日本財団補助金による
２０００年度日中医学学術交流促進事業

⑤．在留中国人研究者研究助成

(18)プラークー感受性(SUS)と抵抗性(RES)ラット三大唾液腺に於けるアドレナリン性受容体サブタイプ遺伝子の発現動態

プラークー感受性 (SUS) と一抵抗性 (RES) ラット三大唾液腺におけるアドレナリン性受容体サブタイプ遺伝子の発現動態

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要旨（日本語）

アドレナリン受容体（AR）は哺乳類の唾液腺における唾液成分の分泌を調節することに関わる。ARには分子クーロンニングにより3種類のα_1 ARs (α_{1a}, α_{1b} and α_{1d})，3種類のα_2 ARs (α_{2A}, α_{2B} and α_{2C}) 及び3種類のβ ARs (β_1, β_2 and β_3)の合計9種類のサブタイプの存在が報告されている。今までの研究では、ODUラット(SUSとRES)の三大唾液腺におけるARサブタイプ遺伝子mRNAとHouse-keeping gene—G3PDHの発現を検索した。更に耳下腺におけるARサブタイプ遺伝子mRNAとG3PDHの発現量を詳細に検討してきた。その結果は：

1. RT-PCRにより5種類のARサブタイプ-- α_{1a}, α_{1b}, α_{2A}, β_1 及び β_2 とG3PDHの増幅産物がODUラット（SUSとRES）の顎下腺、耳下腺及び舌下腺の全てに検出された。なお、α_{1d}, α_{2B}, α_{2C}及びβ_3サブタイプについてもRT-PCRを行ったがその増幅産物は三大唾液腺にいずれも検出されなかった。
2. SUSとRESラットの耳下腺におけるARサブタイプ遺伝子発現量のCompetitive RT-PCRによる比較:
   （1） G3PDHに対する相対値：SUSとRESとで比較すると、α_{1a}, α_{2A} 及びβ_1 ARサブタイプmRNAの発現量はRESより、SUSが顕著に低く、α_{1b} ARサブタイプmRNAの発現量はRESより、SUSが顕著に高く、有意差が認められた。β_2 ARでは両者に有意差が認められなかった。
   （2） 総 AR ($\alpha_{1\alpha}+\alpha_{1\beta}+\alpha_{2A}+\beta_1+\beta_2$) に対する相対値：SUSとRESとで比較すると、α_{1a}, α_{2A}及びβ_1 ARサブタイプmRNAの発現量はRESより、SUSが顕著に低く、α_{1b}及び β_2 ARサブタイプmRNAの発現量はRESより、SUSが顕著に高く、両者に有意差が以上の5種類のARサブタイプ遺伝子mRNA全てに認められた。
   （3） SUSとRESの総 ARの発現量：総 ARの発現量を両者で比較すると、RESよりSUSが顕著に低く、有意差が認められた。
   （4） SUSとRESのARサブタイプの遺伝子mRNA発現量の順位： SUS；$\beta_2 > \alpha_{1\alpha} > \alpha_{1b} > \beta_1 > \alpha_{2A}$, RES； $\alpha_{1a} > \beta_2 > \beta_1 > \alpha_{1b} > \alpha_{2A}$であり， 両者の順位は異なっていた。

以上のことから，ARサブタイプ遺伝子の発現量はSUSとRESとの遺伝子レベルでの鑑別に重要な役割を果たす可能性が示唆された。

Key words: Adrenergic receptor subtype, Quantitative competitive reverse transcription-polymerase chain reaction (RT-PCR), three major salivary glands, ODU plaque-susceptible (SUS) and -resistant (RES) rats,

mRNA Expression of Adrenergic Receptor Subtypes in ODU Rat Salivary Glands

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Key words: Adrenergic receptor subtype, Quantitative competitive reverse transcription-polymerase chain reaction (RT-PCR), three major salivary glands, ODU plaque-susceptible (SUS) and -resistant (RES) rats, mNRA expression

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Running title: mRNA expression of AR subtypes

Abbreviations: dNTP, deoxynucleoside triphosphate; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction; ODU, Osaka Dental University; SUS, ODU plaque-susceptible rat; RES, ODU plaque-resistant rat; SG, salivary gland.

Abstract

Adrenergic receptors (ARs) are involved in mediating saliva secretion in mammalian salivary glands (SGs). Nine AR subtypes, including three α_1-ARs (α_{1a}-, α_{1b}- and α_{1d}-ARs), three α_2-ARs (α_{2A}-, α_{2B}- and α_{2C}-ARs) and three β-ARs (β_1-, β_2- and β_3-ARs), have been identified through molecular cloning. The five subtype genes, α_{1a}-, α_{1b}-, α_{2A}-, β_1-, and β_2-ARs, were expressed in the three major SGs of ODU Plaque-susceptible (ODUS/Odu/SUS) and plaque-resistant (RES) rats. In contrast, the other four subtype mRNAs, α_{1d}-, α_{2B}-, α_{2C}- and β_3-ARs, were not detected by reverse transcription-polymerase chain reaction (RT-PCR). The steady-state mRNA expression for the five AR subtypes in the Parotid glands (PGs) of the ODU rats at 12 weeks of age was measured by quantitative competitive RT-PCR used synthetic DNA fragment (subtype-specific DNA competitor) as an exogenous internal standard. The intensities of the mRNA expression of AR subtypes in the PG were significantly different between SUS and RES groups-- the levels of transcripts for α_{1a}-, α_{2A}- and β_1-AR subtypes were significantly lower, whereas that for α_{1b}-AR subtype mRNA was significantly higher in SUS group in relation to that for a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH), and to that for the total AR (α_{1a}+ $\alpha_{1\beta}$+ α_{2A}+ β_1 + β_2). On the other hand, the level of transcript for the β_2-AR subtype mRNA was not significant between SUS and RES in relation to that for G3PDH, but not to that for the total AR. Moreover, the levels of transcripts for total AR subtypes were significantly lower in SUS than in RES. And the relative rank order of AR subtype mRNA expression in SUS and RES was different as: β_2- > $\alpha_{1\alpha}$- > α_{1b}- > β_1- > α_{2A}-AR and α_{1a}- > β_2- > β_1- > α_{1b}- > α_{2A}-AR, respectively. It is revealed although the three major SGs of ODU rat contain the five AR subtype mRNAs, distinct subtype-specific expression is evident between the SUS and RES group in the PGs, and also suggested that some of the AR subtypes in the PGs might especially be involved in discriminating both SUS and RES groups.

1. Introduction

Adrenergic receptors (ARs) are involved in mediating saliva secretion in mammalian salivary glands (SGs). The ARs, as the two types of three classical autonomic neurotransmitter receptors (α-adrenergic, α-AR, β-adrenergic, β-AR, muscarinic-cholinergic, mAChR), are clarified to be a family of G protein-linked signaling proteins and can be functionally and structurally divided into three main subfamilies, designated as α_1-, α_2- and β-ARs, which are generally coupled to evoking a Ca^{2+} signal (α_1-ARs), inhibiting adenylylate cyclase (α_2-ARs) and triggering a G-protein-mediated cAMP signal (β-ARs), respectively (Garrett *et al.*, 1999). Furthermore, multiple subtypes have been identified through molecular cloning within each subfamily: three α_1-ARs (α_{1a}-, α_{1b}- and α_{1d}-ARs) (Voigt *et al.*, 1990; Lomasney *et al.*, 1991; Laz *et al.*, 1994), three α_2-ARs (α_{2A}-, α_{2B}- and α_{2C}-ARs) (Zeng *et al.*, 1990; Lanier *et al.*, 1991) and three β-ARs (β_1-, β_2- and β_3-ARs) (Buckland *et al.*, 1990; Machida *et al.*, 1990; Muzzin *et al.*, 1991).

The strain of ODU Plaque-susceptible (ODUS/Odu/SUS) rats has been breeding, identifying and developing at Osaka Dental University (ODU) of Japan since 1972 (Ito *et al.*, 1975a), which was originally derived from Wistar-kyoto strain and exhibited markedly heavy plaque formation in the lower incisors and developed both periodontal pocket and gingivitis after being fed with commercially available powder diet (MF, Oriental Yeast Co., Tokyo, Japan) per day with water *ad libitum*. Whereas those not forming plaque and gingivitis under the same dietary condition are known as ODU plaque-resistant (RES) rats. It has been demonstrated that ODU SUS rat is a very suitable and useful experimental model for studying plaque formation, naturally occurring gingivitis (Ito *et al.*, 1975) and experimental periodontitis (Ohura *et al.*, 1995) and thought that plaque formation depended more on the hereditary characteristic of the strain of ODUS rats than on the diet (Ito *et al.*, 1976). The some data about the changes of amount and PH of saliva, IgA in the saliva, salivary kallikrein, silalic acid and fibrinolytic activities in saliva were reported in ODU SUS rats (Ohura *et al.*, 1984 and 1986; Takai *et al.*, 1985; Ieiri, 1982; Shinohara *et al.*, 1994; Ohura *et al.*, 1985). The differences of allelic distribution – cell surface alloantigen RT1.A and RT2 between ODU SUS and RES rats in red blood cell were shown and suggested that forming plaque and gingivitis in the lower incisors might account for allelic distribution (Shinohara *et al.*, 1991). But in

the entities of the SGs, especially in that of the three major SGs, the differences of gene expression between SUS and RES rats have not been completely elucidated yet, so in this study we decided the beginning of our experiment from mRNA expression of AR subtypes in the three major SGs of ODU rats, including ODU SUS and RES rats.

Therefore, it is very important to study the differences in the gene expression and levels of AR subtypes in the SGs of ODU rats to elucidate the mechanisms of AR subtypes' mediating saliva secretion in ODU rats and whether there are certain correlations or not between the gene expression of AR subtypes and each of both dental plaque formation in SUS and dental plaque inhibition in RES in the aspect of the hereditary characteristic of the two strains. However, little information is available on the gene expression and levels, the distribution and their functions of the AR subtypes in the SGs, especially in ODU rats' SGs. The present study was thus designed to determine whether there were some differences in the mRNA expression for AR subtypes in the three major SGs between ODU SUS and RES rats. Furthermore, the steady-state expression of mRNA for AR subtypes were studied in ODU rat parotid glands (PGs) by quantitative competitive RT-PCR, which is a accurate and highly sensitive technique for quantifying small amounts of mRNA (Gilliland *et al.*, 1990; Siebert and Larrick, 1993; Nishiura and Abe, 1999).

2. Materials and methods

2.1. Total RNA preparation from three major SGs

Male ODU SUS and RES rats (at least 3 rats/group) at 12 weeks of age were killed by exsanguination and the PG, submandibular (SMG) and sublingual (SLG) glands were rapidly excised. Total cellular RNA was isolated by the extraction with guanidinium thiocyanate-acidic-phenol-chloroform according to the procedure of Chomczynaski and Sacchi (1987). After precipitation with isopropanol, the RNA was washed with 75 % ethanol and then resuspended and dissolved in RNase-free water. Subsequently the RNA was purified for complete removal of DNA contamination from RNA by MessageClean kit (GenHunter Co., TN, USA) according to the manufacturer's protocol. The yields of RNA were measured by absorbance at 260 nm.

2.2. Design and synthesis of oligonucleotide primer

Gene-specific oligonucleotide primers for α_{1a}-, β_2- and β_3-AR subtypes, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH), were designed from the reported cDNA sequences using the nucleotide-sequence analysis software (DNASIS: Hitachi Software Engineering Co., Yokohama, Japan), and synthesized by Lifetech Oriental Co. (Tokyo, Japan) or Amersham Pharmacia Biotech (Tokyo, Japan). Also, the primers for α_{1b}- and α_{1d}-ARs, and for α_{2A}-, α_{2B}- and α_{2C}-ARs, and β_1-AR subtype, which were designed by Scofield *et al.* (1995), Gold *et al.* (1997) and Elalouf *et al.* (1993), respectively, were synthesized similarly and utilized. Meanwhile, a portion of the composite primers, relevant to gene-specific primers, for the constitution of corresponding DNA competitor, were designed to be made up of two parts as described in *2.4*, synthesized similarly and utilized. The sequences of gene-specific oligonucleotide primers and relevant composite primers are shown in Table 1.

2.3. RT-PCR

RT-PCR analysis was performed under the conditions showed in Table 2, as well as described previously (Nishiura and Abe, 1999). First-strand cDNA was synthesized in a RT reaction mixture containing 50 ng / μl total cellular RNA, PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 5 mM $MgCl_2$, 1 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 1 unit/μl ribonuclease inhibitor, 2.5 μM random 9-mers and 0.25 unit/μl avian myeloblastosis virus reverse transcriptase (Life Science, FL, USA). The reaction was incubated at 30 ℃ for 10 min and at 42 ℃ for 30 min, and the reverse transcriptase was inactivated by heating to 99 ℃ for 5 min. DNA amplification was performed in a PCR mixture containing cDNA, PCR buffer, 4 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each gene-specific forward or sense and reverse or antisense primer and 0.025 unit/μl recombinant Taq DNA polymerase (Takara shuzo, Tokyo, Japan). The PCR for the AR subtypes and the G3PDH amplification reactions was performed with a Program Temp Control System PC-700 (Astec Co., Fukuoka, Japan) with the following cycle parameters: one pre-denaturation cycle for 2 min at 94 ℃; in succession the every PCR cycle included 1 min of denaturation at 94 ℃, 1 min of annealing at 60 ℃ and 2 min of extension at

72 ℃.

2.4. Construction of competitor for competitive RT-PCR

The DNA competitors for quantifying the mRNA of AR subtypes and G3PDH by competitive RT-PCR were constructed with a MIMIC DNA fragment (574 bp, *Bam*HI/*Eco*RI fragment of the v-*erb*B gene) using a PCR MIMIC Construction Kit (Clontech, CA, USA) according to the manufacturer's protocol or the procedure of Siebert and Larrick (1993). The illustrative construction of DNA competitor is shown as Fig. 1. In order to construct the DNA competitors, two rounds of PCR amplification were performed. In the first PCR reaction, two composite primers were utilized, transcribed as well in Table 1. Each of one set of composite primers including forward and reverse primers, was designed to contain the corresponding target gene-specific primer sequence attached to a short, 20-nucleotide stretch of sequence designed to hybridize to opposite strand of the MIMIC DNA fragment. The desired primer sequences were thus incorporated during the first PCR amplification in the reaction mixture containing 1 ng MIMIC DNA fragment, 0.2 μM of each forward and reverse composite primer, PCR buffer, 4 mM $MgCl_2$, 0.2 mM of each dNTP and 0.025 unit/μl recombinant Taq polymerase. The products were diluted 1:100 in DEPC-treated water and 2 μl was added to 98 μl of PCR mixture containing PCR buffer, 4 mM $MgCl_2$, 0.2 mM of each dNTP, 0.025 unit/μl recombinant Taq polymerase and 0.2 μM of each forward and reverse target gene-specific primer. Both the first and the second PCR were incubated at 94 ℃ for 1 min, at 50 ℃ for 1 min and at 72 ℃ for 2 min and the numbers of the two PCR cycles were 17 and 18, respectively. The amplified DNA competitor was then passed through a CHROMA SPIN+TE-100 column (Clontech, CA, USA) to remove primers and reaction components and was separated on agarose gel electrophoresis. The yield of the competitor was estimated by image analysis as described in the latter *2.6*, and diluted to 100 amol (10^{-18} mol) /μl in 10 μg/ml glycogen and stored at -20 ℃ for use as a stock solution for the competitor dilutions. The competitors gained are non-homologous DNA, which remained complete target gene-specific primer sequences. The size of the competitor differed from the target gene product and could range from 425-606 bp by choosing the appropriate sequence of the MIMIC DNA fragment for the composite primers.

2.5. Assay of amplification kinetics

On the basis of DNA competitor and it target manuscript sharing the same set of primers and containing the same sequence DNA fragment, meanwhile also containing the different sequence fragment resulting in the different size products, it was therefore necessary to achieve the assay of amplification kinetics (amplification efficiency identity) before conducting competitive RT-PCR, by a target cDNA and its competitor amplifying simultaneously in equal molar quantities and under same conditions in respective PCR tubes at various cycle numbers from 19 to 36. The curves of amplification kinetics were drawn in accordance with the band intensities from image analysis after agarose gel electrophoresis by using the calibration standard (Fig. 3). The results of amplification kinetics assay were shown in *3.2.* and Fig. 5. Only after passing through the assay, the harvested competitors could be adopted in competitive RT-PCR.

2.6. Competitive RT-PCR

The competitive RT-PCR experiment was performed by the addition of increasing amount of DNA competitor to a fixed amount of cDNA synthesized by reverse transcription (RT) reaction, and both a target and its competitor were co-amplified in the presence of the same set of appropriate or corresponding gene-specific primers as well as RT-PCR as described previously (Nishiura and Abe, 1999). So the competitor competes with the target for the same primers and materials for synthesizing respective PCR products in the same one PCR reaction system and thus acted as an exogenous internal standard. The concise illustrative instructions for competing process and quantitative method of target manuscript — identifying the amount of target manuscript in PCR samples by the competitor 1:1 with target with NIH-Image and a linear regression analysis are shown as Fig. 2. The practical operation of competitive RT-PCR techniques was in accordance with the detailed protocol of PCR MIMIC™ Construction Kit (Clontech Lab. Inc., USA). The initial levels of the target mRNA (an absolute amounts) were determined by calculating how much of the competitor was required to achieve equal molar amounts of products (Gilliland *et al.*, 1990). The log of the number of molecules of DNA competitor added to the sample before starting amplification was plotted against the log of the ratio of the

molar amounts of the target and its competitor. The lines were drawn from a linear regression analysis and the number of molecules of the target manuscript was calculated by extrapolating from the intersection of the line, the equivalence point, *i.e.*, in which the molar ratio of target and competitor was 1.0 (log = 0). The mRNA levels for AR subtypes and G3PDH in the same cDNA sample were determined by utilizing competitive RT-PCR. The G3PDH is a housekeeping gene and reflects constitutive activity ubiquitous to eukaryotic cells. The amounts of mRNA were expressed as a percentage of transcript relative to G3PDH or other genes in order to correct the efficiency differences of total RNA extraction or/and reverse transcription from RNA to cDNA.

2.7. Agarose gel electrophoresis and determination of DNA band intensities by image analysis

An aliquot of each PCR product, including that of RT-PCR and competitive RT-PCR was separated by horizontal gel electrophoresis at 50 V for 1.2 h on 1.5 % SeaKem GTG agarose gel (FMC BioProducts, ME, USA) in 1xTBE buffer and visualized under UV light after staining with 0.5 mg/ml ethidium bromide (EtBr) for 30 min as described previously (Nishiura and Abe, 1999). In the meanwhile, the DNA molecular weight markers (ϕX 174/*Hae*III digest, Toyobo, Osaka, Japan) with distinct gradient-concentration dilutions were subjected to electrophoresis to utilize as calibration standards for determination of the size or the intensities of the PCR-product bands (Fig. 3). The gels were photographed using a Polaroid MP-4 Land camera with Type 667 film, scanned and digitized on an EPSON digital scanner and preserved as TIFF files. The intensity of PCR products' bands from the scanned images was analyzed by adopting a gel plotting macro of NIH-Image program (Version 1.57) on an Apple Macintosh computer and acquired relative data of the products from RT-PCR and Competitive RT-PCR.

2.8. Statistical analysis

Statistical analyses employed the unpaired Student's *t*-test with a computerized program "StatView". The significant and remarkably significant differences between data were evaluated through $p < 0.05$ and $p < 0.01$.

3. Results

3.1. RT-PCR analysis of the expression of AR subtype and G3PDH mRNAs in three major SGs of ODU SUS and RES rats (Fig. 4.1-3)

Figs. 4.1-3 show the patterns of amplified RT-PCR products after electrophoresis and staining, the patterns of mRNA expression for five AR subtypes, α_{1a}-, α_{1b}-, α_{2A}-, β_1- and β_2-ARs, and G3PDH gene were observed in both SUS and RES groups of the three major SGs, but in the PG of SUS group, β_1-AR subtype mRNA was detected under the condition of 48 PCR cycles. These results verified that every band for the five AR subtype genes, α_{1a}-, α_{1b}-, α_{2A}-, β_1- and β_2-ARs, and G3PDH gene corresponded to the size expected from the primer design (Table 1). In contrast, the other three subtype mRNAs, α_{2B}-, α_{2C}- and β_3-ARs, were not detected by RT-PCR, whereas α_{1d}-AR subtype mRNA was not clearly observed in both SUS and RES groups of the three major SGs (data not shown).

3.2. Amplification efficiency of target cDNA and its competitor (Fig. 5)

The amplification kinetic analysis indicated that the target cDNAs (α_{1a}-, α_{1b}-, α_{2A}-, β_1- and β_2-AR subtypes, and G3PDH) and its competitor pairs amplified with very similar efficiencies.

3.3. Expression levels of AR subtype mRNAs in the PGs of ODU SUS and RES rats

3.3.1. Relative expression of α_{1a}-, α_{1b}-, α_{2A}-, β_1- and β_2-AR mRNAs to G3PDH (Fig. 6)

The amounts of transcripts for, relative to that for G3PDH, α_{1a}-, α_{2A}- and β_1-AR subtypes were significantly lower in SUS group, whereas that for α_{1b}-AR subtype mRNA was higher. On the other hand, the β_2-AR subtype mRNA was not significant between SUS and RES.

3.3.2. Relative expression of α_{1a}-, α_{1b}-, α_{2A}-, β_1- and β_2-AR mRNAs to total AR (α_{1a}+ $\alpha_{1\beta}$+ α_{2A}+ β_1 + β_2) (Fig. 7)

The amounts of transcripts for, relative to that for total AR (α_{1a}+ $\alpha_{1\beta}$+ α_{2A}+ β_1 + β_2), α_{1a}-, α_{2A}- and

β_1-AR subtypes were significantly lower in SUS group, whereas those for α_{1b}- and β_2-AR subtypes were higher.

3.3.3. Comparison of total AR mRNA levels between SUS and RES (Fig. 8A)

The amounts of transcripts for total AR subtypes were significantly lower in SUS than in RES.

3.3.4. The relative rank order of expression of AR subtype genes (Fig. 8)

The relative rank order of AR subtype mRNA expression in SUS and RES was β_2- > α_{1a}- > α_{1b}- > β_1- > α_{2A}-AR and α_{1a}- > β_2- > β_1- > α_{1b}- > α_{2A}-AR, respectively, at both to the levels of G3PDH and total AR mRNA.

4. Discussion

Up to date, the expressions for many AR subtypes, containing the various analyses of gene, RNA, protein expression, receptor and second messenger, transactivator and microscopy, have been investigating and identifying in many various mammalian tissues by adopting various biochemical, parmacological and molecular biological techniques (Machida, 2000). Three distinct α_1-AR subtype genes [α_{1a}- (previously also given an α_{1c}- nomenclature), α_{1b}- and α_{1d}- (previously also given an α_{1A}- or $\alpha_{1a/d}$- nomenclature) ARs] and corresponding proteins have been identified by molecular cloning and pharmacological studies, respectively. Only α_{1A}-AR subtype protein has been identified in rat SMG membranes by receptor binding assay (Michel *et al.*, 1989), whereas it has been demonstrated the presence of α_{1A}- and α_{1B}-AR subtype proteins in rat PG acinar cell membrances by ^{125}IBE binding experiments for subtype selective α_1-AR antagonists (Porter *et al.*, 1992). α_{1a}- and α_{1b}-AR subtype genes were expressed in rat SGs, but α_{1d}-AR subtype mRNA was not discovered by using Northern blot analysis and pharmacological profiles of [^{3}H] prazosin binding (Faure *et al.*, 1994). Moreover, Rokosh *et al.* (1994) mentioned that α_{1a}-AR subtype mRNA was abundant in SMG and at lower levels in PG, and α_{1b}-AR subtype gene was expressed at lower levels, approximately 1:1, but α_{1d}-AR subtype mRNA was

not detected in both SMG and PG of adult rat by RNase protection assay. In the present study, we showed similarly that two α_{1a}- and α_{1b}-AR subtype genes were expressed among SMGs, PGs and SLGs of ODU SUS and RES rats (Figs.4.1-3), but α_{1d}-AR subtype mRNA was not detected among them by using RT-PCR (data not shown). Furthermore, in the PGs, the amounts of transcript for α_{1a}-AR subtype were significantly lower in SUS than those in RES, whereas those for α_{1b}-AR subtype were significantly higher in SUS than those in RES, at both relative to G3PDH and to total AR mRNA by competitive RT-PCR (Figs. 6 and 7). The levels of transcript for α_{1a}-AR subtype were highest in the RES, were second order in the SUS of PGs; those for α_{1b}-AR subtype were lower than those for α_{1a}-AR subtype, respectively fourth and third order in the RES and SUS to both the levels of G3PDH and total AR mRNA. Bylund *et al.* (1982) and Nishiura *et al.* (2001) individually reported the changes of α_1-AR protein and α_1-AR mRNA in the developing SMGs of Sprague-Dawley rats, respectively. However, the expression patterns of α_1-AR protein (Bylund *et al.* 1982) were similar to those of α_{1a}-AR mRNA (Nishiura *et al.*, 2001), different merely in the time of approaching adults levels or reaching a maximum.

α_2-AR can now be divided into three AR subtypes, α_{2A}- (previously also given an α_{2D}-nomenclature), α_{2B}- and α_{2C}-AR subtypes. In our present studies, α_{2A}-AR subtype gene was expressed (Figs. 4.1-3), but α_{2B}- and α_{2C}-AR subtypes were not detected (data not shown) in both SUS and RES groups of three major SGs by RT-PCR. The result was identical to that of mRNA expression for α_2-AR subtypes in the SMGs of Sprague-Dawley rats (Nishiura *et al.*, 2001). In the PGs, the amounts of transcript for α_{2A}-AR subtype were significantly lower in SUS than those in RES. Bylund *et al.* (1982) showed that the density of α_2-AR protein in SMGs was nearly undetectable by 6 weeks and Nishiura *et al.* (2001) also showed mRNA expression of α_{2A}-AR subtype in SMGs was a constant low level after 6 weeks of age. But in the PGs of SUS and RES, mRNA expression of α_{2A}-AR subtype was in a lowest level among those for α_{1a}-, β_2-, β_1- and α_{1b}-AR subtype in both SUS and RES groups.

β-AR is divided into β_1-, β_2- and β_3-AR subtypes, of which β_1- and β_2-AR subtype mRNAs were expressed in both SUS and RES groups of three major SGs by RT-PCR (Figs. 4.1-3). In the PGs, the amounts of transcript for β_1-AR subtype were significantly lower in SUS than those in RES at both relative to G3PDH and to total AR mRNA (Figs. 6 and 7); Those for β_2-AR subtype were higher in SUS

than those in RES only relative to total AR mRNA (Fig. 7), not relative to that for G3PDH (Fig. 6). Moreover, the levels of transcript for β_2-AR subtype were higher than those for β_1-AR subtype regardless of in SUS or in RES. The rank order of β_2-AR subtype in the PGs, was first and second in SUS and in RES, respectively. Schneyer and Humphreys-Beher (1987) showed that rat SMG had both β_1- and β_2-AR proteins, with the percentages of each being 90 % and 10 %, respectively, by [^{3}H] DHA binding studies. However, Nishiura *et al.* (2001) showed that the amounts of transcript for β_2-AR subtype in rat SMG were about 1.7—3.6 times higher than those for β_1-AR subtype from 2 to 12 weeks of age by competitive RT-PCR. And then we also found that the levels of mRNA expression for β_2-AR subtype were higher than those for β_1-AR subtype in the PGs of both SUS and RES. Nishiura *et al.* (2001) attributed the expression differences of mRNAs and proteins for β_1- and β_2-AR subtypes to the translation efficiency, half-life or instabilities of them.

The relative rank order of AR subtype mRNA expression in SUS and RES was β_2- > α_{1a}- > α_{1b}- > β_1- > α_{2A}-AR and α_{1a}- > β_2- > β_1- > α_{1b}- > α_{2A}-AR, at both to the levels of G3PDH and total AR mRNA, respectively (Fig. 8). In the relative rank orders of AR subtype expression, we could find that two reversion and one identification between SUS and RES, that is, the rank order of β_2- > α_{1a}-AR in SUS was reversed into that of α_{1a}- > β_2- AR in RES; similarly, that of α_{1b}- > β_1- AR in SUS was reversed into that of β_1- > α_{1b}- AR in RES; However, the levels of transcript for α_{2A}-AR subtype was identically ultimate in both SUS and RES groups. Moreover, the relative rank order of AR subtype mRNA expression in RES: α_{1a}- > β_2- > β_1- > α_{1b}- > α_{2A}-AR, is completely identical to that of SD rats of the same age (12 weeks of age) from Nishiura *et al.* (2001) except that rank order: α_{1b}- > α_{2A}-AR is reverse. On the other hand, the amounts of transcripts for total AR subtypes were significantly lower in SUS than in RES.

Besides, α_{1d}-AR subtype mRNA was not clearly observed in both SUS and RES groups of the three major SGs, not detected in rat liver (data not shown) either by RT-PCR, which corresponded to the results of Rokosh *et al.* (1994) by RNase protection assay, Faure *et al.* (1994) by Northern blot analysis and pharmcological profiles of [^{3}H] prazosin binding and Scofield *et al.* (1995) by using Reverse Transcription and a competitive RT-PCR. Up to now, neither study have been found any detectable

mRNA of the α_{1d}-AR subtype in rat liver, but α_{1d}-AR subtype protein in rat liver had been discovered in 1-week-old and adult rats by western analysis using receptor subtype specific polyclonal antibodies (Shen *et al.*, 2000). Thus this appearance fully proved that there were certain differences of expressions in abundance between for AR subtype proteins and for corresponding AR subtype mRNAs found previously. Rokosh *et al.* (1994) also put forward the differences of expressions for β_1- and β_2-AR subtypes, the similar as ours. On the basis of the differences of expressions described as above, we thus doubt in the expression of AR subtypes, whether reverse-transcription efficiency is completely identical to transcription efficiency or not, that is, whether reverse-transcription *in vitro* is precisely reverse to transcription *in vivo* or not, and whether a spurious translation from mRNA to protein might be contained in the protein expression of AR subtypes?! Therefore, further researches are needed to elucidate these knotty problems and determine the extent and level of translation of AR subtype mRNA into corresponding AR subtype proteins in the SGs.

It is not clear now how much, what proportion and functional differences in AR subtype expressions both the parenchyma (including acinar and ductal cells) and stroma of the SGs possess, thus the cellular localization of AR subtype mRNAs and corresponding proteins in the SGs is also a challenge for future work. Furthermore, it is not definite now for each of AR subtypes in SUS and RES rats how to mediate saliva secretion and whether there is a certain correlation or not with dental plaque information or inhibition. Therefore, it is also very important to elucidate the mechanisms of AR subtypes' mediating saliva secretion in ODU rats and the differences of the two strains in the aspect of the hereditary characteristic.

5. Conclusions

In our present study, the five subtype mRNAs, α_{1a}-, α_{1b}-, α_{2A}-, β_1- and β_2-ARs, were expressed in the three major SGs of both SUS and RES groups by RT-PCR. The intensities of the mRNA expression of AR subtypes in the PG were significantly different between SUS and RES groups by competitive RT-PCR and NIH-Image analysis. It is revealed although the three major SGs of ODU

rats contain the five AR subtype mRNAs, distinct subtype-specific expression is evident between the SUS and RES groups in the PG, and also suggested that some of the AR subtypes in the PG might especially be involved in discriminating both SUS and RES groups.

Acknowledgement

This research was supported in part by a grant-in-aid from the Subsidy for Chinese Researchers of Japan-China Medical Association (JCMA).

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Table 1 Sequences, lengths and cDNA localization of oligonucleotide primers, and PCR product size of cellular mRNA and its competitor

Primer		Sequence	Length (mer)	Location[a]	Product size (bp)	
					mRNA	Competitor
a_{1a} and competitor	Forward (F)	5'-AGCCGGCTCCAGAGGATGAGACCA-3'	24	500-523[b]	282	425
	Reverse (R)	5'-GCCTCACTGAGAAGTGAGTCTTATT-3'	25	707-781		
Construction of a_{1a} competitor	F	5'-AGCCGGCTCCAGAGGATGAGACCATGTTATACAGGGAGATGAAA-3'	44			
	R	5'-GCCTCACTGAGAAGTGAGTCTTATTTTGAGTCCATGGGGAGCTTT-3'	45			
a_{1b} and competitor	F	5'-GCTCCTTCTACATCCCGCTCG-3'	21	629-649[c]	300	428
	R	5'-AGGGGAGCCAACATAAGATGA-3'	21	908-928		
Construction of a_{1b} competitor	F	5'-GCTCCTTCTACATCCCGCTCGCAAGTTTCGTGAGCTGATTG-3'	41			
	R	5'-AGGGGAGCCAACATAAGATGAATTTGATTCTGGACCATGGC-3'	41			
a_{1d}	F	5'-CGTGTGCTCCTTCTACCTACC-3'	21	759-779[d]	304	
	R	5'-GCACAGGACGAAGACACCCAC-3'	21	1042-1062		
a_{2A} and competitor	F	5'-GCGCCCCAGAACCTCTTCCTGGTG-3'	24	196-219[e]	338	484
	R	5'-CCAGCGCCCTTCTTCTCTATGGAG-3'	24	510-533		
Construction of a_{2A} competitor	F	5'-GCGCCCCAGAACCTCTTCCTGGTGCAAGTTTCGTGAGCTGATTG-3'	44			
	R	5'-CCAGCGCCCTTCTTCTCTATGGAGTTGAGTCCATGGGGAGCTTT-3'	44			
a_{2B}	F	5'-AAACGCAGCCACTGCAGAGGTCTC-3'	24	613-636[f]	456	
	R	5'-ACTGGCAACTCCCACATTCTTGCC-3'	24	1045-1068		
a_{2C}	F	5'-CTGGCAGCCGTGGTGGGTTTCCTC-3'	24	160-183[g]	426	
	R	5'-GTCGGGCCGGCGGTAGAAAGAGAC-3'	24	562-585		
β_1 and competitor	F	5'-CGCTCACCAACCTCTTCATCATGTCC-3'	26	272-297[h]	376	557
	R	5'-CAGCACTTGGGGTCGTTGTAGCAGC-3'	25	623-647		
Construction of β_1 competitor	F	5'-CGCTCACCAACCTCTTCATCATGTCCCGCAAGTGAAATCTCCTCCG-3'	46			
	R	5'-CAGCACTTGGGGTCGTTGTAGCAGCATTTGATTCTGGACCATGGC-3'	45			
β_2 and competitor	F	5'-AAGTTCGAGCGACTACAAACCGT-3'	23	178-200[i]	406	602
	R	5'-TGAAGAAGTCACAGCAAGTCTCC-3'	23	561-583		
Construction of β_2 competitor	F	5'-AAGTTCGAGCGACTACAAACCGTCGCAAGTGAAATCTCCTCCG-3'	43			
	R	5'-TGAAGAAGTCACAGCAAGTCTCCTTGAGTCCATGGGGAGCTTT-3'	43			
β_3	F	5'-ACAGACCATAACCAACGTGTTCG-3'	23	195-217[j]	481	
	R	5'-GAACACTCGAGCATAGACGAAGA-3'	23	653-675		
G3PDH	F	5'-TGAAGGTCGGTGTCAACGGATTTGGC-3'	26	5-30[k]	983	606
	R	5'-CATGTAGGCCATGAGGTCCACCAC-3'	24	964-987		
Construction of G3PDH competitor	F	5'-TGAAGGTCGGTGTCAACGGATTTGGCCGCAAGTGAAATCTCCTCCG-3'	46			
	R	5'-CATGTAGGCCATGAGGTCCACCACTTGAGTCCATGGGGAGCTTT-3'	44			

[a]Location of the 5' ends of the primers are numbered from the ATG initiation codon of the ARs and G3PDH.

[b]The sequence for rat a_{1a} (Laz et al., 1994); [c]the sequence for rat a_{1b} (Voigt et al., 1990); [d]the sequence for rat a_{1d} (Lomasney et al., 1991); [e]the sequence for rat a_{2A} (Lanier et al., 1991); [f]the sequence for rat a_{2B} (Zeng et al., 1990); [g]the sequence for rat a_{2C} (Lanier et al., 1991); [h]the sequence for rat β_1 (Machida et al., 1990); [i]the sequence for rat β_2 (Buckland et al., 1990); [j]the sequence for rat β_3 (Muzzin et al., 1991); [k]the sequence for rat G3PDH (Tso et al., 1985).

Table 2 Reagents and conditions for reverse transcription and polymerase chain reaction (PCR)

	Reverse Transcription	PCR
PCR buffer (10xbuffer:100mM Tris/500mM KCl, pH8.3)	1 x	1 x
$MgCl_2$	5 mM	4 mM
dNTP	1 mM each	0.2 mM each
RNase inhibitor (human placenta)	1 unit/μl	
random 9 mers	2.5 pmol/μl	
AMV reverse transcriptase (Avian Myeloblastosis Virus)	0.25 unit/μl	
forward primer		0.2 pmol/μl
reverse primer		0.2 pmol/μl
Taq DNA polymerase		0.025 unit/μl

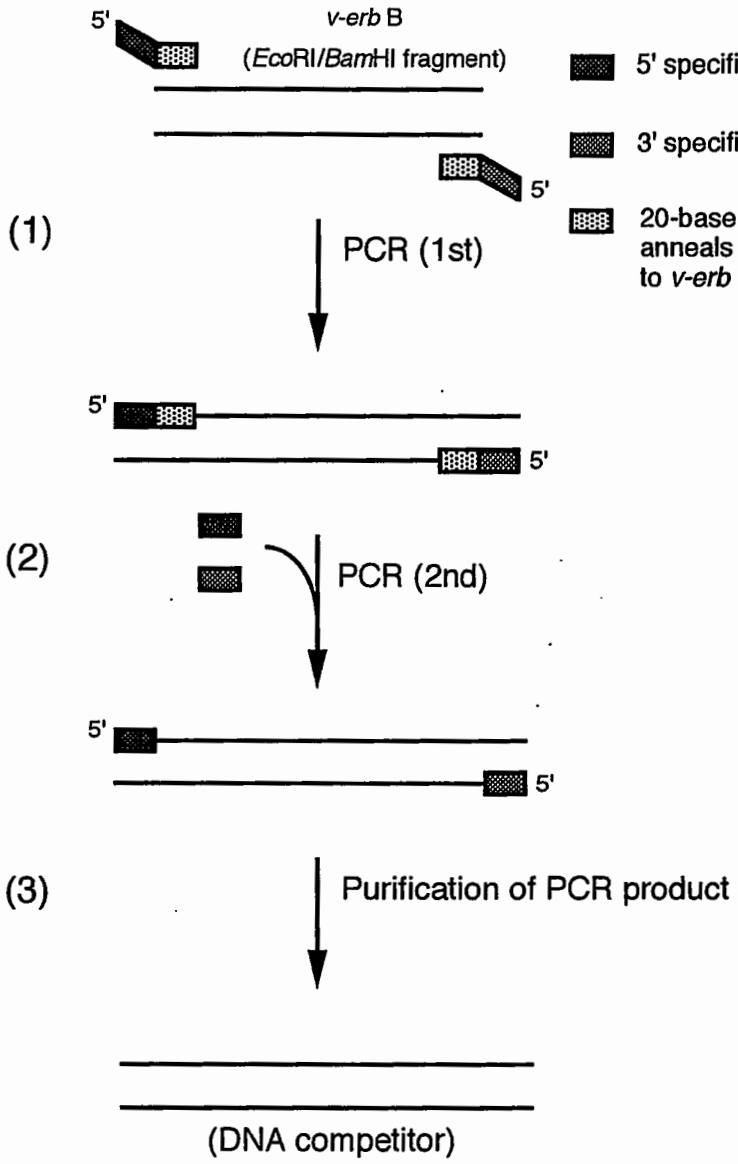

Fig. 1
Construction of DNA competitor.

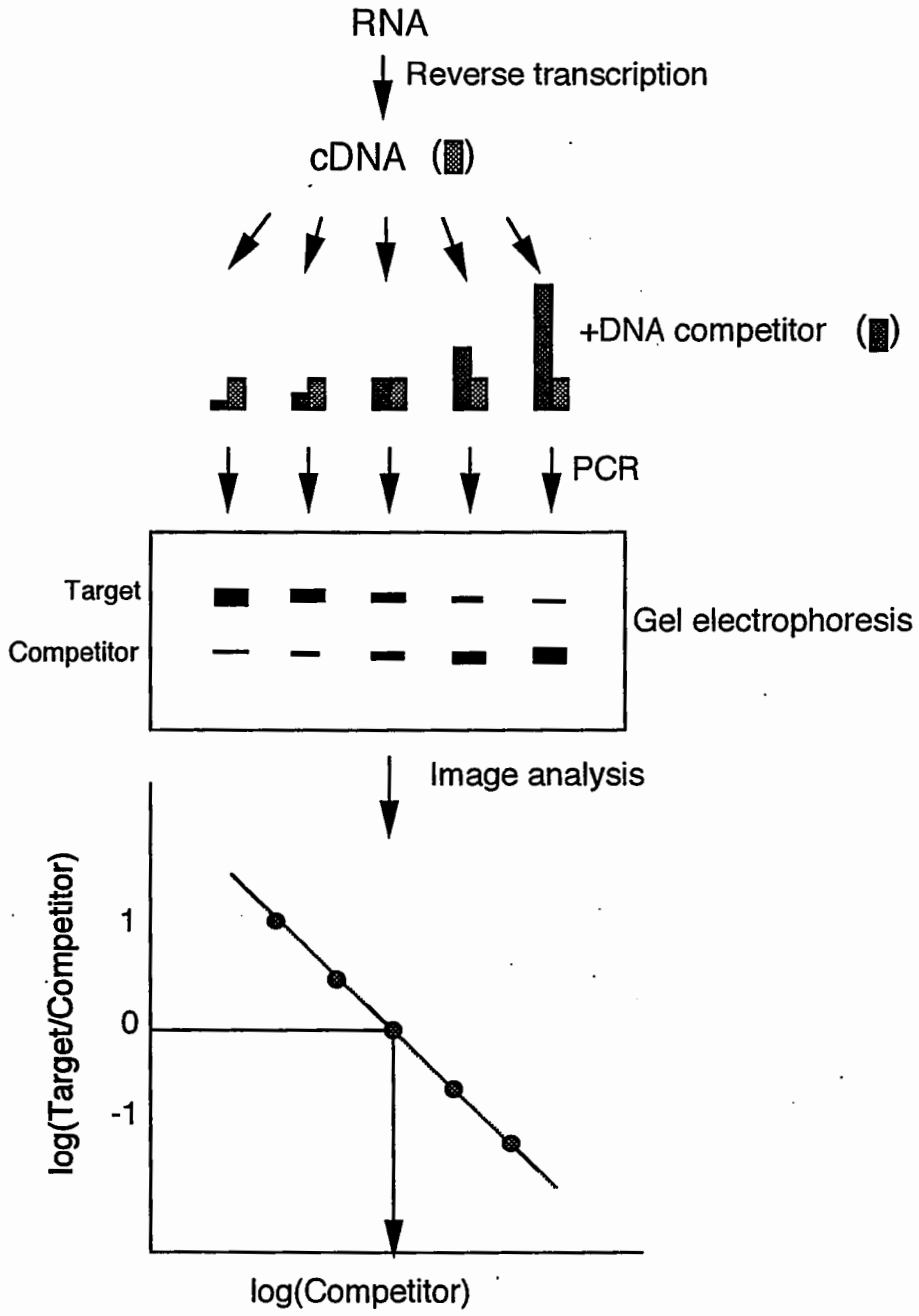

Fig. 2
Competitive RT-PCR.

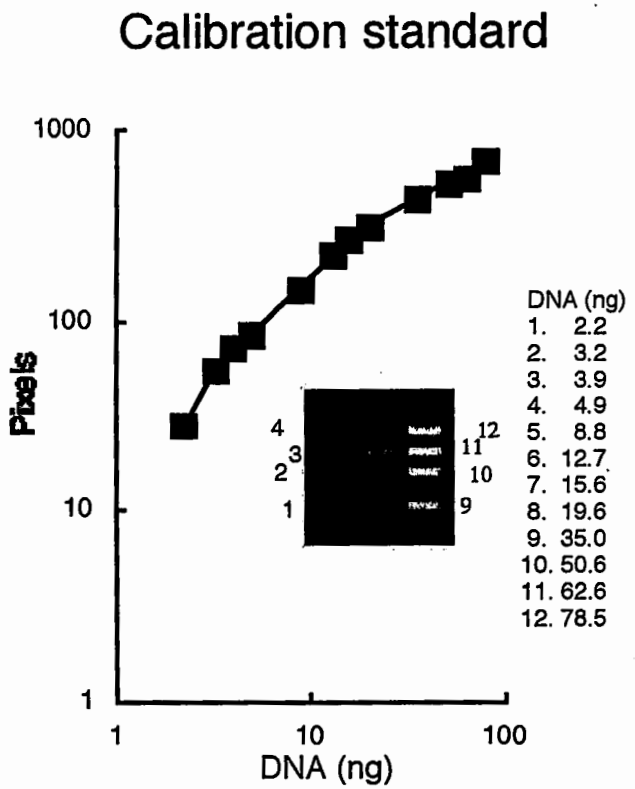

Fig. 3
Calibration standard curve. DNA size marker (*ϕX174/Hae* III digest) was used as a calibration standrad.

PG

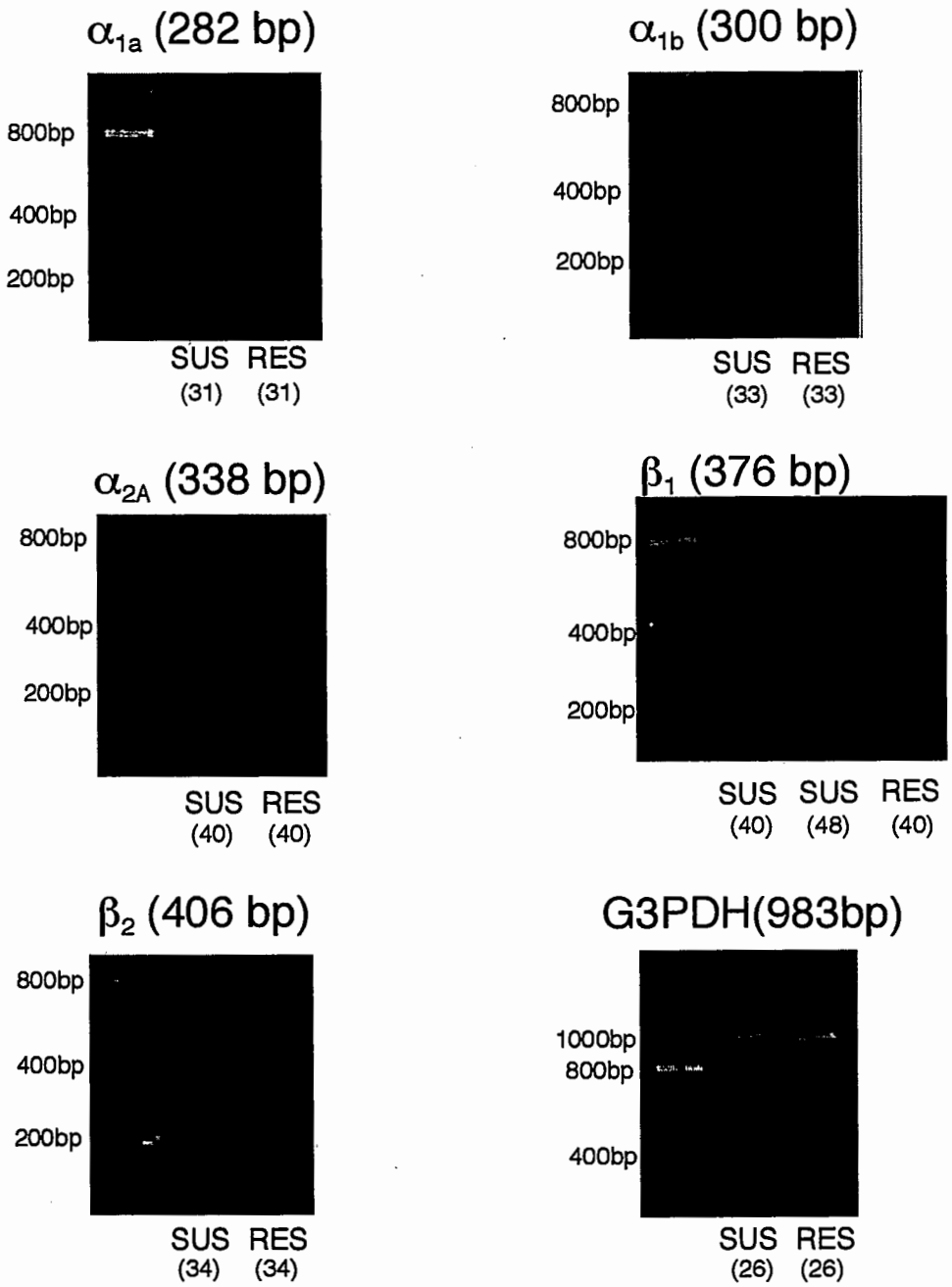

Fig. 4.1
RT-PCR analysis of the expression of α_{1a}, α_{1b}, α_{2A}, β_1 and β_2 AR subtype and G3PDH mRNAs in PGs of ODU SUS and RES rats. Cycle numbers of PCR are shown in parentheses on the bottom of the electrophoretograms.

SMG

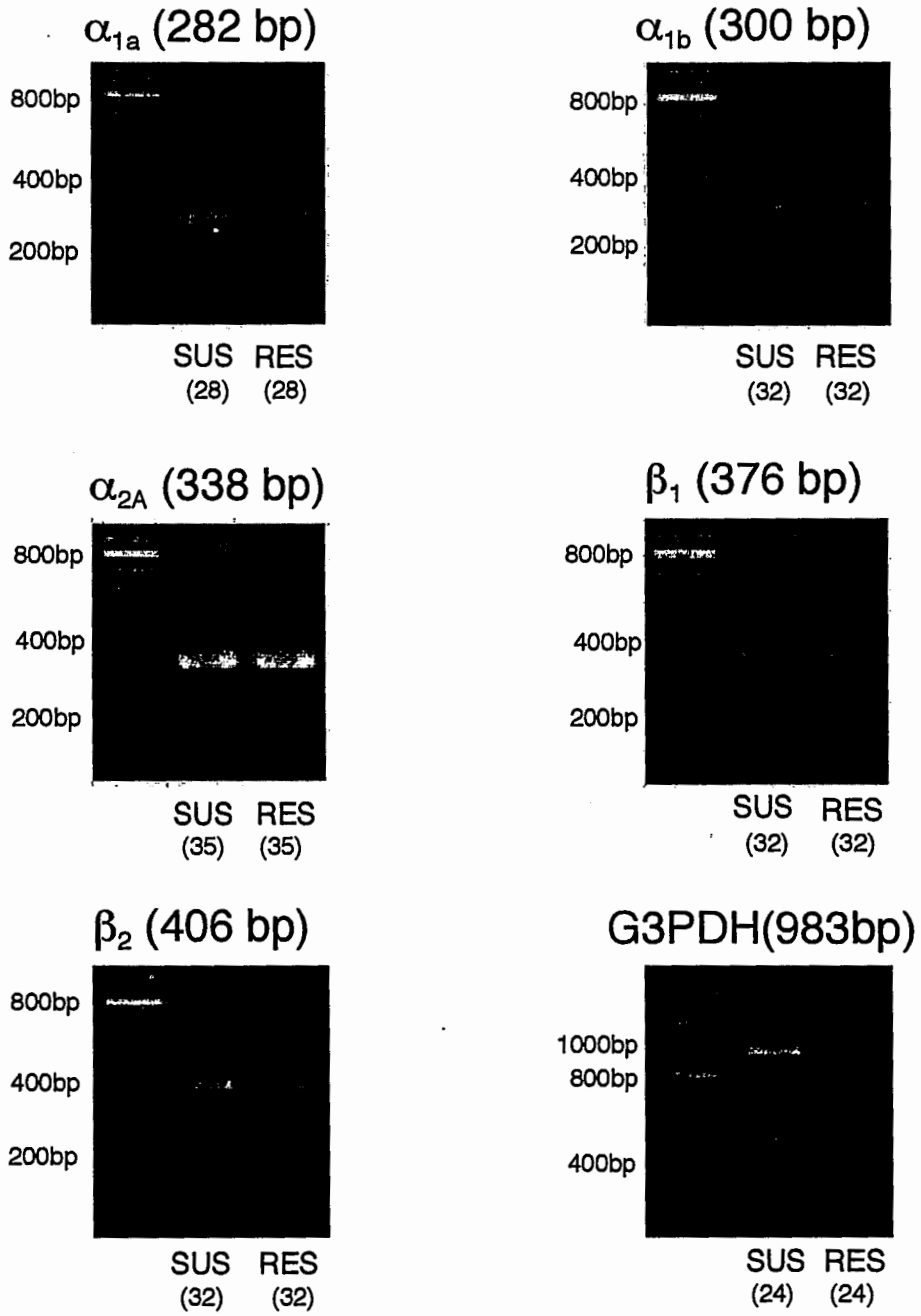

Fig. 4.2
RT-PCR analysis of the expression of α_{1a}, α_{1b}, α_{2A}, β_1 and β_2 AR subtype and G3PDH mRNAs in SMGs of ODU SUS and RES rats. Cycle numbers of PCR are shown in parentheses on the bottom of the electrophoretograms.

SLG

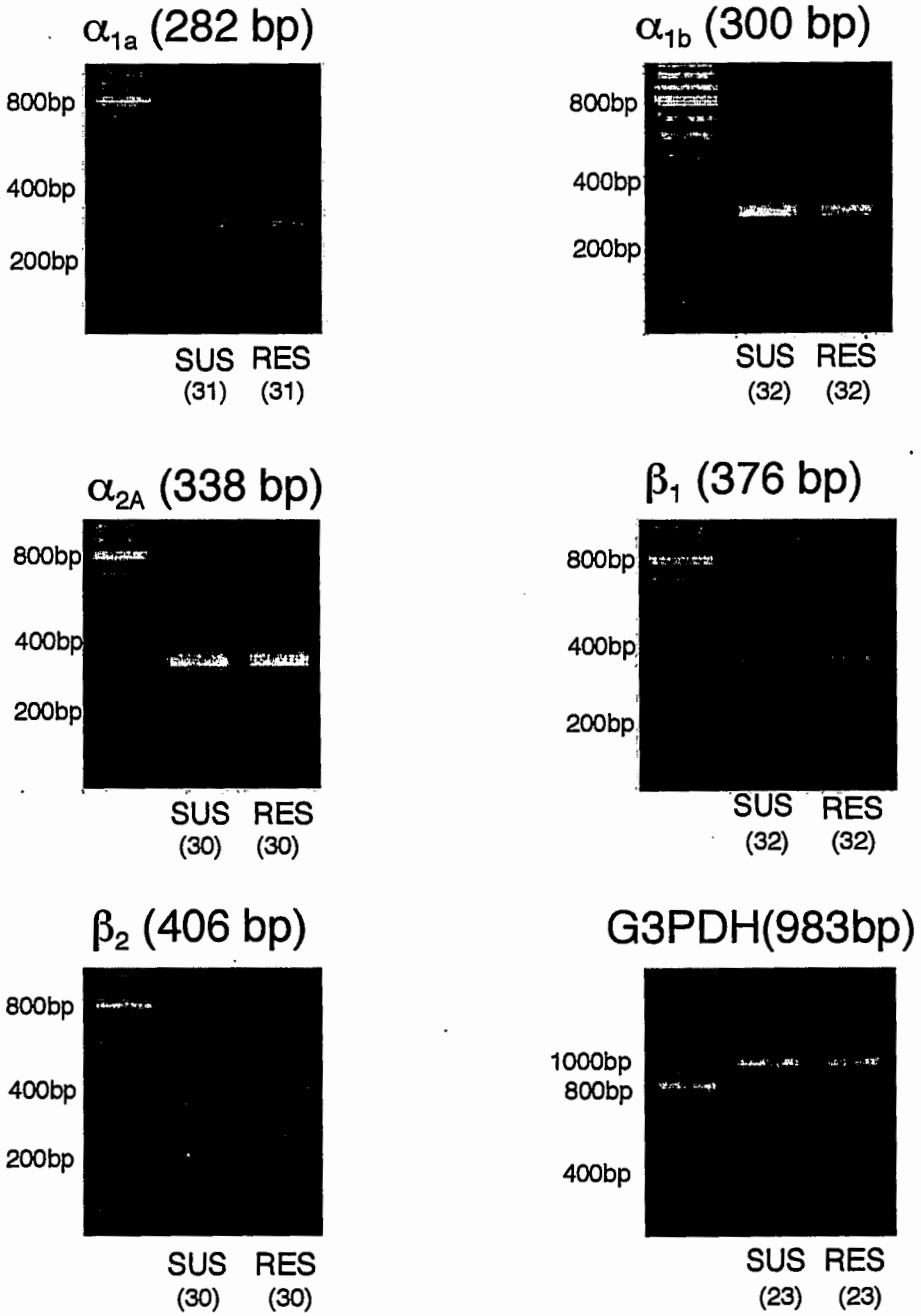

Fig. 4.3
RT-PCR analysis of the expression of α_{1a}, α_{1b}, α_{2A}, β_1 and β_2 AR subtype and G3PDH mRNAs in SLGs of ODU SUS and RES rats. Cycle numbers of PCR are shown in parentheses on the bottom of the electrophoretograms.

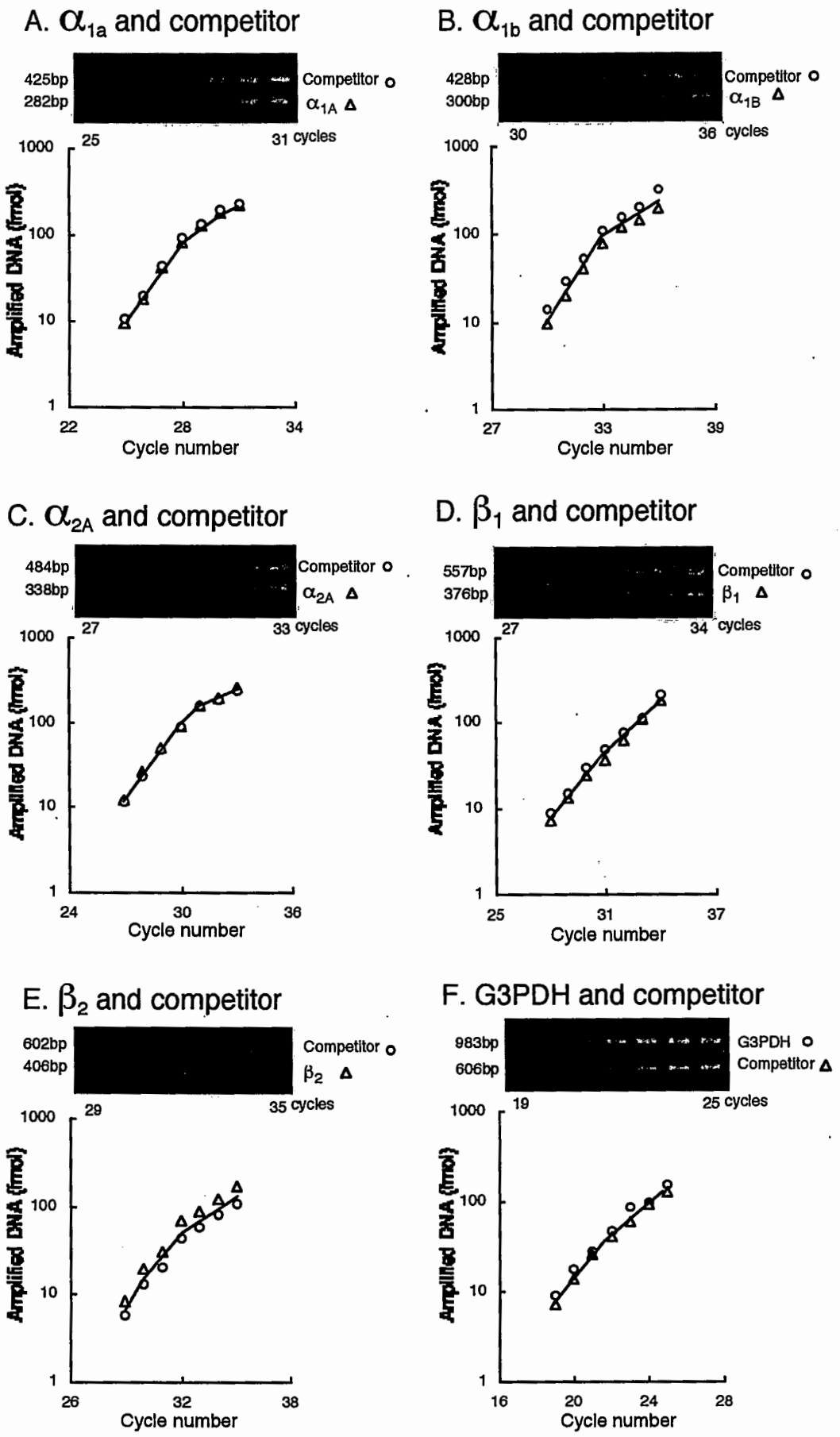

Fig. 5
Kinetics of amplification of α_{1a} (A), α_{1b} (B), α_{2A} (C), β_1 (D), β_2 (E) and G3PDH (F) cDNAs and their competitors . Approximately equal molar quantities of a target cDNA and its competitor were coamplified in a single PCR tube at various cycle numbers and separated on agarose gel. The band intensities were determined by image analysis.

PG

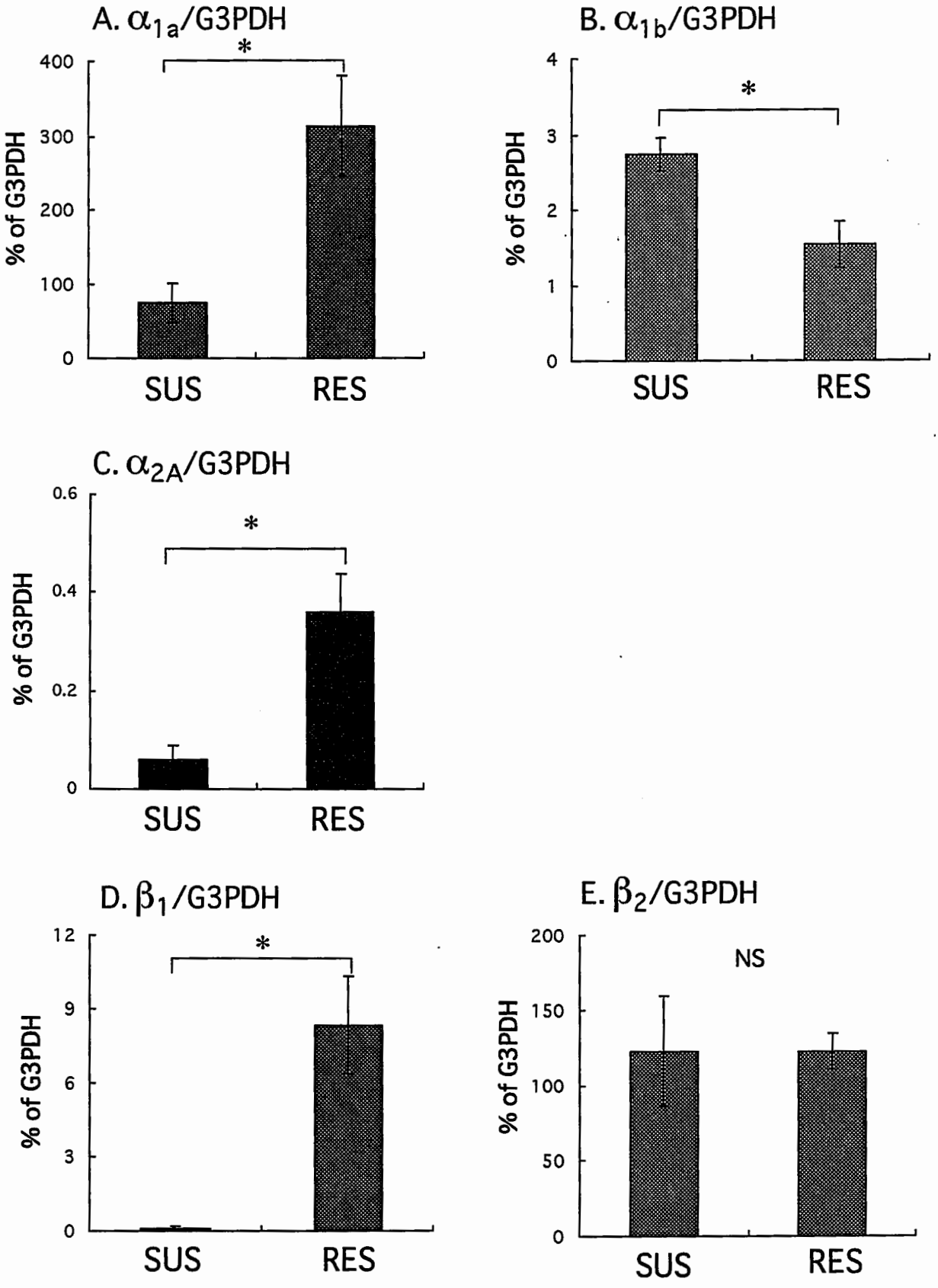

Fig. 6

Relative expression of α_{1a}, α_{1b}, α_{2A}, β_1 and β_2 AR subtype mRNAs to G3PDH in ODU rat PGs at 12 weeks of age. All values are expressed as mean±SEM percentage of transcript concentrations relative to G3PDH. * $p < 0.05$ by unpaired *t*-test between SUS and RES groups. NS, not significant.

PG

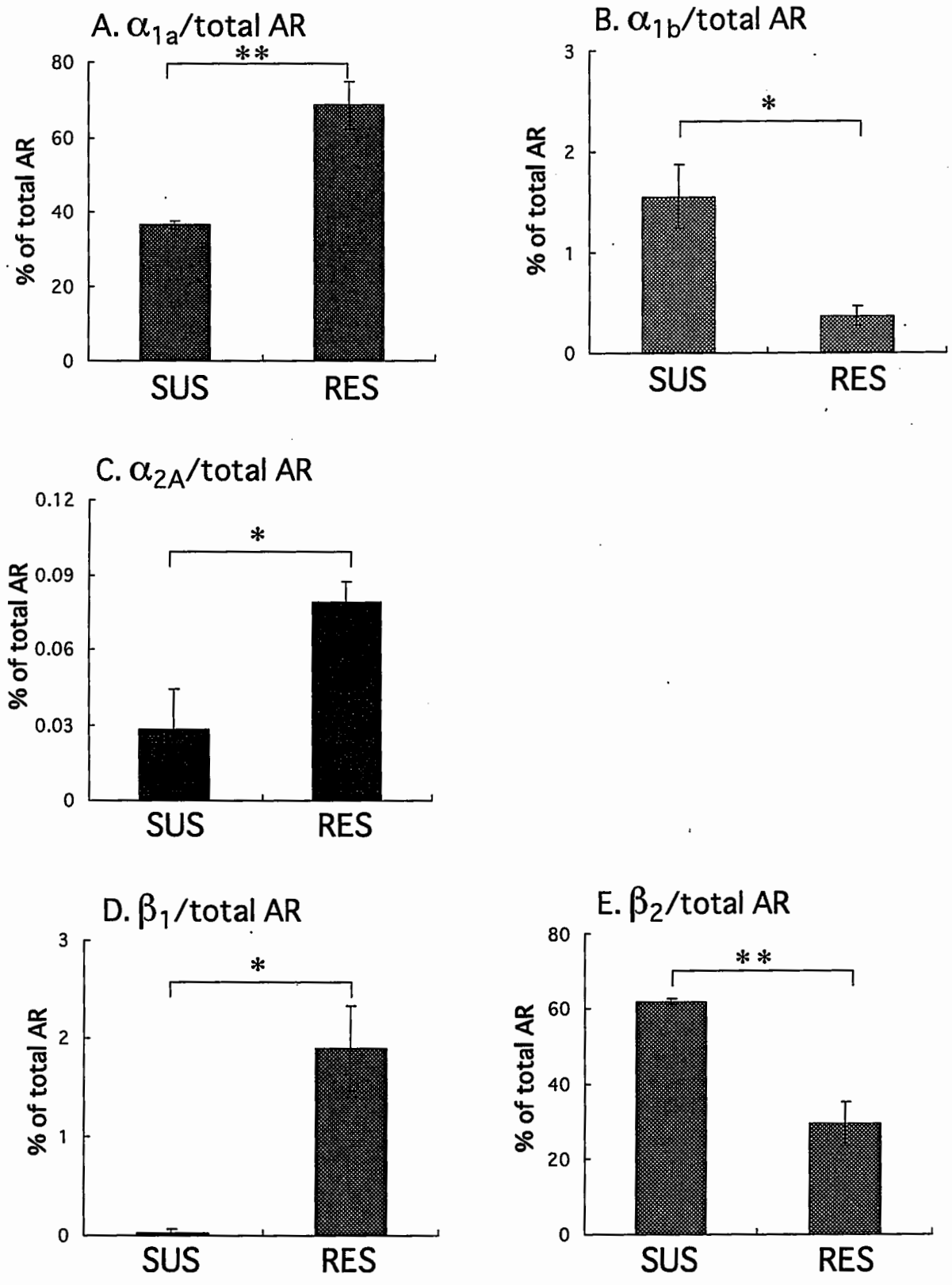

Fig. 7

Relative expression of α_{1a}, α_{1b}, α_{2A}, β_1 and β_2 AR subtype mRNAs to total AR in ODU rat PGs at 12 weeks of age. All values are expressed as mean±SEM percentage of transcript concentrations relative to total AR ($\alpha_{1a}+\alpha_{1b}+\alpha_{2A}+\beta_1+\beta_2$). * $p < 0.05$ and * * $p < 0.01$ by unpaired *t*-test between SUS and RES groups.

PG

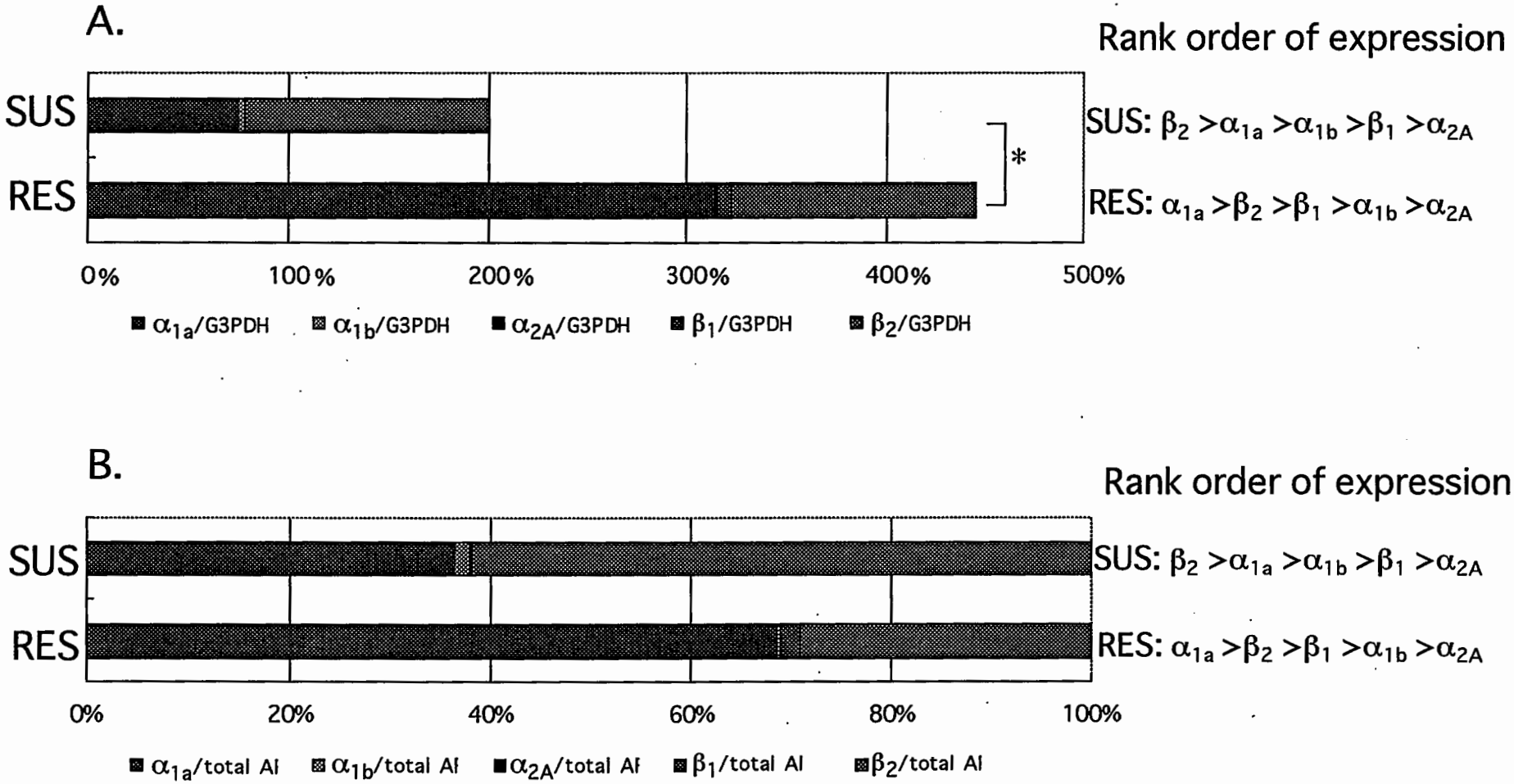

Fig. 8

Relative expression of α_{1a}, α_{1b}, α_{2A}, β_1 and β_2 AR subtype mRNAs to G3PDH (A) and total AR (B) in ODU rat PGs at 12 weeks of age and rank order of expression. All values are expressed as mean±SEM percentage of transcript concentrations relative to G3PDH and total AR ($\alpha_{1a}+\alpha_{1b}+\alpha_{2A}+\beta_1+\beta_2$). * $p<0.05$ by unpaired *t*-test between SUS and RES groups.