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The antagonistic effect of antipsychotic drugs on a HEK293 cell line stably expressing human α_{1A1}-adrenoceptors

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ABSTRACT

Antipsychotic drugs often cause orthostatic hypotension, probably through antagonist action on resistance vessel α_{1A}-adrenoceptors. Here we have tested this possibility directly using cells transfected with a relevant human α_{1A}-adrenoceptor splice variant. To determine a splice variant which was relevant, we used quantitative real-time polymerase chain reaction (qPCR) to determine the prevalence in human subcutaneous small arteries of three of the five splice variants ADRA1A_v1-5, which encode functional protein: α_{1A1}-, α_{1A3}-, α_{1A4}-adrenoceptors. Our statistical analysis showed higher transcription levels of α_{1A1}- than of α_{1A3}- and α_{1A4}-adrenoceptors (1.6 and 5.8 times, respectively). We therefore chose to study the α_{1A1}-adrenoceptor, and the cDNA encoding it was transfected into the Flp-In-293 (modified from HEK-293) cell line to produce a cell line stably expressing a functional form of this splice variant. The expression of recombinant α_{1A1}-adrenoceptor subtype was confirmed by Western immunoblot analysis, and its functionality demonstrated using a Fura-2 assay by a rise in intracellular calcium concentration ($[Ca^{2+}]_i$) when challenged with phenylephrine ($EC_{50} = 1.61 \times 10^{-8}$ M). From Schild analysis, prazosin, sertindole, risperidone, and haloperidol caused a concentration-dependent, rightward shift of the cumulative concentration–response curves for phenylephrine in cells expressing human recombinant α_{1A1}-adrenoceptors to yield pK_B values of 8.40, 8.05, 8.26 and 7.38, respectively. In [7-methoxy-^{3}H]-prazosin binding experiments, high expression was seen ($B_{max} = 48.5 \pm 16.7$ pmol/mg protein, ±S.E.M.) along with high affinity binding to a single site ($K_d = 0.210 \pm 0.034$ nM). The pharmacological profiles of recombinant human α_{1A1}-adrenoceptors in competition binding studies confirmed much higher antagonist affinity of sertindole and risperidone than haloperidol for these receptors. In summary, it can be concluded that there is an approximately 10-fold higher adrenoceptor affinity of risperidone and sertindole for human α_{1A1}-adrenoceptors compared to haloperidol. These findings are consistent with the observation that risperidone and sertindole have a higher incidence of orthostatic hypotension than haloperidol.

1. Introduction

Antipsychotic drugs have been identified as a group of neuroleptic agents effective against symptoms of schizophrenia, a common psychotic disease (Nasif et al., 2000). Extrapyramidal side effects (EPS) in patients under treatment with antipsychotic drugs are mediated via striatal dopamine D_2 receptor blockade (Lidow et al., 1998). Nevertheless, almost universally, these drugs cause cardiovascular side effects such as orthostatic hypotension (Buckley and Sanders, 2000) which may be caused by their antagonist action against α_1-adrenoceptors. Several studies have focused on the importance of the relationship between α_1-adrenoceptors and antipsychotic drugs (Lane et al., 1988; Prinssen et al., 1994; Wadenberg et al., 2000). However, the role of α_1-adrenoceptors with respect to their cardiovascular side effects, especially orthostatic hypotension, has received little attention. Current evidence is based on clinical experiments (Mueck-Weymann et al., 2001; Sramek et al., 1997) or experiments using isolated vascular tissue (Ipsen et al., 1997; Takata et al., 1999; Jarajapu et al., 2001), or animal *in vivo* experiments (Cahir et al., 2004; Goudie et al., 2004; Nasif et al., 2000). Due to the multiplicity of α_{1A}-adrenoceptor splice variants (Hawrylyshyn et al.,

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2004), the interpretation of such experiments can be equivocal. Therefore, we decided to investigate which α_{1A}-adrenoceptor splice variant was most prevalent in human resistance vessels, and then to develop a cell line expressing a functional form of this receptor.

α_{1A}-Adrenoceptors are members of the G-protein-coupled receptor family which mediate various actions of the peripheral sympathetic nervous system through the binding of catecholamines, particularly in the cardiovascular system where they are responsible for regulating vascular tone and peripheral resistance (Chen et al., 2006; Michelotti et al., 2000). Molecular cloning has identified three subtypes of the α_1-adrenoceptor family in several species including humans (Hirasawa et al., 1993; Schwinn et al., 1995; Weinberg et al., 1994), and these subtypes have been classified α_{1A}- (previously α_{1C}), α_{1B}- and α_{1D}-adrenoceptors (Hieble et al., 1995; Michelotti et al., 2000). While most of the genes encoding G-protein-coupled receptors are intronless, those encoding the three α_1-adrenoceptors are interrupted by a single intron (Graham et al., 1996). The gene encoding α_{1A}-adrenoceptor (denoted ADRA1A) located on human chromosome 8 (Hawrylyshyn et al., 2004; Schwinn et al., 1990) consists of two exons separated by a large intron. Exon 1 contains coding sequence from the N-terminus through the extracellular portion of transmembrane domain six (TM6), whereas exon 2 encodes the third extracellular loop, transmembrane domain seven (TM7) and all of the C-terminus (Kilpatrick et al., 1999). To date, 16 splice variants transcribed from the α_{1A}-adrenoceptor gene have been reported. Five of them, denoted ADRA1A_v1-5, encode complete and functional seven transmembrane α_{1A}-adrenoceptor isoforms. Other isoforms of α_{1A}-adrenoceptors are truncated and do not possess all seven intact transmembrane domains (Hawrylyshyn et al., 2004). The complete and functional α_{1A}-adrenoceptor isoforms differ from each other only in their C-terminal ends and display similar pharmacology in terms of ligand binding and noradrenaline-induced intracellular calcium concentration ($[Ca^{2+}]_i$) responses (Chang et al., 1998; Coge et al., 1999; Hirasawa et al., 1995).

In the present study, human resistance vessels were taken from the subcutis, and within these the α_{1A1}-adrenoceptor was found to be the most prevalent of the splice variants investigated at the transcriptional level and the expression of native protein was confirmed by Western immunoblot analysis. This splice variant was then stably transfected into Flp-In-293 (modified from HEK-293) cells, where it was found to mediate a rise in $[Ca^{2+}]_i$ in response to challenge with phenylephrine. The ability of antipsychotic drugs to antagonize these responses was then investigated along with α_{1A1}-adrenoceptor competition binding studies using [7-methoxy-^{3}H]-prazosin. The drugs used were haloperidol (the most commonly used typical antipsychotic drug), as well as risperidone and sertindole (as atypical antipsychotic drugs). The antagonist potency of prazosin as a non-selective α_1-adrenoceptor antagonist was also determined as a positive control. Our hypothesis was that the affinity of the antipsychotic drugs to the transfected α_{1A1}-adrenoceptors would correlate to the clinically observed prevalence of orthostatic hypotension when treated with these drugs.

2. Materials and methods

2.1. Quantitative real-time polymerase chain reaction of human subcutaneous small arteries

Human subcutaneous small arteries were isolated from biopsies of samples of subcutaneous fat tissues from six subjects who were undergoing plastic surgery operations. All subjects gave their written consent, and the investigation was approved by the Local Ethics Committee. Samples were collected in cold L-15 medium and transported to the laboratory under ice-cold conditions. Subcutaneous small arteries were dissected out under a microscope within an hour. The dissected vessels were kept in RNAlater-ICE (Ambion, TX, USA) solution at 4 °C and RNA purification was done immediately following dissection.

To construct cDNA, total RNA was extracted from the vessels using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA samples were converted to randomly primed first strand cDNA using the Superscript III First-strand Synthesis System (Invitrogen, CA, USA) for reverse transcriptase polymerase chain reaction (RT-PCR). The resulting cDNA solutions were stored at −20 °C. The expression of different isoforms of α_{1A}-adrenoceptors was determined by quantitative real-time polymerase chain reaction (qPCR) using the iCycler iQ Detection System (Bio-Rad, CA, USA). The PCR master mix contained a hot start version of a modified *Thermus brockianus* DNA polymerase, SYBR Green I dye, optimized PCR buffer including 5 mM $MgCl_2$, and dNTP mix dUTP from DyNAmo™ HS SYBR® Green qPCR Kit (FINNZYMES, Finland). Oligonucleotide primers were constructed based on annotated sequences from the Entrez nucleotide database for α_{1A1} (NM 000680), α_{1A3} (NM 033302) and α_{1A4} (NM 033304). Sequences of the forward and reverse primers were as follows: For α_{1A1}-AR: 5′-CAG CCT TGA CAA GAA CCA TCA AGT TC-3′ (position 1759–1784) and 5′-GCT CCA AAC TTA GAG TGT GTG CTC A-3′ (position 2210–2186). For α_{1A3}-AR: 5′-CAC ACC CAT GAC ATG AAG CCA GCT-3′ (position 1711–1734), and 5′-CCC TAC ACG TGG CTG ATG ATT CTC A-3′ (position 2083–2059). For α_{1A4}-AR: 5′-GAA TGG ATT GTA GAT ATT TCA CCA AGA-3′ (position 1711–1736), and 5′-TGC CTG CCC AAC CAA ATA GTT CCT-3′ (position 1989–1966). The primers for β-actin (NM 001101) that was used as reference gene were: 5′-GCC TGA CGG CCA GGT CAT CAC CAT-3′ (position 799–822) and 5′-TCC GCC TAG AAG CAT TTG CGG TGG A-3′ (position 1206–1182). The α_{1A2}- and α_{1A5}-adrenoceptor isoforms were not included as they share sequence with the other variants. In the sequence where they differ from the other variants, they have an Alu-Sc repetitive sequence. This feature makes it difficult to design primers that could distinguish them from Alu repeats.

We followed the manufacturer's protocol using 6 pmol of each primer and 2 μl of cDNA for each reaction. A control without cDNA was included in each run. The thermal cycling protocol consisted of a 15 min (95 °C) polymerase activation step followed by 40 cycles of denaturation (94 °C; 10 s), annealing (50 °C; 20 s), and elongation (72 °C; 1 min). Melting curves were obtained for each α_{1A}-adrenoceptor splice variant. The relative amount of transcripts, measured during the exponential phase of reactions, was determined by the comparative threshold cycle (C_T) method (Livak and Schmittgen, 2001). The statistical analysis on threshold cycles (C_T) for different variants of α_{1A}-adrenoceptors compared with β-actin was performed using a mixed model with systematic effect of day of experiment, subjects, type of isoforms and random effect of subject * type and subject * day.

2.2. Experimental procedures to establish a cell line stably expressing human α_{1A1}-adrenoceptors

The full-length cDNA encoding human α_{1A1}-adrenoceptor (constructed in our lab) was cloned by RT-PCR from total cellular RNA isolated from human liver (RNeasy Mini Kit, QIAGEN, Germany). The cDNA was prepared from purified RNA using random hexamer and oligo (dT) primers. Oligonucleotide primers for α_{1A1}-adrenoceptor cDNA synthesis were synthesized based on the cDNA sequence NM 000680. Sequences of the primers were as follows: α_{1A1}, 5′-CGG ATC CCT CCA GCC GAG ACC TTT TGA TTC-3′ (sense, positions 330–355) and 5′-CCT CGA GCC CTT TCC TCT GCA TCT TTC CTG TC-3′ (antisense, positions 1838–1862). The predicted size of the amplified human α_{1A1}-adrenoceptor cDNA was 1532 bp. PCR was carried out using *Herculase* DNA polymerase (Stratagene, CA, USA) for 30 cycles in a Techne ProGene thermocycler TC-312. The amplification program consisted of denaturation (96 °C; 30 s), primer annealing (60 °C; 30 s), and extension (72 °C; 3 min). PCR products were initially cloned into the pCRII-TOPO vector (Invitrogen, CA, USA) and verified by sequencing using the DYEnamic™ ET terminator kit (Amersham Biosciences, NJ, USA). A BamHI/XhoI cDNA fragment from the human α_{1A1}-adrenoceptor cDNA clone was ligated into the pcDNA5/FRT mammalian expression vector (Invitrogen, CA, USA).