Ten commercial antibodies for alpha-1-adrenergic receptor subtypes are nonspecific

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Abstract

Commercial antibodies are used widely to quantify and localize the α1-adrenergic receptor (AR) subtypes, α1A, α1B, and α1D. We tested ten antibodies, from abcam and Santa Cruz, using western blot with heart and brain tissue from wild-type (WT) mice and mice with systemic knockout (KO) of one or all three subtypes. We found that none of the antibodies detected a band in WT that was absent in the appropriate KO or in the KO that was null for all α1-ARs (ABDKO). We conclude that the antibodies we tested are not specific for α1-ARs. These results raise caution with prior studies using these reagents. For now, competition radioligand binding is the only reliable approach to quantify the α1-AR subtype proteins. Receptor protein localization remains a challenge.

Keywords

Alpha-1-adrenoceptor; Antibodies; Subtypes

Introduction

Alpha-1-adrenergic receptors (α1-ARs) mediate smooth muscle contraction, activate cardiac muscle cell physiological growth and protection from injury (O'Connell et al. 2003; O'Connell et al. 2006; Simpson 2006), and are present at high levels in the brain (Simpson 2006). For reviews see Hein and Michel (2007), Koshimizu et al. (2007), and Simpson (2006). The crucial physiological roles of α1-ARs emphasize the importance of measuring tissue levels and cell-type localizations of the proteins representing the three cloned α1-AR subtypes, α1A, α1B, and α1D. Many papers have used commercial α1-AR antibodies to quantify α1-AR subtype protein levels by western blot and to localize the subtype proteins by immunohistochemistry. The commercial suppliers typically cite validation of these antibodies by detection of a band of

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appropriate size in western blot of a cell line with subtype overexpression and/or by elimination of a band with the blocking peptide used to raise the antibody.

However, α1-AR subtype antibodies have not been validated using the most rigorous negative control, knockout (KO) mice with the subtype genetically deleted. In this study, we used heart and brain tissues from mice with systemic KO of all three subtypes to test ten commercial antibodies. We reasoned that valid antibodies would detect a band of the appropriate size in wild-type (WT) mice with all subtypes present and this band would be absent in KO mice with deletion of that subtype. We found that none of the antibodies tested met this criterion.

Materials and methods

Table 1 lists the antibodies tested, one reactive with all subtypes (pan-α1) and nine advertised as subtype-selective. The sequence of the epitope used to make the antibody is known for the pan-α1 and three others, the rest only the general receptor domain (Table 1). Table 1 lists other advertised properties of the antibodies, including type, species reactivity, size of the predicted product, and suggested applications.

The KO mice are described elsewhere as follows: the single AKO (Rokosh and Simpson 2002), the single BKO (Cavalli et al. 1997), the single DKO (Sadalge et al. 2003), the double ABKO (O'Connell et al. 2003; O'Connell et al. 2006), and the triple ABDKO, bred from the single KOs in our lab (unpublished data). All mice used in this study were genotyped. KO and WT mice were all adults, age 3–4 months, congenic in the C57Bl/6J background.

The brain and heart were removed rapidly from mice under deep isoflurane anesthesia, cleaned of all blood, flash frozen in liquid nitrogen, and homogenized with a Polytron at speed 7 out of 10. Lysates were diluted to equal concentrations in RIPA buffer with protease inhibitors, and were run on precast 12.5% SDS-PAGE gels (Criterion). The proteins were transferred to either nitrocellulose or PVDF membranes (Biorad), blocked in either 5% milk or 5% bovine serum albumin (BSA), and incubated in primary antibody diluted in 5% BSA. Secondary antibodies (sc-2020 rabbit anti-goat HRP for all Santa Cruz primary antibodies except sc-10721; Cell Signaling 7074 goat anti-rabbit HRP for ab3462 and sc-10721; and abcam ab6753 rabbit anti-chicken HRP for ab15851) were diluted in 5% milk. Blots were developed using an ECL reagent (Sigma). All results were replicated in samples from at least two mice.

Results

We tested ten antibodies (Table 1), based largely on the frequency with which they are cited. We tested the antibodies using western blot with tissues from WT mice and from KO mice with genetic deletion of one (AKO), two (ABKO), or all three subtypes (ABDKO). DNA analyses, mRNA assays, and radioligand binding have validated each of these KOs.

Heart from WT mouse has about 15 fmol/mg protein total α1-ARs by radioligand binding, with 30% α1A, 70% α1B, and no detectable α1D (Simpson 2006). Brain from WT mouse has about 140 fmol/mg protein total α1-ARs, with 55% α1A, 35% α1B, and 10% α1D (Simpson 2006). Accordingly, we tested each antibody with heart and brain tissue, to provide a range of levels of each subtype protein.

Figure 1 shows western blots representative of each of the ten antibodies. None of the antibodies detected a band of the appropriate size (Table 1), or a band of larger or smaller size, that was present in WT mouse heart or brain but absent in KO heart or brain.

We consulted technical support for both abcam and Santa Cruz and made extensive efforts to optimize our immuno-blotting protocol for each of the antibodies. We varied the amount of

protein loaded, the concentration of primary and secondary antibody, and the duration and ambient temperature of the incubation steps. We used denaturing and non-denaturing conditions and tried immunoprecipitation (Upstate Catch and Release) prior to western blot. Incubation with the blocking peptides for each of the antibodies produced the expected results (data not shown), though this finding confirmed only specificity for the peptide rather than for the receptor protein itself. We tried multiple lot numbers for four of the antibodies (sc-1475, sc-1476, sc-1477, and ab15851). Despite these modifications, none of the ten antibodies that we tested showed specific reactivity to the α1-AR subtype for which it was designed, or to the other two subtypes (Fig. 1 and data not shown).

Discussion

These results show that the ten α1-AR antibodies we tested, which include those cited most commonly, are nonspecific. They cannot be used to quantify or localize α1-AR subtype proteins, or total α1-ARs. Studies with these reagents need to be viewed with caution.

A web search reveals at least 59 commercial antibodies advertised for α1-ARs, nine of which are pan-α1 raised against the same epitope as ab3462 (Table 1), leaving 50 antibodies said to be selective for an α1-AR subtype. Testing 18% of these in this study was enormously expensive and time-consuming, but it is conceivable that one or more of the others we did not test would prove to be valid. We believe that the KO models should be used in validation, as we have done here.

We conclude that, at present, antibody-based methods are unreliable for α1-ARs. α1-AR subtype mRNAs can be quantified by RT-PCR, although it is essential that the primers cross the large intron (Jensen et al. 2007), which is not done in all studies. Unfortunately, subtype mRNA levels might not accurately reflect subtype protein levels (Jensen et al. 2007). Competition radioligand binding for α1-AR subtype proteins is laborious and difficult when cell or tissue amounts are limiting. Nevertheless, we conclude that radioligand binding using appropriately chosen antagonists remains the only reliable means for quantifying α1-AR subtype proteins. In our lab, we use 5-methylurapidil in competition binding to distinguish the α1A (high affinity, pKi 8) from the B and D (low affinity, pKi 4–5), and BMY-7378 to distinguish the α1D (high affinity, pKi 10–11) from the A and B (low affinity, pKi 5–6) (Jensen et al. 2008; Jensen et al. 2007). Binding is very problematic for receptor localization, which remains a challenge.

Acknowledgments

References

Cavalli A, Lattion AL, Hummler E, Nenniger M, Pedrazzini T, Aubert JF, Michel MC, Yang M, Lembo G, Vecchione C, Mostardini M, Schmidt A, Beermann F, Cotecchia S. Decreased blood pressure response in mice deficient of the alpha1b-adrenergic receptor. Proc Natl Acad Sci U S A 1997;94:11589–11594. [PubMed: 9326654]

Hein P, Michel MC. Signal transduction and regulation: are all alpha1-adrenergic receptor subtypes created equal. Biochem Pharmacol 2007;73:1097–1106. [PubMed: 17141737]

Jensen BC, Swigart PM, Myagmar B-E, Shah S, DeMarco T, Hoopes C, Simpson PC. The alpha-1A is the predominant alpha-1-adrenergic receptor in the human heart at the mRNA but not the protein level (abstract). Circulation 2007;116:II-289.

Jensen BC, Swigart PM, Laden M-E, DeMarco T, Hoopes C, Simpson PC. The alpha-1D is the predominant alpha-1 adrenergic receptor subtype in human epicardial coronary arteries (abstract). Arterioscler Thromb Vasc Biol 2008;28:e142.

Koshimizu TA, Tanoue A, Tsujimoto G. Clinical implications from studies of alpha1 adrenergic receptor knockout mice. Biochem Pharmacol 2007;73:1107–1112. [PubMed: 17141736]

O'Connell TD, Ishizaka S, Nakamura A, Swigart PM, Rodrigo MC, Simpson GL, Cotecchia S, Rokosh DG, Grossman W, Foster E, Simpson PC. The alpha(1A/C)- and alpha(1B)-adrenergic receptors are required for physiological cardiac hypertrophy in the double-knockout mouse. J Clin Invest 2003;111:1783–1791. [PubMed: 12782680]

O'Connell TD, Swigart PM, Rodrigo MC, Ishizaka S, Joho S, Turnbull L, Tecott LH, Baker AJ, Foster E, Grossman W, Simpson PC. Alpha1-adrenergic receptors prevent a maladaptive cardiac response to pressure overload. J Clin Invest 2006;116:1005–1015. [PubMed: 16585965]

Rokosh DG, Simpson PC. Knockout of the alpha 1A/Cadrenergic receptor subtype: the alpha 1A/C is expressed in resistance arteries and is required to maintain arterial blood pressure. Proc Natl Acad Sci U S A 2002;99:9474–9479. [PubMed: 12093905]

Sadalge A, Coughlin L, Fu H, Wang B, Valladares O, Valentino R, Blendy JA. alpha 1d Adrenoceptor signaling is required for stimulus induced locomotor activity. Mol Psychiatry 2003;8:664–672. [PubMed: 12874602]

Simpson, PC. Lessons from Knockouts: the alpha-1-ARs. In: Perez, DM., editor. The adrenergic receptors in the 21st century. Totowa: Humana; 2006. p. 207-240.
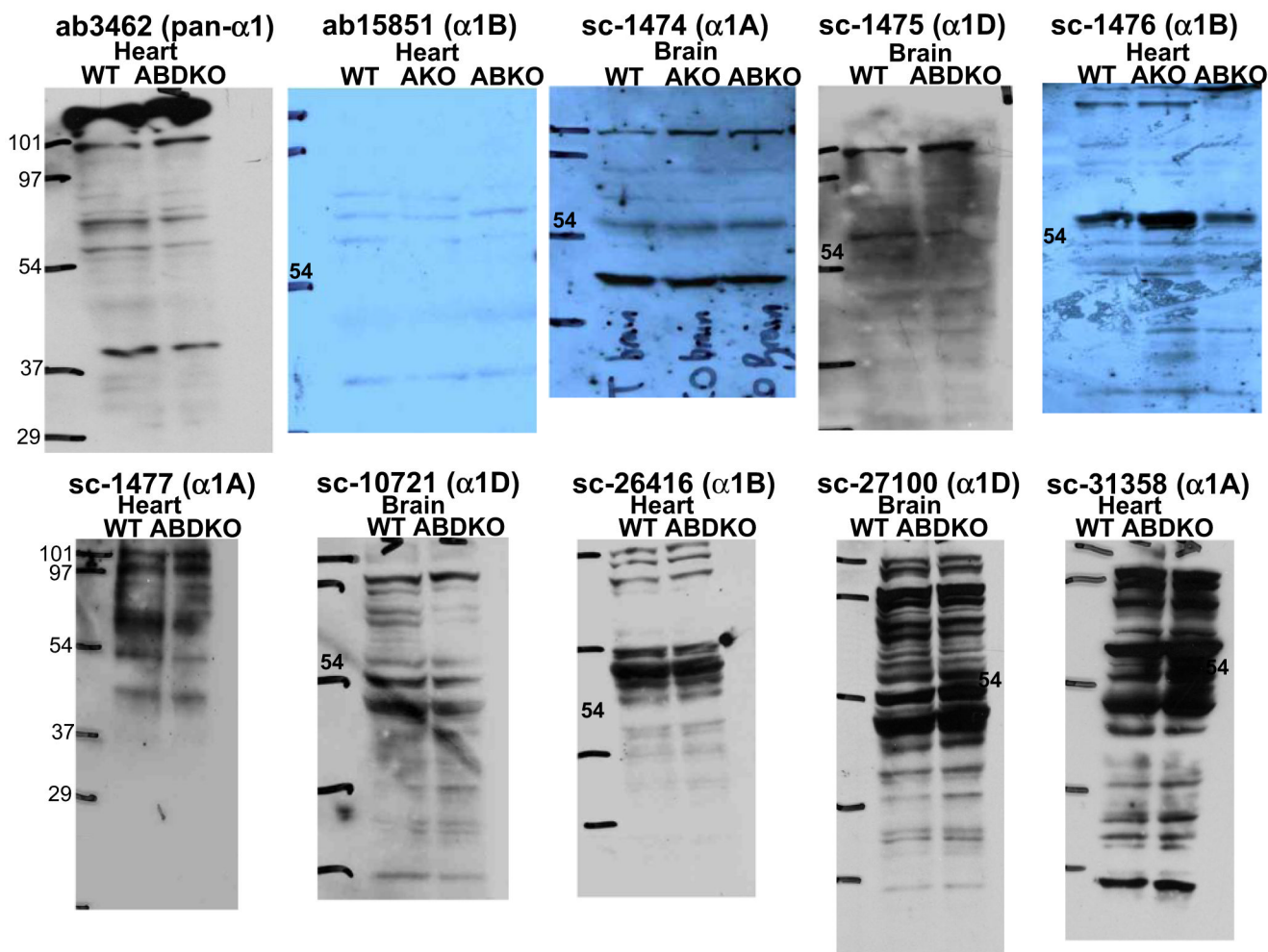
Fig. 1.
Western blots with α1-AR antibodies. Each panel shows the blot for one antibody tested with heart or brain tissue from a WT mouse and a mouse with genetic deletion of α1-AR subtypes: one (AKO), two (ABKO), or all three (ABDKO). Size markers are shown at *far left* and the 54 kDa marker is indicated on each blot. Note that no band with any antibody is present in WT but absent in KO indicating that no band is specific for an α1-AR

Table 1

α1-adrenergic receptor antibodies

Company	Catalog number	Target	Epitope	Ab type	Species	Size (kDa)	Applications
abcam	ab3462	pan-α1	Human 3ICL (339–349, KFSREKKAAKT)	Rabbit pAb	Hu, Ms, Rat, Dm, Zfsh	60	ICC/IF, WB
abcam	ab15851	α1B	Human residues 200–299	Chicken pAb	Hu	62	WB
Santa Cruz Biotechnology	sc-1474	α1A	C terminus (331–466)	Goat pAb	This Ab is no longer available		
Santa Cruz Biotechnology	sc-1475	α1D	Rat C terminus	Goat pAb	Hu, Ms, Rat	70–80, 40	WB, IF, IP, ELISA
Santa Cruz Biotechnology	sc-1476	α1B	Human C terminus	Goat pAb	Hu, Ms, Rat	70–90	WB, IF, IP, ELISA
Santa Cruz Biotechnology	sc-1477	α1A	Human C terminus	Goat pAb	Hu, Ms, Rat, Cow	52	WB, IF, ELISA
Santa Cruz Biotechnology	sc-10721	α1D	Human C terminus (431–572)	Rabbit pAb	Hu, Ms, Rat	70–80	WB, IF, IP, ELISA
Santa Cruz Biotechnology	sc-26416	α1B	Human cytoplasmic domain	Goat pAb	Hu, Ms, Rat	70–90	WB, IF, ELISA
Santa Cruz Biotechnology	sc-27100	α1D	Human cytoplasmic domain	Goat pAb	Hu, Ms, Rat	70–80	WB, IF, ELISA
Santa Cruz Biotechnology	sc-31358	α1A	Human cytoplasmic domain	Goat pAb	Hu, Ms, Rat	52	WB, IF, IP, ELISA

Representative α1-AR antibodies were selected based on the frequency with which they are cited. Species indicates the advertised reactivity (Hu, human; Ms, mouse; Dm, drosophila; Zfsh, zebra fish). Size indicates the advertised size of the protein detected. Applications are as advertised (ICC/IF, immunohistochemistry/immunofluorescence; IP, immunoprecipitation; WB, western blot). pAb, Polyclonal antibody; ICL, intracellular loop