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α2-Adrenergic agonists including xylazine and dexmedetomidine inhibit norepinephrine transporter function in SK-N-SH cells

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HIGHLIGHTS

- We report that α2 adrenergic agonists directly block NET binding of nisoxetine.
- The agents suppressed NET function in an acute and dose-dependent manner.
- This was independent of α2 signaling, and accompanied by reduced NET substrate affinity.
- α_2 agonists are competitive NET inhibitors that interact with the nisoxetine binding site.

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ABSTRACT

α_2-Adrenergic agonists simulate norepinephrine (NE) action on α_2 receptors of sympathetic neurons to mediate feedback inhibition of NE release. These agents are used as valuable adjuncts for management of hypertension and for anesthesia. Their action, equivalent to NE on α2 adrenergic receptors, raises the question whether α2 agonists may also target NE transporters (NETs), another major control mechanism for noradrenergic neurotransmission. We thus investigated the effect of α_2 agonists on transport of the NE analog, ^{131}I-metaiodobenzylguanidine (MIBG). Results from this investigation showed that xylazine and dexmedetomidine dose-dependently blocked [^{3}H]nisoxetine binding in neuron-like SK-N-SH cells. Furthermore, the agents acutely suppressed cellular MIBG uptake in a dose-dependent manner. This effect was uninfluenced by the α2 antagonist yohimbine, but was completely reversed by drug removal. There was no change in membrane NET density by the agents. Moreover, saturation analysis showed that xylazine and dexmedetomidine significantly increased K_m without affecting V_{max}, indicating competitive inhibition of MIBG transport. Thus, the α_2 adrenergic agonists xylazine and dexmedetomidine, acutely suppress NET function through competitive inhibition of substrate transport, likely by direct interaction on a region that over-laps with the nisoxetine binding site.

1. Introduction

α2-Adrenergic agonists are valuable adjuncts for managing various clinical conditions including hypertension, glaucoma, muscle spasticity, and behavior disorders [5]. They are also widely used as anesthetics in perioperative settings for induction of anxiolysis, maintenance of sedation, and management of pain [4,10]. More recently, these agents also show the capacity to attenuate neurological deficits following cerebral ischemia [7,18–20].

α2 agonists simulate norepinephrine (NE) in binding to presynaptic surface autoreceptors, which in turn mediates feedback inhibition of NE release. Another major control mechanism for noradrenergic neurotransmission is termination of signaling by presynaptic NE transporter (NET)-mediated NE reuptake [1,14,23]. The pivotal role of NET in fine-tuning of noradrenergic neurotransmission provides an opportunity for pharmaceutical interventions for such disorders as depression, attention-deficit hyperactivity disorder, and emotional disturbances. NET function also underlies the basis for diagnostic imaging with radiolabeled metaiodobenzylguanidine (MIBG), widely used for evaluation of neuroendocrine tumors [12,21]. Thus, a better understanding of mechanisms that modulate NET function is important for the development of newer psychoactive compounds as well as for optimizing clinical MIBG imaging. The equivalent actions of α2 agonists and NE on α2 adrenergic receptors raise the question whether NET may also be a target for α2 agonists. Interestingly, several injectable anesthetic agents have been observed to cause suppression of NE reuptake [8,9,15,17,24]. Furthermore, there have been a couple of

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previous observations suggesting that such α2 agonists as xyalzine and clonidine may suppress MIBG uptake in neuroblastoma cells. Babich et al. evaluated the effects of various adrenergic receptor ligands on MIBG uptake in SK-N-SH cells and found xyalzine and clonidine to be included among agents with an inhibitory effect [2]. Our group also previously observed a reduction of MIBG uptake in SK-N-SH cells by xylazine while evaluating the effects of anesthetic agents on cellular transport and in vivo biodistribution of MIBG [11]. However, neither of these previous studies attempted to investigate the mechanism underlying this effect. In this study, we thus hypothesized that α_2 agonists can target NET to influence NE reuptake, and further investigated the mechanisms that mediate the response.

2. Materials and methods

2.1. Nisoxetine binding assay

Neuron-like human neuroblastoma SK-N-SH cells were maintained at 37 °C and 5% CO_2 in minimum essential media supplemented with 10% fetal bovine serum and 2 mM glutamine. To evaluate whether α2 agonists compete for nisoxetine binding site, binding assays with [^{3}H]nisoxetine (New England Nuclear, Boston, MA; specific activity, 73 Ci/mmol) was performed at 4 °C for 1 h. Cells were solubilized in 0.1% Triton X-100, mixed with scintillation cocktail, and measured for radioactivity on a Packard beta liquid scintillation counter. Specific binding was calculated by subtracting nonspecific binding measured with 50 μM desipramine from total binding. Nonlinear curve fitting was performed and half-inhibitory concentration (IC_{50}) values were calculated using Prism 3.0.2 Software (GraphPad Software, San Diego, CA).

2.2. MIBG transport assay

NE transport function was measured by incubating cells for 30 min at 5% CO_2 and 37 °C with 37 kBq of ^{131}I-MIBG (Korea Atomic Energy Research Institute; specific activity, 1178 Ci/mmol). MIBG is a structural analog of NE that is taken up by sympathetic neuronal cells through the NET. In this study, we used ^{131}I-MIBG rather than [^{3}H]NE for its wide availability and simple procedure for measuring cell uptake on a γ-counter. Cells were rapidly washed twice with ice-cold phosphate buffered saline (PBS), solubilized in 0.3 ml of 0.05 N NaOH, and measured for bound radioactivity on a high-energy γ-counter (Wallac). Nonspecific uptake was measured with 50 μM of desipramine, and was subtracted from total counts to obtain specific uptake.

To evaluate the effect of agents after their removal from the media, cells treated with α_2 agonists were washed twice with PBS. Cells were then incubated in fresh media for 30 min before adding ^{131}I-MIBG for uptake measurements as above.

For competitive transport assays, MIBG uptake was measured in cells treated with graded concentrations of α2 agonists for 30 min, and specific uptake level was plotted against log drug concentration. Nonlinear curve fitting was performed, and IC_{50} value was calculated. For saturation assays, graded concentrations of ^{131}I-MIBG were added to cells in the presence or absence of α2 agonists, and specific uptake levels were plotted against MIBG concentration. Nonlinear curve fitting was performed, and kinetic parameters of maximum transport velocity (V_{max}) and half-maximum transport (K_m) were calculated.

2.3. Immunoblotting of norepinephrine transporter expression

NET expression in the entire cell and in plasma membrane was evaluated. Total protein obtained from cells was solubilized for 15 min at 4 °C in 500 μl of cold radioimmunoprecipitation assay buffer containing 65 mM Tris (pH 7.4), 150 mM NaCl, 100 mM EDTA, 10% Nonidet P-40, 10% sodium deoxylazinecholate, and protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 500 μM PMSF). After removal of cell debris, 250 μl of the supernatant was incubated for 2 h at 4 °C with 2 μl of anti-human NET monoclonal antibody (NET17-1; Mab Technologies, GA). Protein-A Sepharose CL-4B beads (50 μl; Sigma) was added and samples were incubated overnight at 4 °C. Beads were washed thrice with PBS, and protein was eluted into 50 μl of 2× Laemmli sample buffer for 30 min at room temperature. Plasma membrane protein was obtained by cells solubilized in 500 μl of lysis buffer containing 85.6 mg/ml sucrose, 10 mM HEPES, 1 mM EDTA, 10 mM PMSF, 66.7 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin. After removal of cell debris, supernatants were incubated at 4 °C for 1 h with 1.5 ml of lysis buffer containing 85.6 mg/ml sucrose, 10 mM/ml HEPES and 10 mM/ml $MgCl_2$. The mixture was centrifuged at 45,000 rpm for 60 min, and the pellet was dissolved in a minimum volume of PBS.

Samples containing total cell NET and membrane protein were boiled for 1 min at 90 °C and separated by electrophoresis on a 10% SDS-PAGE gel. Following transfer to a PVDF membrane, the protein was incubated with NET17-1 antibody (1:1000 dilution), followed by an anti-mouse HRP-conjugated secondary antibody (Amersham Bioscience; 1:2000 dilution). Immunoreactive proteins were detected with an enhanced chemiluminescence detection system, and band intensities were measured by a GS-800™ calibrated densitometer with Quantity One® software (Bio-Rad).

2.4. Statistics

Statistical comparisons were done by unpaired Student *t*-tests for two groups, and one-way analysis of variance (ANOVA) with Bonferroni post hoc tests for three or more groups. *P* values of less than 0.05 were considered significant.

3. Results

3.1. α2 adrenergic agonists inhibit SK-N-SH cell nisoxetine binding and mibg uptake

Xylazine and dexmedetomidine dose-dependently inhibited [^{3}H]nisoxetine binding to SK-N-SH cells (Fig. 1A and B). Nonlinear regression showed IC_{50} values for blocking of nisoxetine binding to be 157.5 μM for xylazine and 31.9 μM for dexmedetomidine.

MIBG uptake experiments showed that xylazine and dexmedetomidine also suppress cellular NE transport. Furthermore, all α2 adrenergic agonists tested, including clonidine, lofexidine, tizandine, and guanabenz significantly inhibited MIBG uptake at doses of 100 μM (Fig. 2A). The superselective α2 adrenergic agonists, medetomidine and dexmedetomidine reduced MIBG uptake at a lower dose of 1 μM (Fig. 2B). Competitive analysis demonstrated dose-dependent inhibition of MIBG uptake, with IC_{50} values of 3.4 μM for dexmedetomidine, 108 μM for xyalzine, and 549 μM for clonidine (Fig. 3).

3.2. Rapid-onset, α2 receptor-independence, and reversibility of NET inhibition

Time course experiments revealed rapid onset of inhibitory effects of xylazine and clonidine on MIBG uptake that peaked in less than 30 min (Fig. 4A). The selective α_2 adrenoreceptor antagonist, yohimbine, was unable to block the inhibitory action of xylazine, clonidine and dexmedetomidine on MIBG uptake. Interestingly, we noted that yohimbine itself also has an inhibitory effect on MIBG uptake that appears to be additive to that caused