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Localization of mRNA for Three Distinct α_1-Adrenergic Receptor Subtypes in Human Tissues: Implications for Human α-Adrenergic Physiology

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SUMMARY

α_1-Adrenergic receptors (α_1ARs) are virtually ubiquitous in human tissues and mediate important physiological functions as diverse as smooth muscle contraction, glycogenolysis, and myocardial inotropy. At least three α_1AR subtypes ($\alpha_{1A/D}$, α_{1B}, and α_{1C}) have been described using molecular and pharmacological techniques. The identification of species heterogeneity (rat versus rabbit) in α_1AR subtype distribution has made it imperative to determine the distribution of α_1AR subtypes in human tissues. Accordingly, RNA extracted from human tissues was analyzed using RNase protection assays to determine α_1AR subtype expression. Of the cloned α_1ARs, α_{1C}AR mRNA predominates in many human tissues (heart, liver, cerebellum, and cerebral cortex), in contrast to its restricted distribution in both rats and rabbits. α_{1B}AR mRNA is present in highest concentrations in human spleen, kidney, and fetal brain. $\alpha_{1A/D}$AR mRNA is present in highest concentrations in human aorta and cerebral cortex. Hence, α_1AR subtype mRNA distribution is tissue selective and differs from that reported for rats and rabbits. These results have potentially significant implications for understanding human adrenergic physiology and are important for the rational development of α_1AR subtype-selective drugs.

ARs are G protein-coupled transmembrane receptors that bind the endogenous catecholamines epinephrine and norepinephrine. Recently, molecular cloning techniques have identified significant heterogeneity within this receptor family. A total of nine distinct receptor subtypes have now been idenified, including three subtypes of α_1ARs ($\alpha_{1A/D}$, α_{1B}, and α_{1C}), three α_2ARs (α_{2A}, α_{2B}, and α_{2C}), and three βARs (β_1, β_2, and β_3) (1). Heterogeneity of α_1ARs (α_{1A} and α_{1B}) was first suggested by pharmacological studies based on differential affinity of a variety of agents such as the agonist oxymetazoline and the antagonists WB4101 and phentolamine, differential sensitivity to the alkylating agent chloroethylclonidine, and differing requirements for extracellular calcium in signal transduction (2–4). More recently, the cloning of three distinct cDNAs encoding α_1AR subtypes ($\alpha_{1A/D}$, α_{1B}, and α_{1C}) has been reported (5–8). However, questions have been raised regarding whether the cloned $\alpha_{1A/D}$AR actually represents the true pharmacologically defined α_{1A} subtype or, rather, a fourth, previously unidentified, α_{1D}AR subtype (9). Until this issue is resolved, it has been suggested that the cloned α_{1A}AR be referred to as the $\alpha_{1A/D}$ subtype (10).

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α_1ARs have been localized to many tissues, where they have been shown to play important roles in human physiology, such as their involvement in smooth muscle regulation, myocardial inotropy and chronotropy, hepatic glucose metabolism, uterine contraction, mydriasis, salivary gland potassium and water secretion, prostatic smooth muscle tone, and perhaps growth regulation (11). Although there are only two endogenous ligands, epinephrine and norepinephrine, the physiological responses to catecholamines vary widely. One level of control that may be important in mediating different tissue responses is at the level of the receptors, where different receptor subtypes may regulate very different responses. Therefore, tissue or organ differences in receptor subtype expression may represent an important factor in deterermining AR physiology.

The distribution of α_1AR subtypes has been extensively characterized in rat and rabbit tissues using Northern blot and radioligand binding techniques; however, very little is known regarding the distribution of receptor subtypes in human tissues. Recently, species heterogeneity in the tissue distribution of α_1AR subtypes has been identified (12, 13). Such species heterogeneity in expression of α_1AR subtypes makes it imperative to analyze the localization of α_1AR subtypes in human tissues.

Because neither α_1AR subtype-selective ligands nor specific

ABBREVIATIONS: AR, adrenergic receptor; kb, kilobase(s); PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

antibodies are currently available for investigations of the tissue distribution of α_1AR subtypes, molecular techniques have been used to characterize the tissue distribution of receptor subtypes for a variety of receptors (14). In this study, the ribonuclease protection assay was used to localize mRNA encoding each of the cloned α_1AR subtypes in various human tissues. These results demonstrate, for the first time, that human α_1AR subtype mRNA tissue distribution is distinct from that in other mammalian models such as rats and rabbits. In addition, within the context of cloned α_1ARs, α_{1C}AR mRNA is the predominant subtype expressed in many human tissues.

Materials and Methods

Human tissue preparation and RNA isolation. Human tissue was obtained from the following sources, with appropriate institutional approval: from rapid autopsy (within 1–3 hr of death), from organ procurement procedures (within 30 min of death), or as discarded tissues during surgery. Tissues were snap frozen in liquid nitrogen and stored at −70°. Total RNA was extracted from tissue samples using guanidine isothiocyanate followed by centrifugation through a cesium chloride gradient, as described previously by Chirgwin *et al.* (15). Each RNA sample was quantitated spectrophotometrically, aliquoted into 20-μg samples, and stored as ethanol precipitates at −70° for later use. Additional samples of RNA isolated from human tissues were purchased from Clonetech (Palo Alto, CA). RNA integrity was confirmed by denaturing (formaldehyde) agarose gel electrophoresis or by hybridization to a β-actin probe in a representative number of samples.

Human α_1AR cDNA constructs and generation of RNA probes. Fragments of human α_1AR cDNAs were used as specific probes in RNase protection assays. The human $\alpha_{1A/D}$AR cDNA construct consists of a 0.375-kb (*Eco*RI/*Stu*I) fragment in pSP72 (Promega), initially obtained from Dr. Geoff Johnston, Pfizer Central Research (Sandwich, England), that is identical to the corresponding fragment (nucleotides 449–824) of the previously reported cloned human α_{1A}AR cDNA (16). The human α_{1B}AR construct consists of a 0.670-kb (*Bam*HI/*Xho*I) fragment in pGEM-4Z (Promega) that was obtained originally from a human leukocyte genomic library (Clontech) and is identical to the recently cloned human α_{1B}AR cDNA (nucleotides 91–761) (5). The human α_{1C}AR construct consists of a 0.323-kb (*Hind*III/*Pvu*II) insert in pGEM-4Z (Promega) that was initially cloned from a human leukocyte genomic library (Clontech) (6) and is identical to the recently cloned human α_{1C}AR (nucleotides 1392–1715) (7). Control β-actin cDNA was constructed using a 0.206-kb fragment, corresponding to nucleotides 1858–2064 (17), inserted into pSP73 (Promega). Linearized cDNA constructs were used to synthesize antisense radiolabeled RNA probes with high specific activity using either T7 or Sp6 RNA polymerase and [^{32}P]αCTP (New England Nuclear-DuPont), as described in the *Promega Protocols and Applications Manual* (18).

RNase protection assay and relative quantitation of mRNA. Hybridization of RNA probes to total cellular human RNA was performed as described previously by Zinn *et al.*, with the following modifications (19). In brief, ethanol precipitates of RNA samples (20 μg) were dried in a SpeedVac centrifuge, resuspended in 30 μl of hybridization solution (80% formamide, 40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA) containing an excess of radiolabeled probe (3×10^5 cpm), denatured at 85° for 10 min, and incubated for >12 hr at either 55° (α_{1B}AR, α_{1C}AR, and β-actin probes) or 65° ($\alpha_{1A/D}$AR probes). At the end of this hybridization procedure, 350 μl of RNase buffer (10 mM Tris·HCl, pH 7.5, 5 mM EDTA, 300 mM NaCl, containing 50 μg/ml RNase A and 700 units/ml RNase T1) were added to digest unprotected RNA; samples were incubated for 1.5 hr at 30°. To each sample were then added 50 μg of proteinase K and 10 μl of 20% sodium dodecyl sulfate, followed by vortexing, incubation at 37° for 30 min, phenol/chloroform extraction, and ethanol precipitation with carrier tRNA. After resuspension in 4 μl of loading buffer (80% formamide, 0.1% bromphenol blue, 0.1% xylene cyanol), the samples were separated by electrophoresis on a denaturing 8 M urea/6% acrylamide gel, followed by drying and exposure to XAR-5 (Kodak) film for varying times (24 hr to 2 weeks) at −70°. Autoradiographs were scanned using a laser densitometer (Bio Med Instruments, Fullerton, CA) to quantitate the relative abundance of each of the α_1AR subtype mRNAs in each tissue.

Results and Discussion

Total RNA extracted from a series of human tissues was analyzed by the RNase protection assay, using antisense radiolabeled RNA probes specific for each α_1AR subtype mRNA. The specificity of the RNA probes was verified by hybridization to *in vitro* transcribed sense RNA for each of the three cDNA constructs, and no cross-hybridization was detected (data not shown); furthermore, experiments with human tissues demonstrated a unique tissue distribution for each of the subtypes, thus confirming the specificity of the individual probes. Figs. 1 and 2 are representative autoradiographs (24-hr exposure), demonstrating the distribution of each α_1AR subtype mRNA in various human tissues. As seen in Fig. 1, $\alpha_{1A/D}$AR mRNA is present most abundantly in the cerebral cortex, followed in lower concentrations by spleen, fetal brain, kidney, pancreas, adrenal, and heart. α_{1B}AR mRNA is present most abundantly in human spleen, kidney, and fetal brain, whereas other tissues with lower expression of α_{1B}AR mRNA include human cerebellum, liver, heart, retina, cerebral cortex, and adrenal. Human tissues containing the highest level of α_{1C}AR mRNA include the liver, cerebellum, heart, cerebral cortex, and spleen. Fig. 2 demonstrates the expression of α_1AR mRNA in vascular tissue (aorta) from two patients, which identifies the predominant α_1AR subtype expressed in aorta as the $\alpha_{1A/D}$. These results, along with data collected from other tissues, are summarized in Table 1. From Table 1 it can be appreciated that there is a predominant α_1AR subtype mRNA expressed in many human tissues, such as aorta ($\alpha_{1A/D}$), kidney (α_{1B}), and liver (α_{1C}).

As also seen in Fig. 1, both $\alpha_{1A/D}$ and α_{1B} probes protect a single band of the predicted size ($\alpha_{1A/D}$, 375 nucleotides; α_{1B}, 670 nucleotides), whereas the α_{1C} probe protects three similarly sized bands that are very close to the predicted size (323 nucleotides). Multiple bands could represent either partial digestion of the end of the RNA-RNA hybrid or could simply be due to protection of three nearly identical but separate fragments due to variation in probe length brought about by premature termination of the *in vitro* transcription process. Nevertheless, the protected fragments are specific and are of the appropriate molecular weight.

Functional and pharmacological assays have been used to map the distribution of receptor subtypes; however, for α_1AR subtypes the currently available ligands are only partially selective, leading to confusion regarding tissue expression of these receptors (20). Hence, molecular biological techniques have been relied upon to investigate tissue distribution of receptor subtypes in rats, rabbits, and humans (14, 21–23). Although initial Northern blots failed to localize the α_{1C}AR mRNA to any rat tissue examined, *in situ* hybridization techniques did reveal mRNA for this subtype expressed in localized regions of human brain. However, we have recently reported α_{1C}AR mRNA as the predominant subtype expressed in human prostate (24), and our current study demonstrates α_{1C}AR mRNA expression in many human tissues, which is very different from results reported previously for other species. The functional

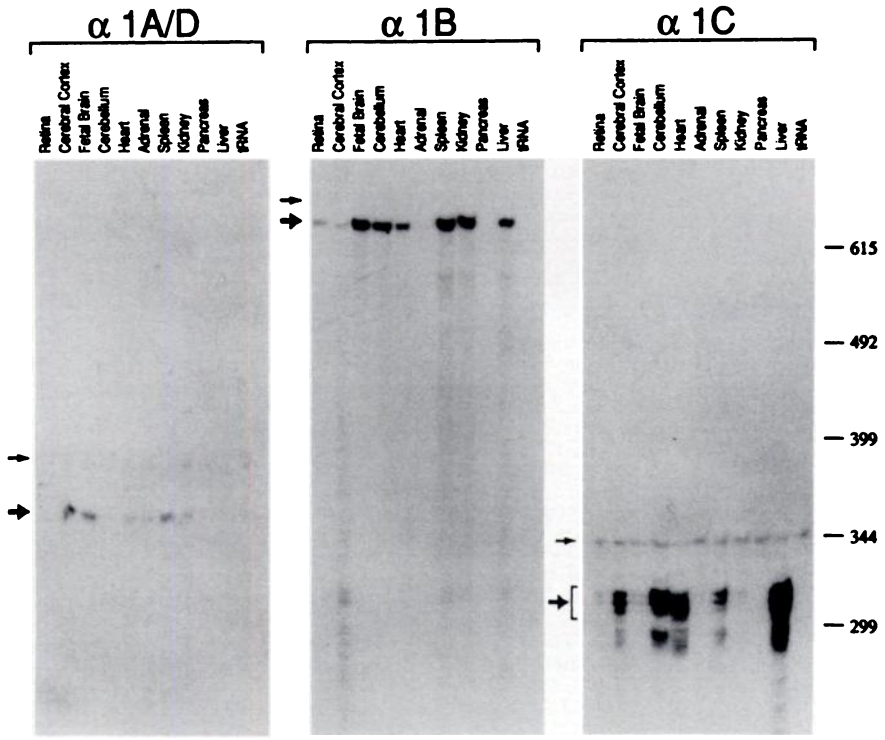

Fig. 1. Representative RNase protection assay of several human tissues. This is a 24-hr autoradiograph that demonstrates the results of a representative RNase protection assay in which 20 μg of total RNA, extracted from several human tissues, were hybridized with each of the radiolabeled antisense RNA probes ($\alpha_{1A/D}$, α_{1B}, and α_{1C}). *Small arrow*, undigested probe; *larger arrow*, specific band(s) of interest. tRNA serves as a negative control. Molecular weight markers are shown on the *right*. Undigested probe (represented by the upper band in each lane) migrates at a higher molecular weight than the protected fragment because it contains a plasmid polylinker region that is not protected from RNase digestion. See text for details.

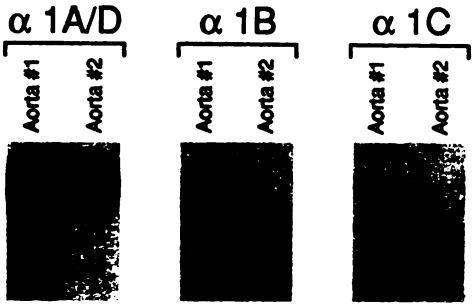

Fig. 2. Representative RNase protection assay of RNA extracted from the aorta. This is a 24-hr autoradiograph that demonstrates the expression level of all three α_1AR mRNAs in 20 μg of total RNA extracted from the aorta of two different patients.

TABLE 1

Relative quantitation and distribution of human α_{1AR} mRNA

Each *n* value represents the number of patients, i.e., *n* = 3 means that tissues were obtained from three different patients. Even in the case of *n* = 1 patient, experiments were performed twice. Quantitation ranges from a minimum of – (not present) to a maximum of ++++. +/– means that on long exposures a weak signal was observed. These values were derived from autoradiographs of protected fragments scanned with a laser densitometer and then normalized for both probe size and maximum possible CTP incorporation (see Materials and Methods).

Tissue	*n*	$\alpha_{1A/D}$	α_{1B}	α_{1C}
Adrenal	3	+	+/–	+/–
Liver	3	+/–	+	++++
Heart	5	+	+	+++
Spleen	3	+	++	+
Kidney	3	+/–	++	+/–
Aorta	3	++	+	+/–
Inferior vena cava	3	+/–	+/–	+
Testis	3	+	+/–	–
Pancreas	3	+/–	+/–	–
Cerebellum	2	+/–	++	+++
Cerebral cortex	3	++	+	+++
Fetal brain	1	+	++	+/–
Retina	1	–	+	+/–

significance of species heterogeneity in α_1AR distribution is not known, but similiar species heterogeneity with regard to α_2AR mRNA expression has recently been identified (22).[1] In support of our findings, cDNAs encoding the human α_{1B}AR and α_{1C}AR have recently been cloned and their expression, as determined by both Northern blot analysis and reverse transcription-polymerase chain reaction techniques, corroborate our findings (5, 7).

Delineation of α_1AR subtype distribution in human tissues, first at the mRNA level but ultimately at the receptor protein level, should lead to a better understanding of the role α_1ARs play in the control of many important physiological processes. Because many human diseases involve imbalances in AR physiology, understanding α_1AR subtype distribution in human tissues may prove important for designing and targeting effective therapeutic modalities for these diseases while minimizing side effects. For example, in the male genitourinary system α_1ARs have been implicated in the development of urinary outflow obstruction by controlling prostatic smooth muscle tone (25). Whereas prostatic hypertrophy has classically been treated surgically, new approaches aimed at relieving bladder outlet obstruction include the use of α_1AR antagonists to relax prostatic smooth muscle tone (26). One problem with this form of therapy is that patients frequently develop side effects (such as postural hypotension), owing to the nonselective nature of currently available α_1AR drugs. We recently reported identification of the α_{1C}AR as the predominant α_1AR mRNA in prostate (24), and recent functional studies by Smith *et al.* (27) have confirmed these findings using new, highly selective, α_1AR subtype-specific ligands. In combination, these data suggest that selective α_{1C}AR antagonists might be useful in the treat-

[1] D. E. Berkowitz, D. T. Price, S. O. Page, and D. A. Schwinn. Localization of mRNA for three distinct α_2-adrenergic recptor subtypes in human tissues: evidence for species heterogeneity and implications for human pharmacology. Submitted for publication.

ment of bladder outlet obstruction associated with benign prostatic hyperplasia. Other urological conditions, such as impotence, priapism, and incontinence, also involve α_1ARs; therefore, α_1AR-selective drugs (either agonists or antagonists) may prove useful in the treatment of these conditions.

More recently, α_1ARs have been localized to cardiac ventricular muscle, where the density of receptors has been shown to be up-regulated during periods of myocardial ischemia (28). Furthermore, these receptors have been implicated as potentially important in the development of ventricular arrhythmias (29). Animal studies demonstrate efficacy of α_1AR antagonists in blocking the development of ventricular arrhythmias during myocardial ischemia (30), suggesting that α_1AR antagonists may have some utility in the treatment of ischemia-induced ventricular arrhythmias in humans. We identify in this study that the predominant α_1AR mRNA in human cardiac ventricular muscle is the α_{1C}AR. Hence, if these results remain consistent at the protein level, then α_{1C}AR-selective antagonists may have utility in the treatment of ischemia-induced arryhthmias.

α_1ARs play a critical role in vascular smooth muscle in maintaining and regulating vascular tone, and hence blood pressure. In the setting of hypotension, α_1AR agonists are commonly used clinically to increase systemic vascular resistance and hence blood pressure via contraction of arteries and arterioles, either as definitive therapy or until fluids or inotropic agents can be applied. Likewise, α_1AR antagonists such as prazosin are sometimes used in the treatment of hypertension. Thus, targeting specific α_1AR subtypes in vascular smooth muscle may have particular utility in the treatment of both hypotension and hypertension. More intense investigation of the distribution of α_1AR subtypes in various vascular tissues may identify further diversity in α_1AR subtype expression, possibly enabling the use of very selective agonists and antagonists for specific vascular beds.

It is important to note that the level of receptor mRNA in a given tissue may not correlate directly with the levels of receptor protein. Hence, in the future α_1AR subtype-selective ligands and antibodies will be necessary to confirm these results. Ultimately, data on both mRNA and receptor protein concentrations will be required to understand mechanisms of how ARs are involved in human pathology. In addition to determining the predominant α_1AR subtype present in a given human tissue, it is also important to remember that each human organ is composed of a heterogeneous population of both specialized and nonspecialized cells, which could express either a mixture or a unique population of α_1AR subtypes. Thus, *in situ* hybridization, receptor autoradiography, and immunohistochemical techniques using α_1AR subtype-selective probes may further identify localized areas of high expression of one particular subtype that may not have been detected in this study because of relatively low abundance. Furthermore, the cloning and expression of the genes that code for the human subtypes may identify important regulatory elements that may also be targeted for therapuetic purposes.

In summary, using RNase protection techniques this study establishes for the first time the distribution of α_1AR subtype mRNA in human tissues. α_1AR subtype mRNA distribution is tissue selective and is very different than previously reported for other species. Of the cloned α_1ARs, α_{1C}AR mRNA predominates in many human tissues (as opposed to a restricted distribution in other species). These results have important implications for understanding human AR physiology and support the need for α_1AR subtype-selective drugs.

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