Interrenal steroid 21-hydroxylase in eels: primary structure, progesterone-specific activity and enhanced expression by ACTH

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Abstract

Cytochrome P450 21-hydroxylase (P450c21) is a key enzyme for corticosteroidogenesis. To understand the regulatory mechanisms of cortisol production in fish, we have cloned a cDNA encoding P450c21, for the first time in non-mammalian vertebrates, from the head kidney of the eel (*Anguilla japonica*). The overall similarity of the deduced P450c21 sequence was modest (41–44% amino acid identity) between the eel and mammals. However, the functional domains for steroid-binding, heme-binding and proton-transfer sites were well conserved (74–100% identity). The eel P450c21 mRNA was expressed abundantly in the anterior quarter of the head kidney, but was undetectable in the remaining three-quarters or in other tissues including the gill, heart, liver, intestine, kidney, immature gonad and skeletal muscle. Functional expression of the cDNA clone in non-steroidogenic COS-1 cells produced a protein with high 21-hydroxylase activity to convert progesterone to 11-deoxycortisterone but not 17 α -hydroxyprogesterone to 11-deoxycortisol, although the latter is a preferred substrate for mammalian P450c21. To examine whether 21-hydroxylated progesterone is actually 17 α -hydroxylated in the eel interrenal, 11-deoxycorticosterone and ³H-corticosterone were respectively incubated with the interrenal-containing anterior quarter of the head kidney. The separation of the steroids produced by two HPLC systems revealed that cortisol was produced from both substrates, showing the 17 α -hydroxylation of 11-deoxycorticosterone and corticosterone in the eel interrenal. ACTH infused at 3 pmol/kg per min for 5 h consistently increased plasma cortisol levels and interrenal P450c21 mRNA levels in seawater eels. These results showed that the interrenal-specific eel P450c21 cloned in this study is involved in cortisol production through conversion of progesterone to 11-deoxycorticosterone in the interrenal-containing anterior quarter of eel head kidney. Furthermore, ACTH stimulates cortisol production in part through enhanced P450c21 expression in the eel interrenal.

Journal of Molecular Endocrinology (2003) **31**, 327–340

Introduction

For the biosynthesis of corticosteroids from cholesterol, several enzymes such as cytochrome P450 cholesterol side-chain cleavage (P450scc), 21-hydroxylase (P450c21), 17 α -hydroxylase (P450c17), 11 β -hydroxylase (P450c11) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) are necessary. Among these enzymes, P450c21 appears especially important, because in mammals it directs the steroidogenic pathway toward the production of corticosteroids principally through conversion of 17 α -hydroxyprogesterone to 11-deoxycortisol

(Crawford *et al.* 1992). However, this is the only enzyme that has not been identified at the molecular level in non-mammalian species. In mammals, corticosteroid production is regulated acutely by the translocation of cholesterol from the outer to the inner mitochondrial membrane through the action of steroidogenic acute regulatory protein (StAR) (Stocco 2001), and chronically by the upregulation of steroidogenic enzymes (Hanukoglu 1992, Omura & Morohashi 1995).

In teleost fish, cortisol is a major corticosteroid secreted from the interrenal tissue. Cortisol acts not only as a glucocorticoid but also as a

mineralocorticoid, and it plays a pivotal role in adaptation to both fresh water (FW) and seawater (SW) environments (for review see McCormick 2001). Thus, the euryhaline eel serves as a good model for examining cortisol actions in fish, especially in relation to osmotic adaptation. Several hormones have been implicated in cortisol secretion (Arnold-Reed & Balment 1991, 1994, Li & Takei 2003), but the mechanisms of these hormone actions are poorly understood in fish. To understand the mechanisms, therefore, it is necessary to identify all factors that are involved in cortisol biosynthesis. In the eel, adrenocorticotrophin (ACTH), angiotensin II (ANG II), atrial natriuretic peptide (ANP) and C-type natriuretic peptide all stimulate cortisol production (Henderson *et al.* 1976, Li & Takei 2003, Li *et al.* 2003). Transfer of eels from FW to SW increases plasma cortisol levels in addition to ANG II and ANP (Tierney *et al.* 1995, Kaiya & Takei 1996, Li *et al.* 2003). With regard to the cortisol-producing enzymes, cDNAs encoding P450c11 (Jiang *et al.* 1996), P450c17 (Kazeto *et al.* 2000) and P450scc (Y Kazeto, S Adachi & K Yamauchi, personal communication) have been cloned in eels. As mentioned above, however, the cDNA encoding P450c21, a key enzyme for cortisol biosynthesis, has not yet been cloned in this fish.

In this study, a cDNA encoding P450c21 was cloned from the head kidney of SW-adapted eels. The tissue distribution of its mRNA was examined by RT-PCR. The cDNA was expressed in COS-1 cells, and the enzymatic activity of the expressed protein was examined using ^3H -progesterone and ^3H -17 α -hydroxyprogesterone conversion. Unexpectedly, only progesterone was converted to 11-deoxycorticosterone by the expressed protein. In mammals, 17 α -hydroxyprogesterone is a preferred substrate of P450c21 for cortisol production, and once progesterone is 21-hydroxylated, 11-deoxycorticosterone is scarcely 17 α -hydroxylated any more (Hanukoglu 1992). We therefore examined the ability of eel interrenal tissues for conversion of 11-deoxycorticosterone to cortisol using an *in vitro* incubation system. We also confirmed the conversion of ^3H -corticosterone to cortisol in the same system, since 11-deoxycorticosterone is readily converted to corticosterone by P450c11 in the eel interrenal (Jiang *et al.* 1998). Finally, changes in P450c21 expression in the interrenal were examined together with

changes in plasma cortisol concentration after ACTH infusion to assess whether the cloned P450c21 is involved in cortisol production after the hormonal stimuli.

Materials and methods

Animals and tissue collection

Cultured immature eels, *Anguilla japonica* (184.8 ± 12.5 g, $n=20$), were purchased from a local dealer. The gonads were too immature to distinguish their sexes by simple visual examination. Eels were maintained under the natural photoperiod in FW or SW tanks for more than 1 or 2 weeks respectively before use. Water in the tank was continuously circulated, aerated and regulated at 18 °C. After anaesthetization of eels with 0.1% (w/v) tricaine methanesulfonate (Sigma, St Louis, MO, USA), tissues (see below) were surgically isolated, immediately frozen in liquid nitrogen and stored at -80 °C.

Isolation of RNA

Total RNA was extracted from various tissues using ISOGEN (Nippon Gene, Toyama, Japan). For cloning of P450c21 cDNA, poly (A) $^+$ -RNA was subsequently isolated from the total RNA extracted from the head kidney of a SW-adapted eel using Oligotex-dT30 (Japan Synthetic Rubber, Tokyo, Japan). RNA was quantified by measuring absorbance at 260 nm in a spectrophotometer (DU, Beckman Coulter, Fullerton, CA, USA).

cDNA cloning by RT-PCR and 5'- and 3'-rapid amplification of cDNA end (RACE)

The double-strand cDNA pool was prepared from 0.5 μg poly (A) $^+$ -RNA using the SMART cDNA library construction kit (Clontech, Palo Alto, CA, USA) according to the supplier's instructions. PCR was then performed to amplify a partial cDNA fragment using degenerate primers S1 (5'-ATHGGNGGNACNGARAC-3') and A1 (5'-CKNGCNCRCANCCRAA-3'), which were designed based on the highly conserved regions of P450c21 peptides of the cow (accession no. P00191), the pig (P15540), the mouse (P03940) and the human (P08686). PCR reactions were started with an initial denaturation at 94 °C for 1 min

followed by 30 cycles at 94 °C for 60 s, 44 °C for 30 s and 72 °C for 45 s, and with a final extension step at 72 °C for 7 min, in a total volume of 50 µl containing 50 pmol of each primer, 40 nmol dNTP, 1 µl template and 1 unit Ex *Taq* DNA polymerase (Takara Shuzo, Kyoto, Japan). The PCR products were isolated using the GeneClean Kit (Obigene Inc, Carlsbad, CA, USA), subcloned into pT7 Blue vector (Novagen, Madison, WI, USA) and sequenced using a 310 DNA sequencer (PE Biosystems, Foster City, CA, USA).

Subsequently, RACE was performed using the PCR protocol as described above. The primers used were gene-specific primers (A2, 5'-GATT GGGGATAATAATGGTGTCT-3' and S2, 5'-TGCCCTGCTTGTCTGCTCTGAT-3') and their respective adaptor primers, 5' PCR primer and CDS III/3' primer (Clontech) (Fig. 1). The PCR products were isolated, subcloned and sequenced as above. Full-length P450c21 cDNA was constructed by aligning the three overlapping cDNA fragments, i.e. the original fragment amplified from primer pair S1-A1, together with the 5' and 3' RACE products. Finally, the full coding region was amplified to confirm the sequence and used for transient expression.

Transient expression of P450c21 in COS-1 cells

Since three possible start codons (ATG) were found at nucleotides 31–33, 43–45 and 247–249 of eel P450c21 cDNA (Fig. 1), two fragments with different lengths were amplified: the shorter one starting from ATG_{247–249} was amplified with primers S4 (5'-CGGAATTCAACATGTTGGA GCTGACACGGG-3') and A3 (5'-GCGGCCG CTCAAGTTAATGGTTCGTG-3') and the longer one starting from ATG_{31–33} was amplified with primers S5 (5'-CGGAATTCGTGATGCCAGT CTGCATGACAT-3') and A3 (Fig. 1). PCR was performed using Advantage 2 DNA Polymerase Mix (Clontech) at 95 °C for 1 min followed by 25 cycles at 95 °C for 30 s, 68 °C for 3 min and finally one cycle at 68 °C for 3 min. The amplified fragments were first subcloned into pT7 Blue and, after confirmation of the sequence, they were inserted at the *EcoR* I/*Not* I site into the expression vector pEGFP-N1 (Clontech). The recombinant expression vectors containing the shorter and longer fragments of eel P450c21 cDNA were named pe21S-N1 and pe21L-N1

respectively. The original pEGFP-N1 was used for control.

For functional expression of P450c21, COS-1 cells (kindly provided by Dr M K Park, University of Tokyo) were plated into three 60 × 15 mm culture dishes at 6.4×10^5 cells per dish in 5 ml Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum and 0.1 mM non-essential amino acids. After incubation at 37 °C for 16–24 h (the cells were 80–90% confluent), the cells were transfected with 5 µg plasmid DNA (pe21S-N1, pe21L-N1 or pEGFP-N1) using LIPO-FECTAMINE PLUS Reagent (Invitrogen) according to the supplier's instructions. After pre-culture of transfected cells for 36–40 h, the medium was exchanged with 5 ml DMEM containing 21 pmol ³H-progesterone and ³H-17 α -hydroxyprogesterone (Amersham International plc, Amersham, Bucks, UK). Medium (1 ml) was taken at 5 and 10 h of incubation and the steroids were extracted with diethyl ether. After drying under N₂, the residues were dissolved in 0.4 ml 40% acetonitrile and 0.1 ml was subjected to reverse-phase HPLC analysis (see section on HPLC conditions).

Tissue distribution of P450c21 mRNA expression evaluated by RT-PCR

P450c21 expression was examined in the head kidney, kidney, brain, heart, liver, skeletal muscle, immature gonad, intestine and gill from five FW eels and two SW eels. Since eel head kidney comprises approximately 23% of the total body length, the tissues were divided antero-posteriorly into four parts; P1, P2, P3 and P4 (Fig. 4). Total RNA (2 µg) was reverse transcribed in 20 µl reaction mixture with Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's protocol. The products were diluted 1:5 with distilled water and kept at –20 °C until use. A negative control was run for each sample without reverse transcriptase to confirm the absence of genomic DNA contamination. PCR was run at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s for 26 cycles with 20 pmol primers S6 (5'-TGTGGATTTGTCAGAGGA CTTC-3') and A4 (5'-CACAGGTCTAAGTCTA AGCACCT-3') (Fig. 1) and 5 µl diluted template. The expression of eel glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal

		gctcttttcgagtgataacctaataatgtg	30
S5	→		
ATGCCAGTCTGCATGACATTGTGGGTGCGGGTACAGCAGTCGTGCTGTGTACTGTGGTTAGTGTTGTT			102
M P V C M T F V G A G T A V V L C V L W L V F V			24
TATTACCCAGACAGACTGATCAGAAGCTATGCTCGCAATCAAACGTTGTTGGTTCCACACTCCTTTACCCA			174
Y Y P R Q T D Q K L C S Q S N V V G S T L L Y P			48
CTCAACCGGCTTCTTCCCCACACTCTCTCCCTCCCTCCCTGGCCCCCCTCCCTCCCTCTTCTGGGCAAC			246
L N R L L P H T L S P S L P G P P S L P L G N			72
S4	→		
ATGTTGGAGCTGACACGGGACCTCCCAATCACCTGACCTCTCTGGECTCGCCACTATGGCAACATCTAC			318
M L E L T R D H L P N H L T S L A R H Y G N I Y			96
AGACTCTACTGTGGCAACACAACCATAGTTGTGTTGAGCAGTGCTGAGATGATCCGGGAGGCCCTGGTGAAG			390
R L Y C G N T T I V V L S S A E M I R E A L V K			120
AAGTGGTCAGACTTTGCTGGGAGGCCACACTCATACACCGGCGATATTGTGTCAGGTGGTGGGCGATCCATC			462
K W S D D F A G R P H S Y T G D I V S G G G R S I			144
TCTTTGGGGACTACAGTGAGGCGTGGCGACACATCGCGCCTGGCCACAGTGCTTTGACAGCCTTGCTCT			534
S L G D Y S E A W R T H R R L A H S A L Q R C S			168
GCGCAGTGCTGCACCTGTCAATTCAGAAGCAGGCTCTCGGCTCAGGCAGGTTCTAATGGACTACAATGAG			606
A Q C L H A V I Q K Q A L G L R Q V L M D Y N E			192
S6	→		
ACTCCTGTGGATTGTGAGAGGACTTCACCGTGGCTGCCAGTAATGTCATCACTACTCTGGTCTTTGGAAAA			678
T P V D L S E D F T V A A S N V I T T L V F G K			216
GAATATGACAAGAGCTCCCTGAGCTGCAGCGTCTGCATGGCTGTCTGAATGAGATCGTGCTCTGTGGGGC			750
E Y D K S S P E L Q R L H G C L N E I V S L W G			240
TCCCCCTGGACTCTCTCCCTGCTCAGGAAGTTGCCCAATCCTCCTTTCTCTCGTTTA			822
S P W I S A L D S F P L L R K L P N P P F S R L			264
CTGAAAGAGGTGGCCAGGAGAGATGACATCATTAGATCACATATCCAAGAATATAAGGAGCTGTGCCAGGAT			894
L K E V A R R D D I I R S H I Q E Y K E L C Q D			288
GAAGGGACGGGGGTACTATAACAGCCTCTCTACTTCAGAGCCTGGATCAGCCACTGGGGGAGAAAGAGAGG			966
E G T G G T I T A S L L Q S L D Q P L G E K E R			312
S1	→		
GTGGCGTTGACAGAACTCATGTTTACATGGCTACAGTGGATCTACTATTGGTGGCACAGAGACCAGTCT			1038
V A L T E T H V H M A T V D L L I G G T E T T A			336
GCCTGGCTGGGCTGGACTGTGGCCTTCTTACTACACAGGCCTGAGGTGCAGTGTAGAGTGTACAGTGAGTTG			1110
A W L G W T V A F L L H R P E V Q C R V Y S E L			360
S2	→		
TGTACAGTGCTGGATACTCGGTACCCACAGTACAGTGATAGGCTCAAGCTGCCCTGCTTGTCTGCTCTGATC			1182
C T V L D T R Y P Q Y S D R L K L P C L S A L I			384
A4	→		
AATGAGGTGCTTAGACTTAGACCTGTGGCTCCACTTGACAGTTCCTCACCGTGCCATTAGAGACAGTAGCATT			1254
N E V L R L R P V A P L A V P H R A I R D S S I			408
A2	→		
GCAGGTACCGTATCCCGAAGGACACCGTTATTATCCCCAATCTGTATGGTGTCTCACCATGACCCAGCAATG			1326
A G Y R I P K D T V I I P N L Y G A H H D P A M			432
TGGATTGACCCCTACTCTTCAAACCGAAGCTTTTGTAGATGGAGGACAGCTTCCACTCGTGCTCTTCTG			1398
W I D P Y A F K P E R F L D G G R A S T R A L L			456
A1	→		
CCATTTGGAGGTGGAGGCCCGCTGTGCCTTGGAGAGTCCATAGCCAAAATGGAGCTATTCTGTTCCTGCTG			1470
P F G G G A R L C L G E S I A K M E L F L F T A			480
TACTTACTTCGTATTTCATTTCTTCCCTGGAAACAAAGAACCTTCCCGACCTGACTGGAGTTGCA			1542
Y L L R D F Q F L P S G N K E T L P D L T G V A			504
A3	→		
AGTGTGTCTTAAAGTCAAACCATTTCAAGTCATAGTGCCTCCACGACCATTAAGTTGAactttcttaaca			1614
S V V L K V K P F Q V I V R P R P L T *			523
gttaagtcgtataacggtttttgtaggtttttttttttttttgtgctatcctgattccctaataactaggat			1686
aataattggcctattgtcctactaataaaatgacctcacacagaatgcattcactgacaggtgttggtacctaca			1758
ataaaaggtagtactgttttttaacagatttggaggtaatggaaaaatcaatggaaaacatttacatctgttaa			1830
atagattttgcacacaacaagatccttagatccacagacgtgtcctagaatgaatttaattgtgtcctcaagct			1902
gatcagatgctcgtgcctacatactgtaagttttccacacacacaaacacaaatttatagtttatttaaacatctc			1974
ggatctgatgactgctgtacattttgtttgatgttagtgtttaactgctttgtagttcatcccttcactt			2046
tggcctttctgtgtgaaaacataattttagtggggaaaagacagatgttatatgtaaaatgtaaaacatata			2118
actcaaatatttgtagtgcattaaaatctatttttaatttctgcttcc(poly A)			2165

Figure 1 Nucleotide and predicted amino acid sequences of eel P450c21 cDNA. Nucleotide and amino acid numbers are shown on the right. Nucleotides in the untranslated region are in lower case. Two possible start codons (ATG), a termination codon (TGA) and two putative polyadenylation signals are underlined. Primer sequences used in this study are indicated by arrows (see Materials and methods).

controls as described previously (Takei *et al.* 2001). PCR was run for 24 cycles at 94 °C for 30 s, 62 °C for 30 s and 72 °C for 60 s. The PCR condition was

within the linear range of amplification for both genes in the interrenal tissue. The PCR products were electrophoresed in 1–1.2% agarose gels

containing ethidium bromide (EtBr), and observed in an FAS-III Electronic U.V. Transilluminator (Toyobo, Osaka, Japan).

***In vitro* incubation of steroids with interrenal tissue**

Conversion of 11-deoxycorticosterone to cortisol

Since expressed eel P450c21 converted progesterone but not 17 α -hydroxycorticosterone, and since the interrenal tissue that expresses P450c21 was exclusively localized in the anterior quarter (P1 segment) of the head kidney, we examined whether 11-deoxycorticosterone is actually converted to cortisol after incubation with interrenal tissue. The head kidney was excised from two FW eels, cut antero-posteriorly into four parts, and the P1 and P4 segments were used for experimental and control respectively. Each segment was minced with iris scissors, washed twice in sterile isotonic saline and incubated in 5 ml sterile Hank's balanced salt solution (HBSS; Life Technologies, Grand Island, NY, USA) at 27 °C with gentle agitation. After 1-h preincubation, 5 μ l 25 mM 11-deoxycorticosterone (Sigma) in ethanol was added (final concentration, 25 μ M), and the incubation continued for a further 1, 3 and 7 h at 27 °C. Neither 3 H- nor 14 C-deoxycorticosterone were commercially available. At each time-point, 200 μ l medium was taken, spun at 14 000 *g* for 5 min, and the supernatant was directly applied to HPLC analysis (see section on HPLC conditions). After the termination of incubation, all minced tissues were collected, freeze-dried and weighed. The P1 and P4 segments weighed 8.30 and 7.84 mg respectively.

Conversion of 3 H-corticosterone to cortisol

Since eel P450c11 cloned from eel interrenal readily converts 11-deoxycorticosterone to corticosterone (Jiang *et al.* 1998), we examined whether eel interrenal can convert corticosterone to cortisol. The P1 and P4 segments from two different FW eels were incubated in 2.5 ml HBSS as above, and 7.5 μ l 3 H-corticosterone (Perkin Elmer Life Sciences, Boston, MA, USA) was added to the medium at a final concentration of 30 nM. After 1, 3, 7 and 21 h of incubation, 100 μ l medium was taken and spun at 14 000 *g*. The supernatant was applied to the HPLC analysis. The elution condition and detection of radioactivity were the

same as for functional expression (see section on HPLC conditions).

HPLC conditions

In the sections 'Transient expression of P450c21 in COS-1 cells' and 'Conversion of 3 H-corticosterone to cortisol', steroids were separated in the reverse-phase column (ODS-120T, 4.6 $\times$ 250 mm; Tosoh, Tokyo, Japan) with a linear gradient of acetonitrile concentration from 40 to 70% over 30 min at 0.7 ml/min. The radioactivity of the eluate was monitored in a radiomatic flow scintillation analyzer (500TR; Perkin Elmer Inc., Shelton, CT, USA). The elution positions of progesterone, 17 α -hydroxyprogesterone, 11-deoxycorticosterone, 11-deoxycortisol, corticosterone and cortisol were determined at 274 nm by authentic steroids (Sigma-Aldrich, St Louis, MO, USA). Since 11-deoxycortisol and corticosterone were indistinguishable by this elution condition, a different system was applied to the experiment in the section on 'Conversion of 11-deoxycorticosterone to cortisol'. In this experiment, the column was eluted with a linear gradient of methanol/acetonitrile concentration in water from 20 to 40% in 30 min at 0.7 ml/min, which was modified from Nunez & Trant (1999). The steroid peaks were detected at 240 nm to minimize the absorption of contaminated proteins. Under this condition, corticosterone and 11-deoxycortisol are apparently distinguishable.

Effects of ACTH on plasma cortisol and P450c21 mRNA

ACTH infusion

Six FW eels were used in this experiment. After anaesthesia in 0.1% tricaine methanesulfonate, polyethylene tubes (SP 10, outside diameter=0.61 mm; Natsume, Tokyo, Japan) were surgically inserted into the ventral aorta for blood sampling and into the dorsal aorta for infusion. After operation, eels were transferred to individual plastic troughs through which aerated and filtered SW was continuously circulated at 18 °C. The troughs were covered with a black vinyl sheet to minimize visual stress during experiments. On the day after surgery, ACTH (human, 1–24; Peptide Institute Inc., Osaka, Japan) dissolved in 0.9% NaCl solution was infused into the eel at 3 pmol/kg per min in a volume of 8.3 μ l/min.

Eel	MPVCMTFVGAGTAVVLCVLWLVFVYYPRQTDQKLCSSQSNVVGSTLLYPLNRLLPHTLSPS	60
Bovine	MVLAGL-LLLTLLA-AH--WGRWK-RNLH-P-L	33
Pig	MVLVWL-LLTTLKA-AR--WGQWK-RNLH-P-L	33
Human	MLLLG-LLLPLLA-AR--WNWWK-RSLH-P-L	32
Mouse	MLLPGL-LLLLLLA-TRW-WGQWK-RKLH-PRL	33
Eel	LPGPPSLPLLGNMLELTRDHLPNHLTSLARHYGNIYRLYCGNTTIVLVSSAEMIREALVK	120
Bovine	V-.....F-H-LQPN--I--L--TQKL-PV---RL-LQEV---N-KRT-E--MIR	84
Pig	V-.....F-H-LQPN--IY-LG-TQKL-P---RL-LQDV---N-KRT-E---R	84
Human	A-.....F-H-LQPD--IY-LG-TQKF-P---HL-LQDV---N-KRT-E--M--	83
Mouse	A-.....F-HFLQPN--IY-LG-TQKL-P---IRL-MQDV---N-NRT-E---IQ	84
Eel	KWSDFAGRPHSYTGDIVSGGGRSISLGDYSEAWRTHRRLAHSALQRCSAQCLHAVIQKQA	180
Bovine	--V-----QIPSYKL--QRCQD-----LL-KA-KK-TR---LLGTRSSMEPVWDQLT	144
Pig	--V-----QIPSYKLA-QHCPD-----LF-KA-KK-TR---LLGVRSSMEPRVCQLT	144
Human	--A-----EPL-YKL--KNYPDL-----LL-KA-KK-TR---LLGIRDSMEP-VEQLT	143
Mouse	--V-----...HMLNGKMDLDL-----LM-KA-KK-SR---MLGMRDSMEPL-EQLT	140
Eel	LGLRQVLMDYNETPVDLSEDFTVAASNVITTLVFCKEYDKSSPELQRLHGCLNEIVSLWG	240
Bovine	QEFCERMVQAGA--TIQKE-SLLTCSI-CY-T--NKE...DTLVHAF-D-VQDLMT-D	201
Pig	QEFCERMRAQAG--TIQKE-S-LTCSI-CC-T--DKE...DTLVHA--D-VQDLMT-D	201
Human	QEFCERMRAQPG---AIE-E-SLLTCSI-CY-T--DKIKD.DNLMPAYYK-IQ-VLKT-S	202
Mouse	QEFCERMRAQAG---AIHKE-SFLTCSI-SC-T--DND...TLV-T--D-VQDLLQA-N	197
Eel	SPWISALDSFPLLRKLENPPFSRLLKEVARDDIIRSHIQEYKELCQDEGTGGTITASLL	300
Bovine	HWS-QI--MV-F--FF--GLW--KQAIEN--HMVEKQLTRH--S.MVA-QWRDM-DYM-	260
Pig	HWS-QI--MV-F--FF-S-GLR--KQAIEN--HLVEKQLRRH--S.MVA-QWRDMLDYM-	260
Human	HWS-QIV-VI-F--FF--GLR--KQAIK--H-VEMLRQH--S.LVA-QWRDMDYM-	261
Mouse	HWS-QI-TII----F---GLQK-KQIQES--H-VKQQLKQH--S.LVA-QWKDMIDYM-	257
proton-transfer site		
Eel	QSLDQPLGEKERVALTETHVHMTVDLLIGGTETTAAWLGWTVAFLLHRPEVQCRVYSEL	360
Bovine	-GVGRQRV-EGPGQ-L-G---SV---F-----ST-S-A-----H--I-R-LQE--	320
Pig	-EAGRQRV-EGQGQ-L-G---SV---F-----NT-S-A-VY---H--I-W-LQE--	320
Human	-GVA--SM-EGSQ-L-G---A-----NT-S-A-V---H--I-Q-LQE--	321
Mouse	-GVEKQRDG-DEER-H-G---SV---F-----TT-S-A-----H--I-K-LQA--	317
steroid-binding site		
Eel	CTVLDTR...YPOYSDRLLPCLLSALINEVLRLPVAPLAVPHRAIRDSSIAGYRIPKD	416
Bovine	DRE-GPGASCSRV-T-K--AR--L-N-T-A-----V---L---TT-P---F--D--EG	380
Pig	DRE-GPGAAGSRVP-K--AR--L-N-T-A-----V---L---T-P---F--D--EG	380
Human	DHE-GPGASSRVP-K--AR--L-N-T-A-----V---L---TT-P---S--D--EG	381
Mouse	DLK-GP...GSQLL-RN-MQ--L-M-T-A-----V---L---T-A---S--D---374	374
heme-binding site		
Eel	TVIIPNLYGAHHPAMWIDPYAFKPERFLDGGRASTRALLPFGGGARDCLGESIAKMLFF	476
Bovine	M-V-----Q--L-ETV-EQ-HE-R-D---EP-ANPS..A-A--C--VC---L-R-L--	438
Pig	--V-----Q--IL-ETV-EQ-HE-R-D---AP-ANPS..A-A--C--VC---PL-RL---	438
Human	-----Q--L-ETV-ER-HE-W-D---EP-KN-R..A-A--C--VC---PL-RL---	439
Mouse	M-----IQ--NL-EMV-EL-SK-W-D---EP-KNPR..TPS--C--VC---PL-RL---	432
Eel	LFTAYLLRDFQFLPSGNKETLPDLTG..VASVVLKVKPFQVIVRPRPLT.....	523
Bovine	VVLTR--QA-TL--PP.VGA--S-QPDPYCG-N--Q---RLQ--GVEAGAWESASAQ	496
Pig	VVLVQ--QA-TL--P..EGA--S-QVPYSGIN--Q---RLQ--GGRGEGPGPR...	492
Human	VVLTR--QA-TL---.GDA--S-QPLPHC--I--MQ---RLQ--GMAHSPGQNQ..	494
Mouse	VVL-R--QA-TL--PP.DG--S-QPQPY-GIN-PIP---RLQ--N-APQDQGERP..	487

Figure 2 Comparison of amino acid sequences of eel P450c21 with those of mammals. Sequences were aligned by Clustal W in DDBJ website at <http://www.ddbj.nig.ac.jp>. The accession numbers were: bovine (P00191), pig (P15540), human (P08686) and mouse (P03940). Amino acid numbers are shown on the right. Dashes represent identical amino acid residues and dots represent gaps. Putative proton-transfer, steroid-binding and heme-binding sites are boxed. The cysteine residue in the heme-binding sites which, it is suggested, co-ordinates with the heme-prosthetic group, is highlighted by a black box.

Infusion of vehicle alone served as control. Human ACTH is only one amino acid different in its structure from eel ACTH (accession no. AF194969) (Dewied 1990) and exhibits full steroidogenic activity

in fish (Young 1993). Blood (approximately 30 µl) was collected into a capillary before and every hour after the start of infusion. The blood volume removed was replaced immediately by a vehicle injection.

After centrifugation, plasma was stored at -20°C for subsequent cortisol determination. Five hours after infusion, eels were anaesthetized and the anterior part (P1 segment) of the head kidney was collected from each eel for RNA extraction. All fish were killed at the same time of the day.

Comparison of P450c21 mRNA expression

The P450c21 mRNA in the P1 segment of head kidney was compared between ACTH- and saline-infused eels by RT-PCR as described previously (Li *et al.* 2003). The RT-PCR protocol was the same as that used for examining tissue distribution. PCR cycles were 26 for P450c21 and 24 for GAPDH (internal control). We confirmed in the pilot experiments using serially diluted templates that the signal intensity of PCR products and the amount of template exhibited linear relationship under the current PCR conditions (data not shown). Each PCR product (10 μl) was electrophoresed in 1.2% agarose gels, stained with EtBr and quantitated using an imaging analyzer (FLA-2000; Fuji Film, Tokyo, Japan).

Cortisol measurement

Plasma cortisol was measured by enzyme immunoassay after extraction with ethyl ether as described previously (Li & Takei 2003). Cross-reactivity of the anti-cortisol serum used in the assay was 31% with 11-deoxycortisol, 6.0% with cortisone, 3.6% with corticosterone, 0.1% with progesterone and <0.1% with oestradiol. Measurements were carried out in duplicate.

Statistics

All data are presented as means $\pm$ S.E.M. Paired *t*-test was used to determine statistical differences of plasma cortisol concentrations before and after ACTH or saline infusion in the same fish. Student's *t*-test was applied for comparison of plasma cortisol and P450c21 mRNA levels between ACTH- and saline-infused eels. Significance was set at $P < 0.05$.

Results

Nucleotide and amino acid sequences of eel P450c21 cDNA

Using a combination of RT-PCR and RACE, a P450c21 cDNA (accession no. AB095111) of 2165 bp length (excluding the poly (A) tail) was

isolated from the eel head kidney (Fig. 1). The open reading frame (ORF) started from nucleotide 31 and encoded 523 amino acids. Methionine codons were also located at nucleotides 43–45 and 247–249 (Fig. 1). The 3'-untranslated region had 563 bp, where two possible AATAAA polyadenylation signals were found. The predicted eel P450c21 protein displayed 41–44% overall identity with those of mammals, and contained all functional domains for steroid binding, heme binding and proton transfer in which the similarities were 77%, 74% and 100% respectively (Fig. 2).

21-Hydroxylation activity of eel P450c21 expressed in COS-1 cells

The COS-1 cells transfected with pe21L-N1, containing the whole ORF (starting from ATG_{31–33}) of eel P450c21 cDNA, converted most progesterone to 11-deoxycorticosterone in 5 h (Fig. 3). However, no obvious conversion of 17 α -hydroxyprogesterone was observed even after 10 h. The COS-1 cells transfected with pe21S-N1, containing the partial ORF (starting from ATG_{247–249}), converted neither progesterone nor 17 α -hydroxyprogesterone (data not shown), as observed in controls transfected only with the vector (Fig. 3).

Tissue distribution of eel P450c21 mRNA

P450c21 mRNA displayed consistent tissue-specific expression in all FW and SW eels. Abundant P450c21 mRNA was detected only in the head kidney (Fig. 4). More detailed analyses of the head kidney located the expression only in the anterior quarter (P1). The negative tissues included the kidney, brain, heart, intestine, liver, gill, immature gonad and skeletal muscle (Fig. 4).

Conversion of 11-deoxycorticosterone to cortisol in eel interrenal

Incubation of 11-deoxycorticosterone with the P1 segment of head kidney containing interrenal tissue produced cortisol and its intermediate products, 11-deoxycortisol and corticosterone, depending on the incubation time (Fig. 5). However, incubation with the P4 segment scarcely converted 11-deoxycorticosterone to any other steroid including cortisol (Fig. 5). Thus the P1 segment contained both P450c17 and P450c11.

Figure 3 Radiochromatograms showing specific conversion of ^3H -progesterone (P_4) to 11-deoxycorticosterone (DOC). ^3H - P_4 and ^3H -17 α -hydroxyprogesterone (^3H -17 α -OH- P_4) were incubated together with COS-1 cells transfected with vector only (control) or vector containing the whole ORF of eel P450c21 cDNA (experimental). Incubation medium was collected after 5 and 10 h. The steroids produced were separated by reverse-phase HPLC using an ODS-120T column (4.6 $\times$ 250 mm) with a linear gradient of acetonitrile concentrations from 40 to 70% in 30 min at 0.7 ml/min, and radioactive peaks were detected in a radiomatic flow scintillation analyzer.

Incubation of ^3H -corticosterone with the P1 segment time-dependently produced cortisol (Fig. 6), showing that interrenal P450c17 can act on corticosterone. After 21 h of incubation, the major product was cortisol among others (Fig. 7). However, incubation with the P4 segment failed to produce any cortisol (Fig. 6), and a significant amount of corticosterone still remained after 21 h of incubation (Fig. 7).

Effects of ACTH on plasma cortisol and P450c21 mRNA levels in head kidney

A sustained increase in plasma cortisol concentration was observed in eels infused with ACTH at

3 pmol/kg per min for 5 h compared with the preinfused level or with the level of saline-infused controls (Fig. 8). The expression of P450c21 mRNA in the P1 segment of head kidney also increased after 5 h of ACTH infusion.

Discussion

Structural characteristics of eel P450c21

To date, the cDNAs encoding P450c21 have been cloned in various mammalian species (e.g. Yoshioka *et al.* 1986, Crawford *et al.* 1992, Zhou *et al.* 1997). In fish, the presence of P450c21 activity in the interrenals of teleosts has been suggested (Sampath-Kumar *et al.* 1996, Blom *et al.* 2001), as

Figure 4 Tissue distribution of P450c21 mRNA examined by RT-PCR. The expression of eel GAPDH was used as internal control. Specific expression in the P1 segment of head kidney occurred in all samples from five FW and two SW eels. The head kidney was divided equally into four parts, and named P1–P4 from the anterior to the posterior direction.

well as in elasmobranchs (Nunez & Trant 1995). However, a P450c21 cDNA has not yet been cloned in any non-mammalian vertebrate, including fish.

In this study, a P450c21 cDNA was cloned from the eel head kidney. At the 5' region of its cDNA, possible start codons (ATG) were found at three positions. The studies of functional expression showed that the 21-hydroxylase activity was detected only with the cDNA that contains the longest ORF starting from ATG_{31–33}, and not with that starting from ATG_{247–249}. This result showed that the first or second ATG are used as the start codon, and that the N-terminal region of eel P450c21 is essential for its activity as demonstrated for human P450c21 (Hsu *et al.* 1993). The similarity of P450c21 protein between eels and mammals was low (41–44%). Nevertheless, the putative functional domains for steroid binding, heme binding and proton transfer, as identified in mammalian P450c21s (John *et al.* 1986, Crawford *et al.* 1992, White & Speiser 2000), displayed high identity (74–100%) between eels and mammals, suggesting their importance for enzyme activity.

Tissue-specific expression of eel P450c21

The interrenal steroidogenic tissue in fish is homologous to mammalian adrenal cortex, and is the site for corticosteroid biosynthesis (Chung *et al.* 1986, Nunez & Trant 1995, White & Speiser 2000). In the eel, the interrenal cells are closely associated with blood vessels of the head kidney (Sandor *et al.* 1966, Takashima & Hibiya 1995). The head kidney of the eel is a long tissue that extends from the kidney to the heart and spans

more than 20% of the total body length. Since the head kidney is essentially a haematopoietic tissue in fish, we chose to determine which part of the head kidney contains interrenal cells. In the present study, P450c21 mRNA was expressed exclusively in the anterior quarter (P1) of the head kidney, suggesting that the interrenal steroidogenic cells are represented only in this part. The expression of P450c21 mRNA has also been detected exclusively in the adrenal cortex in mammals (John *et al.* 1986, Zhou *et al.* 1997). On the other hand, other eel proteins related to corticosteroidogenesis such as P450c17 and P450c11 and StAR are expressed in the gonads and/or interrenal (Jiang *et al.* 1998, Kazeto *et al.* 2000, Li *et al.* 2003).

Enzymatic activity of eel P450c21 expressed in COS-1 cells

In mammals, P450c21 expressed in COS cells converts both progesterone and 17 α -hydroxyprogesterone to their 21-hydroxylated products (Lorence *et al.* 1989, Crawford *et al.* 1992, Zhou *et al.* 1997). In the *in vitro* incubation of bovine adrenocortical homogenates, however, the presence of two 21-hydroxylases have been suggested, one for a 17-deoxy substrate (e.g. progesterone) and the other for a 17-hydroxylated one (e.g. 17 α -hydroxyprogesterone) (Kahnt & Neher 1972). Since P450c21 cDNA has not yet been cloned in non-mammalian species, their substrate specificity is not known. However, indirect evidence from *in vitro* studies has indicated that in elasmobranchs, amphibians, reptiles and birds, progesterone is the major substrate of P450c21 because of the absence of P450c17 in the adrenal/interrenal of these groups (Lofts & Bern 1972, Nunez & Trant 1995). In teleosts, limited studies suggest that both progesterone and 17 α -hydroxyprogesterone may be 21-hydroxylated in the interrenal (Sandor *et al.* 1966, Idler & Truscott 1972). However, the preferential substrate of P450c21 might differ with species, developmental stage and season (Lofts & Bern 1972, Sangalang & Uthe 1994).

In this study, cloned P450c21 actively converted progesterone to 11-deoxycorticosterone, displaying the characteristic enzyme activity. However, the cloned enzyme did not convert 17 α -hydroxyprogesterone, i.e. it lacked 17 α -hydroxyprogesterone 21-hydroxylase activity. It is possible that COS cells lack specific factor(s)

Figure 5 Time-course of conversion of 11-deoxycorticosterone after incubation with eel head kidney. The steroid was incubated either with the anterior P1 segment of head kidney (experimental) or the posterior P4 segment (control). While 11-deoxycorticosterone (d) was hardly converted in the control group, it was time-dependently converted to produce cortisol (a), 11-deoxycortisol (b) and corticosterone (c) in the experimental group. Other peaks without arrows did not change with time. The steroids produced were separated by reverse-phase HPLC using an ODS-120T column (4.6×250 mm) with a linear gradient of methanol (MeOH)/acetonitrile (AcCN) concentrations from 20 to 40% in 30 min at 0.7 ml/min.

needed for this activity. In a previous study, however, ovine P450c21 expressed in COS cells catalysed both progesterone and

17 α -hydroxyprogesterone (Crawford *et al.* 1992). Thus, the progesterone-specific activity seems to be intrinsic to the enzyme found in this study.

Figure 6 HPLC profiles showing conversion of ^3H -corticosterone (a) to ^3H -cortisol (b) after 21 h of incubation with eel head kidney. After incubation, radioactive steroids were separated by reverse-phase HPLC in the same conditions as in Fig. 3. While corticosterone still remained in considerable amounts after incubation with the P4 segment (control), cortisol was a major product after incubation with the P1 segment (experimental). Most radioactive peaks of unknown products occurred in both control and experimental groups.

In vitro tissue incubation experiments have shown that progesterone is converted to 11-deoxycorticosterone in the interrenal of the European eel (Sandor *et al.* 1966). Therefore, an enzyme activity similar to that of the cloned P450c21 also appears to be present in the interrenal of this species of eel.

Biosynthetic pathway of cortisol in eel interrenal

Since the interrenal-specific P450c21 cloned in this study specifically converted progesterone to 11-deoxycorticosterone, the latter should be converted to cortisol in the interrenal. In fact, 11-deoxycorticosterone incubated with eel interrenal

Figure 7 Time-course of conversion of ^3H -corticosterone (●) to ^3H -cortisol (○) after incubation with eel head kidney. Cortisol was not produced after 21 h of incubation with the P4 segment of head kidney (control), but it was time-dependently produced after incubation with the P1 segment (experimental).

was converted to cortisol in this study. Both corticosterone and 11-deoxycortisol were detected after incubation. Thus both pathways through these intermediate products are active in the eel interrenal. Jiang *et al.* (1998) showed that the P450c11 enzyme that is abundantly expressed in the interrenal of Japanese eel converts both 11-deoxycorticosterone and 11-deoxycortisol to corticosterone and cortisol respectively. Judging from their data, however, 11-deoxycorticosterone appears to be a preferential substrate. Actually, the eel interrenal tissue actively converted ^3H -corticosterone to cortisol in this study. 17 α -Hydroxylation of corticosterone to produce cortisol has previously been reported in fish including the European eel (Sandor *et al.* 1966). Together with the present data, it seems that the route for cortisol biosynthesis from progesterone through

Figure 8 Effects of ACTH infusion on (A) plasma cortisol concentration and (B) P450c21 mRNA levels in the head kidney in SW eels. P450c21 transcripts were quantified by RT-PCR after 5 h of infusion of ACTH or saline, and were normalized by GAPDH transcripts. Values are means $\pm$ S.E.M. ($n=6$). ** $P<0.01$, *** $P<0.001$ compared with saline-infused controls.

11-deoxycorticosterone is present in fish, although the major route in mammals is thought to be through 17α -hydroxyprogesterone (Hanukoglu 1992). However, since interrenal P450c11 converts 11-deoxycortisol to cortisol (Jiang *et al.* 1998), there is a possibility that another P450c21 is also present in the eel interrenal that can 21-hydroxylate 17α -hydroxyprogesterone.

Upregulation of P450c21 expression by ACTH

ACTH is a major stimulator for corticosteroid production in vertebrates. However, the

mechanisms of its action are poorly understood in fish. In this study, infusion of ACTH increased both plasma cortisol levels and interrenal expression of the P450c21 gene in eels. This suggests that the increase in plasma cortisol level is mediated, at least in part, by the increased expression of P450c21 gene as already observed in mammals (John *et al.* 1986, Provencher *et al.* 1992). In addition, other enzymes responsible for corticosteroid synthesis may also be activated by ACTH as reported in rats (LeHoux *et al.* 1998). Our recent results have shown that injection of ACTH increased StAR gene expression in the interrenal within 1.5 h after injection; this was accompanied by an increase in plasma cortisol levels (Li *et al.* 2003). Taken together, ACTH may stimulate cortisol production by enhancing the expression of StAR and steroidogenic enzymes including P450c21 in eels.

Perspectives

Cortisol is an important hormone for SW adaptation in teleosts (McCormick 2001). Other hormones are also implicated in SW adaptation and some of them, such as ANP, ANG II and urotensin, have been shown to increase cortisol production in eels and other teleost fishes (Henderson *et al.* 1976, Arnold-Reed & Balment 1991, 1994, Li & Takei 2003). To delineate the whole picture of hormonal control of SW adaptation in teleosts, it is necessary to examine how cortisol production is regulated by osmotic adaptation in relation to these peptide hormones. Cloning of eel P450c21 cDNA, together with previously cloned eel P450c11, P450c17 and StAR cDNAs, now allows us to examine how these steroidogenic factors are involved in cortisol production with respect to osmotic adaptation.

Acknowledgements

We thank Dr Christopher A Loretz of the State University of New York at Buffalo for critical reading of this manuscript, and Dr M K Park of the University of Tokyo for kindly providing COS-1 cells and advice for cell culture. We also thank Dr S Hyodo of the Ocean Research Institute, University of Tokyo, Dr K Ukena of Hiroshima University, Dr T Kobayashi of the National Institute for Basic

Sciences, and Drs S Adachi, Y Kazeto and K Yamauchi of Hokkaido University for their advice. This work was supported by grants-in-aid for Creative Basic Research (12NP0201) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and for Scientific Research (A) (13304063) from the Japan Society for the Promotion of Science to Y.T. Y-Y L is a recipient of a Postdoctoral Fellowship from the Japan Society for the Promotion of Science.

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Received in final form 3 June 2003

Accepted 3 July 2003