Maturation-Dependent Changes of Angiotensin Receptor Expression in Fowl

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An angiotensin (Ang) receptor homologous to the type 1 receptor (AT₁) has been cloned in chickens (cAT₁). We investigated whether cAT₁ expression in various tissues shows maturation/age-dependent changes. cAT₁ mRNA levels detected in renal glomeruli (in situ hybridization) and kidney extract (RT-PCR) are significantly (P < 0.01) higher in 19-d embryos (EB) than in chicks (CH, 2-3 wk) and pullets/cockerels (PL/CK, 14-16 wk). The levels in adrenal glands (concentrated in subcapsular regions) is high in EB and further increased in CH and PL/CK. cAT₁ mRNA is also detectable in smooth muscle (SM)/adventitia of EB and CH aortae, and in the adventitia, but not SM, from PL/CL aortae. The endothelia from small arteries and arterioles, but not from aorta, express cAT₁ mRNA (ISH). In all age groups, Ang II induces profound endothelium-dependent relaxation of abdominal aorta, partly (37-47%) inhibitable (P < 0.01) by Nω-nitro arginine methyl ester (L-NAME, 10⁻⁴ M), suggesting the presence of Ang receptor in endothelium. L-NAME-resistant Ang II relaxation, examined in a limited number of EB or CH aortae, was reduced by 125 mM K⁺ or apamin plus charybdotoxin. The results suggest that: 1) cAT₁ is present in kidney, adrenal gland, and vascular endothelium (heterogeneity exists among arteries) of EB, CH, and PL/CK, and in aortic SM/adventitia of EB/CH but only in adventitia of PL/CK, 2) levels of cAT₁ gene expression change during maturation in tissue-specific manner, and 3) Ang II-induced relaxation may be partly attributable to nitric oxide and potassium channel activation.

Key Words: angiotensin receptor subtype, angiotensin receptor mRNA, endothelium-dependent relaxation, chick embryo, EDRF, EDHF, potassium channel
THE RENIN-ANGIOTENSIN SYSTEM (RAS) and angiotensin (Ang) receptors evolved during the early phylogeny of vertebrates, and at least part of the RAS has been biochemically or pharmacologically identified in representative species of all vertebrate classes (9, 23, 25, 39). Ang receptors with considerable homology to the mammalian Ang type 1 receptor (AT₁) protein/nucleotide sequences have been cloned in several species of nonmammalian vertebrates (for review, 14, 23). In birds (15, 21), AT₁ homologue receptors (359 amino acid residues) cloned from chicken and turkey adrenal glands (cAT₁ and tAT₁), show 75% amino acid identity with the mammalian AT₁ receptor. These avian receptors possess the motifs conserved among G-protein-coupled receptors and stimulate the inositol phosphate pathway. The cAT₁ is detected by in situ hybridization (ISH) in adrenal glands, kidney, heart, and endothelia of small renal arteries from adult fowl (15), in mesonephric kidneys, and extrarenal structures of the developing embryos (14). We reported previously that vascular walls of domestic fowl express three types of Ang receptors: 1) vascular endothelial receptors that mediate vasorelaxation via the release of endothelium-derived relaxing factor (EDRF), partly nitric oxide (NO)/cyclic GMP (8, 26, 45); 2) saturable and specific vascular smooth muscle (VSM) receptors/binding sites that have a currently unknown function (37, 40); and 3) Ang receptors that are presumably localized at adrenergic nerve endings and mediate norepinephrine release and vasoconstriction in vivo (22, 24). It is not as yet clear, however, whether these receptors are the same as cloned cAT₁.

In endothelium-denuded fowl aortae, Ang II neither causes contraction/relaxation nor evokes Ca²⁺ signaling (23, 42, 45).

In fowl, Ang II stimulates thymidine incorporation into cultured aortic SM cells from chicks (CH), indicating that Ang II promotes growth (35). This effect decreases, however, with maturation; no growth-stimulatory effect of Ang II is seen in VSM cells (either primary cultures or subcultures) from adult chickens (35), whereas specific Ang II binding sites exist in aortae (40) and cultured aortic SM cells (42). We therefore hypothesized that modulation of Ang receptor expression occurs during maturation.

The aim of this investigation was twofold. We intended to determine whether 1) cAT₁ is expressed and 2) maturation-dependent changes occur in cAT₁ mRNA signals, in kidneys, adrenal glands, and aortae from embryos (EB), CH, and pullets/cockerels (PL/CK), using ISH,
and RT-PCR analyses. We also examined whether endothelium-dependent Ang II-induced relaxation of aortae agrees with the levels of cAT₁ mRNA.

MATERIALS AND METHODS

Animals and Maintenance

Fertile Lohman-selected White Leghorn eggs obtained from a commercial breeder ('t Anker; Ochten, NL) and CH (both sexes) hatched and grown at the University of Maastricht, Maastricht, NL, were used for both functional and molecular studies. The eggs were incubated at 38°C, air humidity of 60%, in a commercial incubator (Polyhatch, Brinsea Products Ltd.; Sandford, UK) with automated rotation (usually 21-d incubation). CH were kept in brooders in temperature/humidity-controlled animal quarters (10-20 CH per brooder) for 4 wk. Chickens over 4 wk old were kept in groups in large indoor pens with wood dust-seated floors in a well-ventilated, temperature/photoperiod-controlled room (22-24°C; 12:12 h light-dark cycle). Age groups used included: 1) EB, day 19, 2) CH, 2-3 wk-old, 3) PL/CK, 14-16 wk-old. Birds were maintained on fowl laboratory chow (Kenner Opfokkorrel, Agri Retail bv; Arnhem, NL; 18% protein, 1% Ca²⁺) and water ad libitum.

In Vitro Isometric Tension Measurement of Aortic Rings

The EB (19 d of growth) was removed from an egg into a Petri dish containing warm Kreb’s Ringer bicarbonate (KRB) buffer solution consisting of (in mM) 113.5 NaCl, 5.0 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.2 KH₂PO₄, 25.0 NaHCO₃, and 5.5 glucose (pH 7.4, 37°C). The Petri dish was coated with Sylgard 184 (mixed with hardener and dried by air suction; Dow Corning Corp.; Midland, MI), on which the embryo’s legs and wings were fixed with fine pins at positions suitable for surgical incision. The lower segment of the abdominal aorta was carefully isolated, devoid of stretching, from the decapitated EB under a dissecting microscope and cut into aortic rings (~1.6 mm, 2 rings/EB). After removal of surrounding connective tissues, aortic rings were
mounted in a myograph organ bath (37°C; Model 610M, Danish Myotechnology by J. P. Trading; Aarhus, Denmark) containing aerated (95% O₂ and 5% CO₂) KRB solution and were placed between an isometric force transducer and displacement device using two stainless steel wires (40 µm diameter) (41). Abdominal aortic rings (1.6~1.8 mm, 4 segments/bird) were also excised from decapitated CH and PL/CK, cleared of surrounding tissue, and vertically mounted in water-jacketed organ chambers (5 ml, 37°C) containing the aerated 5-ml KRB solution.

Isometric tension was recorded on a Power Lab/8SP (AD Instrument; Castle Hill, Australia) through a force-displacement transducer (UF1, Pioden Controls Ltd.; Canterbury, UK). All aortic rings were equilibrated 90-120 min in KRB buffer and subjected to incremental stretching until maximal contractile responses were obtained (previously determined in several rings).

Endothelium was denuded by gently rubbing the internal surface with a ruffled cotton string. Aortic rings were contracted with a thromboxane mimetic, U46619 (9, 11-dideoxy-11α, 9α-epoxymethano-prostaglandin F2α) (2 x 10⁻⁷ M, Sigma, St. Louis, MO), and used for one of the following four treatments: 1) solvent control; 2) Nω-nitro arginine methyl ester (L-NAME), 10⁻⁴ M; 3) L-arginine, 10⁻⁴ M (Sigma), plus L-NAME, 10⁻⁴ M; or 4) endothelium denudation. The relaxing effects of [Val⁵]Ang II (10⁻⁶ M) and acetylcholine (ACh, 10⁻⁸ M ~ 3 x 10⁻⁵ M) were determined in all treatments and age groups. Location of aortic segments and treatments were randomly matched. Because aortae show a tachyphylactic response to Ang II, only one dose of Ang II was examined per tissue. The dose-response study of [Val⁵]Ang II indicated that relaxation induced by 10⁻⁶ M is considerable but not maximal (45). At the end of the experiment, 63 mM K⁺ and papaverine (10⁻⁴ M, Sigma) were respectively applied to examine the viability of the aortic rings and to induce complete relaxation (base line).

The transducer was calibrated with a known weight, and contractile responses were expressed as active wall tension (force divided by twice the segment length, N/m). The relaxing effects of Ang II and Ach were expressed as the % decrease in tension from the stable level prior to drug application; pre-drug application levels were calculated from the completely relaxed basal level obtained at the end of each experiment. Because of tachyphylaxis, however, Ang II relaxation (control) and L-NAME plus Ang II were examined in different rings from the same
aorta. The part of the Ang II-induced relaxation that was not inhibitable by L-NAME (10^{-4} M) was considered the “L-NAME-resistant component of Ang II-induced relaxation.”

In a limited number of EB or CH, the L-NAME-resistant component of Ang II-induced relaxation was pharmacologically characterized. The following K⁺ channel inhibitors (6, 29-31, 43) were used for examining the possible involvement of endothelium-dependent hyperpolarization factor (EDHF): tetraethylammonium (TEA; Sigma; nonselective K⁺ channel inhibitor and large conductance Ca^{2+}-activated K⁺ channel inhibitor), barium (conventional inwardly rectifying K⁺ channel inhibitor), apamin (Sigma; selective blocker for small conductance Ca^{2+}-activated K⁺ channel), and charybdotoxin (Sigma; intermediate/large conductance Ca^{2+}-activated K⁺ channel inhibitor). Aortic rings collected from the same bird (3 or 4 rings from CH, 2 rings from EB) were used for control (L-NAME plus Ang II) and experiments (L-NAME, K⁺ channel inhibitor(s), and Ang II). The protocol was repeated in 2-3 birds for each inhibitor.

**In situ hybridization**

Sense and antisense riboprobes complementary to cAT1 mRNA were prepared by in vitro transcription of cloned cAT1 cDNA templates (15). Transcription and radioactive labeling (³⁵S-UTP; Amersham, SJ1303; Piscataway, NJ) were conducted from the T3, T7, or SP6 RNA polymerase promoter site of the plasmid vector after linearization of the plasmid with appropriate restriction enzymes as previously described (12, 15, 36). Kidneys, adrenal glands, and aortae from EB, CH, and PL/CK were fixed by in vivo perfusion of (or immersion into) 4% paraformaldehyde/KRB, and the tissue sections (5-6 μm in thickness) from three organs from three groups were hybridized simultaneously with sense or antisense riboprobe (~4 x 10⁵ cpm/section). The first observation on Biomax-MR film (Kodak; Rochester, NY) showed macroscopic tissue distribution of the cAT1 mRNA (1-3 d autoradiography). The process was completed by dipping the slides in liquid emulsion for 2-5 wk (exposure). We determined cAT1 mRNA levels in renal glomeruli by counting positive grains in the glomerular tufts (6 glomeruli/slice, 3 kidneys/age group), excluding Bowman’s capsule. The number of grains was
also counted in the renal tubule area (background). The values were normalized by the area (NIH 1.62 image program).

**Tissue isolation and RNA preparation**

Following decapitation of the bird, kidneys, adrenal glands, and abdominal aortae were quickly removed, freed from surrounding connective tissues, and washed in chilled aerated KRB buffer solution. Tissues were snap-frozen in liquid nitrogen and placed on dry ice. Total RNA was extracted by the method of Chomczynski and Sacchi (5). Briefly, the frozen tissues were homogenized by a homogenizer (Omni International; Waterbury, CT) on ice in 4 M guanidium thiocyanate (Fluka Chemical; St. Louis, MO) containing 25 mM Na citrate (pH 7), 0.5% Na lauryl sarcosinate, and 0.1 M beta-mercaptoethanol (Sigma; St. Louis, MO). RNA was extracted from the supernatant of centrifugation (17,000 g, 4°C, 30 min) by sequential mixing with 2 N Na acetate, water-saturated phenol (Invitrogen; Carlsbad, CA), and chloroform and then precipitated in isopropanol (-20°C 16-20 h). The RNA was washed, reprecipitated with isopropanol (-20°C, 2 h), and reconstructed. The concentration of RNA was determined by measuring the absorbance at 260 nm, and its quality was assessed by the absorbance ratio of 260/280 nm (1.8-2.0) and by gel electrophoresis, scanning through the integrity of 28S and 18S ribosomal bands.

**Reverse Transcription Reaction**

To synthesize single-stranded cDNA, the total RNA of each organ (adrenal, 0.1 μg; kidney, 0.6 μg; aorta, 0.6 μg) was reverse-transcribed in incubation mixture (20 μl) that contained 20 U ribonuclease (RNase) inhibitor (Promega; Charbonnieres, France), 100 pmol of random hexamer (Pharmacia; Orsay, France), and 200 U MuLV reverse transcriptase (Roche; Meylan, France), in the presence of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 5 mM dithiothreitol, and 1.25 mM 2'-deoxynucleoside 5'-triphosphate. The mixture was incubated at 37°C for 90 min (12). The reaction was stopped by heating samples for 5 min at 65°C. Since
the coding regions of the cAT₁ receptor gene comprise only one exon, we examined the possible contamination of sample RNA with genomic DNA by incubating the sample RNA without reverse transcriptase. Both series were simultaneously processed for PCR amplification.

**PCR amplification.**

Double-stranded cDNAs were synthesized and amplified by incubating (total volume, 25 µl in duplicate) the reverse transcription (RT) reaction product (3 µl) with 1 U of Taq polymerase (Roche) and 5 pmole each of 5’-and 3’-primer pairs (sense position [Ss], 72-100; antisense position [As], 600-578) in the presence of 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 3.5 mM MgCl₂, and 0.5 mM dNTP for 28 cycles (adrenal), 29 cycles (kidney), or 30 cycles (aorta) at 94° (denaturation), 65° (primer annealing), and 72°C (extension/synthesis), respectively, for 30, 30, and 60 s. A trace amount of [α-³H]dCTP (3 µCi; Amersham) was included in the PCR reaction for quantification of the PCR product. Twenty µl of the PCR products were electrophoresed on a low-melting-temperature agarose gel (1.5 %). After solubilization, radioactivities of the bands in the PCR gel were counted by scintillation counter. The G3PDH mRNA obligatorily expressed in the same RT reaction was similarly determined. To confirm that the amplicon was specific to cAT₁, the PCR product was electrophoresed and transferred to a nylon membrane for Southern blot analysis. The product was hybridized with [α-³²P]ATP-labeled oligonucleotide, internal to the amplicon. The radioactive fragment coincided with the length of the cAT₁ amplicon (data not shown).

To conduct RT-PCR within the exponential phase of the reaction, the cycle number, primer-annealing step, and polymerization step were optimized (12). The Mg concentration (1.5-5.0 mM) that optimally produces PCR product was determined and selected (3.5 mM). The yield of PCR products from total RNA (0.1-1.0 µg) and the effect of the number of PCR amplification cycles (25-30 cycles) were examined, and those representing the mid-linear part of the log dose-response curves were selected for kidneys (0.6 µg, 29 cycles), adrenals (0.1 µg, 28 cycles), and aortae (0.6 µg, 30 cycles). To minimize variability among samples, tissues collected from the three age groups (EB, CH, and PL/CK) were simultaneously processed for
RT-PCR. Radioactivities ([α-3H]dCTP) of the gel were counted and normalized by the radioactivity of G3PDH mRNA derived from the same RT reaction. PCR incubations from which the RT reaction product was deleted and to which the cAT1 plasmid (~30 pg) was added are designated, respectively, negative and positive control. Pooled RNA was also used as an inter-assay control.

Statistical Analysis

All data are shown as means ± SE. For statistical analysis, a single- or two-factor analysis of variance (ANOVA) was used, followed by, when applicable, the Tukey HSD unbalanced or the Newman-Keuls multiple comparison test. The difference was considered significant at a P-value of less than 0.05.

RESULTS

Angiotensin II-induced Relaxation of Abdominal Aorta

The responses (N/m) of CH aortae (endothelium intact) to U46619 (3.24 ± 0.16, n = 18) (2 x 10^-7 M, beginning of experiment) and to 63 mM K⁺ (3.78 ± 0.35, n = 18) (end of experiment) were significantly larger (P < 0.05, ANOVA) than those of EB (U46619, 2.65 ± 0.23, n = 21; 63 mM K⁺, 1.82 ± 0.13, n = 17) or PL/CK (U46619, 1.55 ± 0.11, n = 19; 63 mM K⁺, 2.53 ± 0.12, n = 17). Within the age groups, the responses of the aortic rings to U46619 and to 63 mM K⁺ were similar among the four groups of birds subsequently used for different treatments.

[Val⁵]Ang II (10⁻⁶ M) induced profound relaxation of abdominal aortic rings from EB (embryonic day 19; n = 13; BW, 26.0 ± 0.9 g), CH (2-3 wk of age; n = 7; BW, 184.5 ± 16.5 g), and PL/CK (14-16 wk; n = 7; BW, 1308 ± 108 g) (Figs. 1 & 2). There was no significant difference in the magnitude of relaxation (% decrease in tension: EB, 69.3 ± 5.1; CH, 74.3 ± 2.8; PL/CK, 65.4 ± 4.9). In all groups, Ang II-induced relaxation was significantly inhibited by L-
NAME (10^{-4} M) (P < 0.01), whereas inhibition was restored by pretreatment with L-arginine (10^{-4} M). Removal of endothelium thoroughly eliminated Ang II-induced relaxation (Figs. 1 & 2).

The L-NAME-resistant component of Ang II-induced relaxation (see Methods) was not reduced by TEA (3 x 10^{-3} M), but barium (3 x 10^{-5} M) plus ouabain (5 x 10^{-7} M) or apamin (10^{-7} M) plus charybdotoxin (10^{-7} M) nearly completely eliminated L-NAME-resistant Ang II relaxation (Fig. 3). Precontracting the rings with 125 mM K^+ instead of U46619 or using a higher dose of L-NAME (10^{-3} M instead of 10^{-4} M) inhibited Ang II-induced relaxation more clearly (64.1 ± 0.1%, n = 3), whereas a combination of 125 mM K^+ and L-NAME (10^{-3} M) completely inhibited Ang II relaxation (Fig. 3). [Sar^{1}, Ile^{8}]Ang II, a peptide Ang II antagonist, also completely eliminated the L-NAME-resistant component of Ang II-induced relaxation (data not shown).

ACh induced a concentration-dependent relaxation of aortic rings in all three age groups (P < 0.01, ANOVA). Higher doses (5 x 10^{-5} M or higher) often caused biphasic responses (relaxation followed by contraction, data not shown). L-NAME only modestly shifted the dose-response curves (ED_{50}) of ACh to the right in EB (control, 2.21 x 10^{-7} M; L-NAME, 1.30 x 10^{-6} M) and CH (control 1.08 x 10^{-7} M; L-NAME, 2.57 x 10^{-7} M) and more clearly in PL/CK (control 9.38 x 10^{-8} M; L-NAME, 2.89 x 10^{-7} M), whereas ACh-induced relaxation was nearly completely abolished by 125 mM K^+ and 10^{-3} M L-NAME and was totally inhibited by endothelium denudation (Figs. 1 & 3).

**Chicken Angiotensin Receptor mRNA Detection by In Situ Hybridization**

**Kidney.** cAT, mRNA was detected by ISH in metanephric kidneys from day-19 EB (n = 9; BW, 28.2 ± 0.7 g), 2- to 3-wk-old CH (n = 4; BW, 127.8 ± 16.6 g), and 14- to 16-wk-old PL/CK (n = 3; BW, 1231 ± 72 g). In EB, dense silver grains forming clusters are markedly seen in the center area of glomeruli, possibly on mesangial cells (Fig. 4A). The silver grains are less dense in CH glomeruli (Fig. 4B) and are only weakly detected in PL/CK (Fig. 4C). Semiquantification of cAT, mRNA in glomeruli is shown in Table 1. The number of glomeruli per unit area is highest in EB (P < 0.01) and decreases with maturation. The density of silver grains (normalized by glomerular area) is similar in EB and CH, but significantly (P < 0.05) lower in
PL/CK; therefore, total signal per kidney slice is highest in EB. The density of silver grains in
renal tubules is low and approximately the same as that of sense probe controls (Fig. 4D, Table
1). cAT1 mRNA is also expressed in the endothelia of small renal arteries and arterioles (Fig. 5).
Labeling of endothelial cells is readily observed in endothelia of CH and PL/CK (Fig. 5A and
5B), whereas labeling is hardly above background level in EB (not shown). In all three groups,
no concentration of silver grains was seen in VSM or adventitia.

Adrenals. The cAT1 mRNA riboprobe was hybridized in adrenals from EB, CH, and
PL/CK (Fig. 6). In EB (Fig. 6A) and CH adrenals (Fig. 6C), silver grains are localized with
highest density in the subcapsular zone, forming clusters, but only weak labeling is seen in the
inner regions; in PL/CK adrenals, cAT1 mRNA signals are seen more diffusely over wider areas
(Fig. 6D). cAT1 mRNA was also detected in ganglia (Fig. 6B). In adrenals, endothelia from
small arteries express cAT1 mRNA (Fig. 5C & 5D), although the number of positive arteries is
lower than in the kidney.

Abdominal aortae. In EB (day 19 and 20), silver grains were localized at the outer edge
of aortic SM layers and adventitia close to the media (Fig. 7A and 7B). No such concentration
of cAT1 mRNA signals was found in either CH or PL/CK aortae. Likewise, no localized silver
grains were detected in aortic endothelia from any age group. The results were the same,
regardless of the method of tissue fixation (in vivo perfusion of, or in vitro immersion into, 4%
paraformaldehyde/PBS).

Chicken Angiotensin Receptor mRNA Examined by RT-PCR

Agarose gel electrophoresis revealed amplified fragments of 525 bp, as expected from
the primer locations, in the kidney, adrenal, and aorta from all three age groups. Examples of
gel electrophoresis (kidney) are shown in Fig. 8. The RT-PCR products from kidneys, adrenals,
and aortae of day-19 EB (n = 13; BW, 28.3 ± 0.7 g ), 2- to 3-wk-old CH (n = 9; BW, 129.8 ± 6.1
g), and 15-wk-old PL/CK (n = 5; BW, 973 ± 81 g) are summarized in Figs. 9 and 10. We
expressed the levels of RT-PCR products in two ways: 1) as radioactivities of gel products (not
shown) and 2) radioactivities normalized by radioactivities of G3PDH gel products. Results
were similar for the two methods. cAT\textsubscript{1}, mRNA was high in embryonic kidneys (metanephros) and became significantly lower ($P < 0.01$) in CH and PL/CK (Fig. 9). cAT\textsubscript{1}, mRNA was clearly detected in adrenals from EB, despite the fact that a lower amount of total RNA (0.1 \(\mu\)g) and fewer PCR amplification cycles (28 cycles) were used for adrenals than for kidneys (0.6 \(\mu\)g, 29 cycles); cAT\textsubscript{1}, mRNA further increased in CH and PL/CK adrenals ($P < 0.01$) (Fig. 9).

Because we could not obtain a sufficient amount of RNA from endothelia of abdominal aortae, the PCR products were compared between abdominal aortae with (E+)/without endothelium (E-). In both EB and CH aortae, there was no significant difference in the PCR products between E+ and E- (Fig. 10). In aortae from PL/CK, we manually dissected VSM layers (endothelium deleted) from adventitia. cAT\textsubscript{1}, mRNA was detected ($P < 0.01$) in adventitia but not in VSM (Fig. 10).

No DNA contamination was detected in RNA preparations from adrenals or kidneys of any age group. A trace amount of DNA contamination was noted in RNA from VSM of EB (3 of 10), CH (3 of 8), and PL/CK (3 of 5); therefore, the radioactivity of the product incubated without reverse transcriptase was subtracted from the radioactivity of the corresponding RT-PCR products. This slight DNA contamination is presumably due to the fact that more vigorous homogenization was used for the aortae than kidneys or adrenals, during which nuclei may also be broken. No contamination was seen in RNA from PL/CK adventitia.

DISCUSSION

Angiotensin II-Induced Relaxation of Abdominal Aortae and Signaling

For precontracting aortic rings, we used U46619 because this thromboxane mimetic induces a stable contraction in all age groups, whereas phenylephrine-induced contraction is low and inconsistent in EB, and 63 mM K\textsuperscript{+} often failed to maintain a plateau level in PL/CK. It is unclear why the aortic contractile response to U46619 and 63 mM K\textsuperscript{+} is slightly higher in CH than in EB or PL/CK. Since we expressed the Ang II-induced relaxation as a percent change from the respective level preceding Ang application (calculated from the completely relaxed
basal level), the effect of variable vascular reactivities among age groups should be minor. Within the same age group, there was no significant difference among the four treatment groups in the aortic responses to U46619 and to 63 mM K^+; indicating that the observed effects of the treatments are not due to different degrees of responsiveness or viability of aortae. We examined only one dose (10^{-6} M) of [Val^5]Ang II (submaximal dose [45]) because fowl aortae show strong tachyphylaxis in response to Ang II. Ang II-induced relaxation was equally clear in the three age groups examined in the present study, suggesting that endothelium-dependent relaxation may be an important vascular function during maturation.

Earlier, we examined the pharmacological properties of endothelium-dependent Ang II- and ACh-induced relaxation in adult chickens (8, 45). The endothelium-dependent substance is transferable (7); and the relaxation is accompanied by a rapid increase in cGMP and is not inhibitable by inhibitors of cyclooxygenase, lipoxygenase, or cytochrome P450 monoxygenase (8, 45). The relaxation and depressor effects are not due to prostacyclin release because fowl aorta does not synthesize 6-keto PGF_1α (38). Thus, previous and present studies suggest that a part of Ang II-induced relaxation is attributable to an endothelium-derived NO mechanism.

In limited numbers of EB and CH aortae, we examined whether EDHF and K^+ channels may be involved in Ang II-induced relaxation. We chose EB and CH because L-NAME-resistant Ang II-induced relaxation is more clearly seen in EB and CH than in PL/CK. While Ang II-induced relaxation partly remains after treatment with either high K^+ (125 mM) or a higher dose of L-NAME (10^{-3} M) alone, a combination of the two eliminates Ang II-induced relaxation completely, suggesting that both NO and an EDHF/K^+ channel may be involved. We noted that L-NAME-resistant Ang II-induced relaxation was not inhibitable by TEA, but was considerably inhibited by a combination of barium plus ouabain and by apamin plus charybdotoxin. We used a combination of apamin and charybdotoxin because, in the rat aorta (30) and rat mesenteric artery (6), charybdotoxin plus apamin inhibited ACh-derived hyperpolarization via an endothelial mechanism, whereas these drugs showed no inhibitory effect when applied separately (6, 30). It is therefore unlikely that this inhibition is an additive effect of inhibitors selective for small-(SKca; apamin) and intermediate/large-(IKca and BKca; charybdotoxin) conductance Ca^{2+}-activated K^+ channels; the presence of a K^+ channel isoform, enhanced binding of charybdotoxin
by apamin, etc., has been suggested (6, 30). In chick aortae, the involvement of BKca is unlikely since TEA, a nonselective K⁺ channel antagonist that also inhibits Bkca, shows no inhibitory effect. A higher dose of TEA may be necessary to see a nonselective K⁺ channel inhibitory effect of TEA in chick aortae. In rat renal arterioles, ACh-induced relaxation is nearly completely inhibited by apamin plus charybdotoxin, but not by TEA (43).

The combination of ouabain and barium, a selective inhibitor for conventional inwardly rectifying K⁺ channels (Kir), considerably inhibited Ang II-induced relaxation, suggesting that Kir may be involved in Ang II-induced relaxation. In adult chickens, ouabain (10⁻³ M) alone inhibited the relaxation of isolated aortic rings precontracted with phenylephrine by approximately half (45). Further study is needed to determine the type(s) of K⁺ channels involved and the site of interaction of Ang II with K⁺ channels. It is possible that K⁺ channels in fowl are not specifically differentiated or that the selectivity of antagonists is lower than in mammals. [Sar¹, Ile⁸]Ang II, a peptide Ang II antagonist, eliminates Ang II-induced relaxation completely, indicating that the relaxation is mediated by Ang receptors.

ACh-induced relaxation of the aorta is also endothelium-dependent, but its inhibition by L-NAME is only modest (8 and present study). L-NAME inhibits ACh-induced relaxation substantially in carotid and femoral arteries (19) and in intrapulmonary arteries (41) from CH and EB when arteries are precontracted with K⁺; hence, K⁺-induced depolarization abolishes the vascular response to EDHF, and thus the L-NAME-inhibitable component of ACh-induced relaxation may have been more clearly exhibited. A detailed analysis of ACh-induced relaxation of avian aortae is, however, beyond the scope of the present study.

cAT₁ Receptor in Kidney

Positive signals detected by ISH and the RT-PCR products derived from total RNA suggest that cAT₁ mRNA and cAT₁ receptors exist in fowl kidneys. The cAT₁ mRNA levels detected by both methods are higher in EB and decrease with maturation. ISH signals are even higher in mesonephric glomeruli (data not shown). Strong expression in glomerular tufts, presumably mesangial cells, during development may indicate that the cAT₁ receptor plays a
role in the mesangial cell growth and contraction that help to filter fluid out of glomerular capillaries to Bowman’s capsule under the low-pressure system of fetal kidneys (A. Gomez, personal communication). Ang II stimulates glomerular mesangial cells in human fetal kidneys (13). In neonatal rat kidneys (2-5 d after birth), AT, receptor binding and mRNA (ISH) are seen in immature glomeruli (1). Ang II is necessary for normal kidney development, and the targeted inactivation of a component of the RAS or AT, receptor induces morphological and functional abnormalities (10, 20). In mammalian fetal tissues, the type-2 Ang receptor (AT,2) is widely expressed, specifically in areas of active mesenchymal differentiation (4). The cAT, homologue receptor is, however, not AT,2, although both are strongly expressed in embryonic kidneys. First, the molecular properties of cAT, are distinctly different from those of mammalian AT,2 (15). Second, the site of expression of cAT, in the kidney differs from that of mammalian AT,2; AT,2 receptor mRNA is detected in undifferentiated nephrogenic mesenchymal tissue but not in immature or mature glomeruli or tubules (34), whereas we found that cAT, mRNA is expressed in glomeruli of both embryonic and mature chickens.

cAT, Receptor in Adrenals

Likewise, cAT, mRNA was detected with high intensity in adrenal glands in all age groups examined by ISH and RT-PCR analyses, indicating that the cAT, receptor is present in fowl adrenal glands of different maturation stages. cAT, mRNA expressions are densely localized in subcapsular regions of EB and CH adrenals; in more mature chickens, hybridized signals are more widely distributed in subcapsular and inner zones, and the cAT, mRNA levels measured from whole adrenal glands by RT-PCR are higher. The bird is evolutionarily the first vertebrate whose adrenal cortex has two distinct zones, although the zonation is not as clearly seen as in mammalian species. The subcapsular zone (aldosterone and corticosterone synthesis) comprises interrenal cells, and an inner zone contains both interrenal cells (corticosterone, but less aldosterone, synthesis) and chromaffin cells (11). Superfusion of [Asp5, Val5]Ang II, native avian Ang II, enhanced the secretion of aldosterone, but not corticosterone, from the subcapsular zone of adrenal slices of the duck (16). The high intensity
of cAT1 mRNA signals in subcapsular regions of adrenals in EB, CH, and PL/CK in the present study agrees with the distribution of aldosterone-secreting cells in birds. The high levels of expression, however, may not be solely attributable to Ang II’s action on aldosterone synthesis because, in birds, ACTH stimulates both aldosterone and corticosterone secretion, whereas Ang II is a rather weak stimulator of mineralocorticoid secretion (16). The detection of cAT1 mRNA by ISH in neuronal ganglia agrees with functional evidence that Ang II stimulates catecholamine release in nonmammalian vertebrates (2, 22, 24, 44).

**cAT1 Receptor in Arteries**

We reported earlier that a specific Ang II binding site exists in aortic endothelia of adult chickens (26, 38) that is displaced by the 8th amino acid-replaced Ang II peptide antagonist, but not by mammalian nonpeptide AT1 antagonist (losartan) or AT2 antagonist (PD123319) (26). The pharmacology of the Ang II receptor in chicken aortic endothelia in the previous (8, 26, 38, 45) and present studies resembles that of the cloned cAT1 receptor transfected to COS-7 cells (15). Although a cAT1 mRNA riboprobe was not hybridized in situ in the aortic endothelia, endothelium-dependent Ang II-induced relaxation of abdominal aortae is clearly seen in all age groups, indicating that Ang receptor protein is present in aortic endothelia. The cAT1 mRNA was detected in the endothelia of small arteries and arterioles of the kidney and, less extensively, of the adrenals. The mechanism of this tissue-dependent heterogeneity in cAT1 mRNA hybridization is not clear at present, but the properties of endothelia from conducive arteries and resistance vessels may differ. In mammalian aortic endothelium, AT1 mRNA is unable to be visualized by ISH for unknown reasons (J.-M. Gasc, personal communication), whereas Ang II stimulates NO production in cultured rat aortic endothelial cells via the AT1 receptor (28). Chicken aortic endothelial receptors also mediate the production of NO/cGMP (8, 45, and present study).

The findings that cAT1 mRNA was detected by RT-PCR in EB and CH aortae without endothelia and that cAT1 mRNA is detectable by ISH in the outer layer of SM and adventitia of the embryonic aortae suggest that cAT1 may be expressed in aortic SM/adventitia during
development. This agrees with the finding by Le Noble (17) showing that specific binding sites for $^{125}$IAng II (and, hence, AT receptor protein) are seen predominantly in the adventitia and, to a lesser extent, in the media of chorioallantoic membrane (CAM) arteries from day-10 chicken EB. Furthermore, we noted that cAT$_1$ mRNA is detected in the adventitia, but not SM, of PL/CK aortae. This supports our previous studies reporting that Ang II stimulates thymidine incorporation into cultured aortic SM cells from CH, whereas in adult birds, Ang II stimulates neither aortic SM cell growth nor contraction (35, 45), nor does it induce cytosolic Ca signaling (42). The aortae of fetal and neonatal rats express both AT$_2$ and AT$_{1A}$ receptor mRNA (33); potent and lower levels of expressions are noted, respectively, in the tunica adventitia and tunica media of developing aortae at fetal day 10 and postnatal day 0.

The membrane fractions of aortic SM cells derived from adult chickens exhibit specific Ang II binding sites that have high- ($K_d$, 0.5 nM) and low-affinity sites ($K_d$, 8 nM). These Ang II binding sites/receptors are saturable and completely displaceable by Ang II agonists, but not enhanced by calcium, and binding dissociation is not inhibitable by GTP (37). Neither the cAT$_1$ mRNA probe (15) nor the mammalian AT$_2$ probe (T. Inagami, personal communication) hybridizes to this medial Ang II receptor, suggesting that this receptor may represent a subtype different from cAT$_1$ (23).

**Maturation-dependent changes in cAT$_1$, expression and functional implication**

The present study indicates that the sites and levels of Ang receptor expression in fowl change during maturation and that these maturation-dependent changes differ among tissues/organs. The cAT$_1$ mRNA levels are high in renal glomeruli and decrease with maturation. cAT$_1$ mRNA is detected in VSM/adventitia from EB/CH aortae, but not from aortic SM from more mature chickens. It is therefore possible that Ang II plays a significant role in glomerular mesangial cell and VSM cell growth and differentiation during development/maturation, whereas cAT$_1$ mRNA and growth-promoting action remain in the adventitia in adult birds. Ang II increases vascular density in the CAM of chicken EB (18). Endothelium-dependent Ang II-relaxation indicates that Ang II receptor is present in aortic
endothelium in all age groups examined. We therefore hypothesize that Ang II exerts a dual action in fowl aorta: 1) in intact vascular walls, Ang II helps maintain vascular wall integrity via an endothelium-dependent NO production or plays a role in regulating renal blood flow, whereas 2) Ang II stimulates aortic SM/fibroblast growth during development/maturation. It is possible that Ang II also stimulates the growth/mobilization of adventitial cells in adults, particularly in injured vascular walls, leading to NP formation (27). Endothelium-dependent relaxation is attenuated in adult chickens with aging, presumably due to exposure to sustained elevation of BP and subendothelial hyperplasia (8, 27). In contrast, cAT1 expression in the adrenals is consistently high in both EB/CH and more mature birds. The particularly dense Ang II expression in the subcapsular zone in EB may indicate that Ang II plays a role in the growth/differentiation of adrenal cortex. In human adrenal glands, AT1 receptors are detected at the peripheral zone after 16 wk of gestation (3).

Ang receptors sharing part of the AT1 receptor protein/nucleotide sequences have been identified in several nonmammalian species, including teleost fish, amphibians, and birds (for review, see 23, 32). Homology to the AT1 receptor increases with vertebrate advancement (~50% in teleosts, 60-65% in Xenopus, and 75% in birds). The AT1-homologue receptors are G protein-coupled and stimulate the formation of inositol triphosphate (15) and cytosolic Ca\(^{2+}\) release (23, 32). The molecular properties and signaling of Ang receptors suggest that a progenitor receptor evolved during early vertebrate evolution and that during the phylogenetic advancement of vertebrates, multiple AT1-homologue isoforms evolved with a gradual increase in homology to AT1. In summary, present results suggest that levels of cAT1 gene expression change during maturation in a tissue-specific manner and that cAT1 may have a role in growth promotion. The mechanism of changes in sites and level of cAT1 expression and whether modulation occurs at transcriptional or post-transcriptional levels remain to be determined. In the aortae of fowl of all ages examined, Ang II (10\(^{-6}\) M) induces endothelium-dependent relaxation partly inhibitable by L-NAME; a K\(^+\) channel may be involved in the L-NAME-resistant component of Ang II-induced relaxation.
FOOTNOTE

Preliminary studies were presented at the Annual Meeting of the Federation of American Societies for Experimental Biology, 2002. The presented studies were conducted at Institut National de La Santé et de la Recherche Médicale (INSERM) and College de France, Paris, France, and Department of Pharmacology, Universiteit Maastricht, Maastricht, Netherlands, during the sabbatical leave of Hiroko Nishimura. Address for reprint requests: Hiroko Nishimura, M.D., Department of Physiology, University of Tennessee - Memphis, Memphis, TN 38163 (nishimur@physio1.utm.edu).
ACKNOWLEDGEMENTS

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21. **Murphy TJ, Nakamura Y, Takeuchi K, and Alexander RW.** Accelerated Communication: A cloned angiotensin receptor isoform from the turkey adrenal gland is


FIGURE LEGENDS

**Fig. 1.** Representative recordings of isometric tension of abdominal aortic rings from day-19 embryo. [Val]^5^ angiotensin II ([Val]^5^Ang II) and acetylcholine (ACh, 10^-8 M ~ 3 x 10^-5 M) induced rapid relaxation of aortae precontracted with U46619 (2 x 10^-7 M) (A). Ang II-induced relaxation is partly inhibited by L-NAME (10^-4 M) (B), and restored by pretreatment with L-arginine (10^-4 M) (C). Removal of endothelium completely eliminated Ang II-induced relaxation (D). Vertical bar, 1.96 mN (0.2 g)

**Fig. 2.** [Val]^5^Ang II-induced relaxation of abdominal aortae precontracted with U46619 (2 x 10^-7 M) in embryos (number of rings for each treatment) (EB, n = 4-7), chicks (CH, n = 6), and pullets/cockerels (PL/CK, n = 5-7) and the effects of L-NAME, L-arginine plus L-NAME, and endothelium denudation. Values are means ± SE. Relaxation is expressed as % decrease in tension induced from the level preceding Ang application. Magnitude of relaxation is compared among treatments and age groups (ANOVA). *P < 0.01, between treatments; ^0.01 <P < 0.05 and ^P < 0.01 indicate significant differences from respective controls. No significant difference was noted among age groups within the same treatment. The increases in forces induced by U46619 (precontraction) or K^+^ are similar among aortic rings used for different treatments within the same age group.

**Fig. 3.** Isometric tension recording of abdominal aortic rings from 1.5-wk-old (panels A and D) and 2.5-wk-old (panels B and C) chicks. The L-NAME-resistant component of [Val]^5^Ang II-induced relaxation was characterized after pretreatment with U46619 (2 x 10^-7 M) plus L-NAME (10^-3 M) (A, B, & C) for possible EDHF/K^+^ channel involvement. Ang II-induced relaxation is nearly completely inhibited after treatment with barium plus ouabain or apamin plus charybdotoxin. The combination of 125 mM K^+^ and L-NAME (10^-3 M) abolished Ang II-induced relaxation completely and acetylcholine (ACh)-induced relaxation nearly completely (D). Simultaneous recording of two rings in D. KRB solution containing a high K^+^ concentration was prepared by replacing NaCl with KCl; the osmolality of the KRB remained the same.
**Fig. 4.** The cAT, shown by in situ hybridization (ISH) in the kidney from an embryo (A, day 19, EB), a chick (B, 2 wk old, CH), and a pullet (C; 14 wk old, PL). The mRNA is detected as clusters of silver grains in the center area of the glomerular tuft in EB and CH and, to a lesser extent, in PL. The labeling of the glomerulus is the same as the background in sense probe controls (D). No selective hybridization signals are seen on renal tubules at any ages. Horizontal bar, 20 μm.

**Fig. 5.** cAT, mRNA probes hybridized in situ in the small arteries and arterioles of kidneys from a chick (A, CH) and a pullet (B, PL) and in arterioles from adrenals (C and D). In CH and PL, silver grains are localized in the endothelium, but not in the vascular smooth muscles or adventitia. In both kidneys and adrenals, the labeling of the endothelium is more clearly seen in CH and PL than in EB (not shown). Horizontal bar, 20 μm.

**Fig. 6.** cAT, mRNA signals detected by in situ hybridization (ISH) in adrenal glands from an embryo (A & B, day 19, EB), a chick (C; 2 wk of age, CH), and a pullet (D; 14 wk of age, PL). In EB and CH adrenals, silver grains are localized in the subcapsular region (A) and less strongly in ganglia (B). Signals are more diffusely seen in adrenals from PL. Insets show macroscopic autoradiographs of adrenals after in situ hybridization with specific cAT, antisense (left) and sense (right) 35S-labeled riboprobes. Dark bands are seen at subcapsular regions. Horizontal bar, 100 μm.

**Fig. 7.** Abdominal aortae of embryo (EB) and chick (CH) incubated with cAT, mRNA sense (Ss) and antisense (As) probes. Silver grains are localized in the outer edge of smooth muscle layers and adjacent adventitia in EB aortae (A & B; As). No such focal localization is seen in EB Ss or CH As. Horizontal bar, 20 μm.
Fig. 8. Representative gel electrophoresis of RT-PCR products from embryo (EB), chick (CH), and pullet (PL) kidneys. The amplified fragments are of 525 bp, as expected from the location of specific primers (see method). Duplicate determinations from each animal indicate that the densities of the 525-bp bands from EB are higher than those from CH or PL. All RNA preparations are free from DNA contamination (no band from reverse transcriptase-minus incubation). NC, negative control in which RT reaction mixture was deleted. PC, positive control (plasmid cDNA).

Fig. 9. cAT, mRNA levels (normalized by G3PDH) in kidneys (kid, left) and adrenals (adr, right) from three age groups determined by RT-PCR. To minimize analytical variations, the RNA samples from all age groups were reverse-transcribed and amplified (in duplicate) in the same incubation. Total RNA (μg) (adr, 0.1; kid, 0.6) and PCR cycles (adr, 28; kid, 29) needed to detect PCR product are lower in adrenals. Values are mean ± SE. Significance was determined with ANOVA.

Fig. 10. A. PCR products from abdominal aortae with/without endothelium (E+/E-) in embryos and chicks. In both groups, the cAT, mRNA levels (normalized by G3PDH) from E+ and E-aortae are not significantly different. B. In aortae from pullets (PL)/cockerels (CK), vascular smooth muscle layers (VSM; endothelium denuded) were isolated from the adventitia. cAT, mRNA was detected in the adventitia but not in VSM. C. Gel electrophoresis of RT-PCR products from PL/CK adventitia detecting the fragments of 525 bp. No DNA contamination is present. NC, negative control. PC, positive control (plasmid cDNA).
Table 1. *cAT1*, mRNA signals hybridized in situ in renal glomeruli

<table>
<thead>
<tr>
<th></th>
<th>Embryos</th>
<th>Chicks</th>
<th>Pullets/Cockerels</th>
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<tbody>
<tr>
<td>No. of glomeruli/mm²</td>
<td>30.1 ± 2.9</td>
<td>13.5 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.7&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>(N = 4)</td>
<td>(N = 4)</td>
<td>(N = 2)</td>
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<tr>
<td>No. of grains/mm²</td>
<td>0.101 ± 0.1</td>
<td>0.099 ± 0.09</td>
<td>0.019 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>(n = 24)</td>
<td>(n = 24)</td>
<td>(n = 12)</td>
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</tbody>
</table>

Values are means ± SE. N: number of birds. n: number of glomeruli.

<sup>a</sup>The number of *in situ* hybridization signals was counted in six glomeruli (glomerular tuft only) from 2-4 kidneys.

Background was subtracted, and the values were normalized by glomerular area.

<sup>b</sup>P < 0.01 compared to embryo (ANOVA).
<table>
<thead>
<tr>
<th>Control</th>
<th>L-NAME (10^{-4} M)</th>
<th>L-Arginine (10^{-4} M) L-NAME (10^{-4} M)</th>
<th>Endothelium -denuded</th>
</tr>
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<tr>
<td>[Val^{5}]AngII 10^{-6} M</td>
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<td>Ach (M)</td>
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<td>3x10^{-6}</td>
<td>10^{-6}</td>
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</table>

Fig 1
Fig 2

[Graph showing the decrease in tension (%)]

- Control
- L-NAME (10^{-4} M)
- L-Arg (10^{-4} M) + L-NAME
- Endothelium denuded

Treatments:

- Embryo (day 19)
- Chick (2-3 wk old)
- Pullet/Cockrel (14-16 wk old)

[Val^{5}]Ang II, 10^{-6} M

Decrease in tension (%)

Treatment
Fig 3

A. [Val^5]AngII 10^{-6} M
   Ach 3 \times 10^{-5} M
   Barium 3 \times 10^{-7} M
   Ouabain 5 \times 10^{-7} M
   [Val^5]AngII 10^{-6} M
   Ach (M)
   10^{-7}
   3 \times 10^{-7}
   10^{-6}
   3 \times 10^{-6}

B. [Val^5]AngII 10^{-6} M
   Ach (M)
   10^{-7}
   3 \times 10^{-7}
   10^{-6}
   3 \times 10^{-6}

C. [Val^5]AngII 10^{-6} M
   Ach (M)
   10^{-7}
   3 \times 10^{-7}
   10^{-6}
   3 \times 10^{-6}

D. [Val^5]AngII 10^{-6} M
   Ach (M)
   10^{-5}
   3 \times 10^{-5}
   5 \times 10^{-7}
   L-NAME 10^{-3} M

Pretreatment: U46619 (2 \times 10^{-7} M) + L-NAME (10^{-3} M)

Pretreatment: 125 mM K^+

= 4.9 mN
Fig 4

A. Embryo day 19
B. Chick 2 wks
C. Pullet 14 wks
D. Embryo day 19
Fig 5

A. Chick 2 wks
B. Pullet 14 wks
C. Chick 2 wks
D. Pullet 14 wks
Fig 6

A. Embryo day 19

B. Embryo day 19

B. Chick 2 wks

C. Pullet 14 wks
Fig 7

A. Embryo day 19
B. Embryo day 20
C. Embryo day 20
D. Chick 2 wks
Fig 9

Bar chart showing the comparison of cAT/G3PDH ratio between different age groups (Embryo, Chick, and Pullet) for Kidneys and Adrenals, with statistical significance indicated by $P < 0.01$.
Fig 10

A

B

C

Reverse Transcriptase (-)

525 bp