th>NADP+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>9.3 ± 1.0</td>
<td>157.7 ± 8.0</td>
</tr>
<tr>
<td>Small intestine</td>
<td>0.9 ± 0.4</td>
<td>72.6 ± 5.9</td>
</tr>
<tr>
<td>Colon</td>
<td>0.3 ± 0.0</td>
<td>92.0 ± 11.1</td>
</tr>
<tr>
<td>Oviduct</td>
<td>5.4 ± 0.5</td>
<td>20.8 ± 2.7</td>
</tr>
<tr>
<td>Liver</td>
<td>0.1 ± 0.0</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Brain</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Ovary</td>
<td>0.7 ± 0.2</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>Testis</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.0</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. of 5–10 animals; n.d., not determined. Data are given in picomoles of corticosterone oxidized to 11-dehydrocorticosterone and 11-dehydro-20-dihydrocorticosterone per hour and milligram of protein. The 11HSD2 activity in oviduct homogenates was measured in chickens treated with DES.
Fig. 3 – Protein sequence alignment of chicken 11HSD2 with other known 11HSD2s. The sequences around the putative catalytic site (Tyr-X-X-X-Lys) and the binding sites for the cosubstrate (Gly-X-X-X-Gly-X-Gly motif) are boxed. The numbers of important amino acid residues are taken from the human sequence. Amino acid residues are numbered on the right. (*): All amino acid residues in this column are identical in all sequences in the alignment, (>): conserved substitutions have been observed, (.) semi-conservative substitutions were observed (according to UniProKB/Swiss-Prot).

In summary, we have partially cloned cDNA for chicken 11HSD2. The enzyme shows nearly 60% homology with that of mammals and is not only widespread in classical mineralocorticoid target tissues such as the kidney and intestine but also in some other tissues such as the ovary. The good correspondence between 11HSD2 mRNA and NAD+/NADH) in the cell might be a signal that is able to inhibit or activate the complex of glucocorticoids with the mineralocorticoid receptors [39,40]. The sensitivity of transcription factors to redox state and particularly to NAD+ has been described in various systems [41,42] including glucocorticoid receptor-mediated responses [43].
Fig. 4 – Protein sequence homology of chicken 11HSD2 with known sequences of mammals and fish expressed in percentage of identical amino acid residues.

Fig. 5 – Tissue distribution of chicken 11HSD2 mRNA normalized to total RNA and β-actin. The 11HSD2 mRNA abundance in the oviduct was measured in chickens treated with DES (for further details, see Experimental). Values are means ± S.E.M. of 5–10 animals.

Fig. 6 – Oxidation of corticosterone to 11-dehydrocorticosterone in tissue slices. Activity in the oviduct was measured in chickens treated with DES (for further details see Experimental). Values are means ± S.E.M. of 7 animals.

NAD⁺-dependent 11β-oxidase activity clearly suggests that ch11HSD2 is expressed in some avian tissues.

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