Steroid Sensitivity of Norepinephrine Uptake by Human Bronchial Arterial and Rabbit Aortic Smooth Muscle Cells

Gabor Horvath, Thomas Lieb, Gregory E. Conner, Matthias Salathe, and Adam Wanner

Division of Pulmonary and Critical Care Medicine, and Department of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, Florida

We have shown that an inhaled glucocorticosteroid (GS) causes α_1-adrenergic antagonist–blockable, rapid, and transient bronchial vasoconstriction in healthy and asthmatic subjects. Steroids inhibit norepinephrine (NE) uptake by non-neuronal cells, thereby increasing NE concentration at α-adrenergic receptor sites. This could explain the GS-induced bronchial vasoconstriction. We therefore studied expression of the steroid-sensitive extraneuronal monoamine transporter (EMT) and steroid sensitivity of NE uptake in human bronchial artery and rabbit aorta (as a substitute for the limited supply of human bronchial artery). NE uptake was measured using a semiquantitative, sucrose–potassium phosphate–glyoxylic acid fluorescence method that we newly adapted for use in single cells. Both human bronchial arteries and rabbit aorta expressed messenger RNA for EMT, and steroids blocked NE uptake into freshly dissociated human bronchial arterial and rabbit aortic smooth-muscle cells (SMCs). In the latter, inhibition of NE uptake by steroids was not altered, either by a protein synthesis inhibitor (cycloheximide) or by a transcription inhibitor (actinomycin D), and corticosterone made membrane-impermeant by conjugation to bovine serum albumin inhibited NE uptake equipotently. These data show that NE uptake into bronchial arterial and rabbit aortic SMCs is sensitive to steroids, possibly mediated by EMT, and suggest a mechanism for GS-induced bronchial vasoconstriction.

The sympathetic nervous system provides the main nervous control of the bronchial vasculature that is the principal vascular supply to the airway wall (1). Norepinephrine (NE) released from sympathetic nerve endings leads to bronchial vasoconstriction via α-adrenergic receptors. The time that extracellular NE occupies its receptor is a critical determinant of the magnitude and duration of bronchial vasoconstriction and is determined mainly by specialized cellular uptake processes (2). These mechanisms include neuronal and non-neuronal membrane transporters, which are thus responsible for the functional inactivation of NE (3, 4). The NE metabolizing enzymes, namely catecholamine-O-methyltransferase (COMT) and monoamine oxidase (MAO), reside intracellularly. Therefore, they are not involved in NE's initial, functional inactivation. Three non-neuronal NE transporters have been cloned and characterized: organic cation transporter (OCT) 1 (5–7), OCT2 (8–10), and the extraneuronal monoamine transporter (EMT or OCT3) (11–13). Although these NE transporters and their functions have been studied in detail in other tissues (14–16), little is known about transporter expression and non-neuronal NE uptake mechanism in the human bronchial vasculature.

We have shown that an inhaled glucocorticosteroid (GS) causes a rapid, α_1-adrenoceptor–mediated decrease in bronchial blood flow *in vivo* (17), the mechanism of which is unknown. We hypothesized that GSs inhibited NE uptake by bronchial arterial smooth-muscle cells (SMCs), thereby increasing the concentration and prolonging the duration of NE at α-adrenergic receptors and causing vasoconstriction. This hypothesis was supported by the facts that inhibition of non-neuronal NE uptake has been shown to increase NE at its active site *in vitro* (18) and that EMT, a steroid-sensitive NE uptake transporter, has been found in several systemic arteries (19–22).

To examine the presence of a steroid-sensitive, non-neuronal NE uptake in the human bronchial circulation, we evaluated whether EMT messenger RNA (mRNA) is expressed in human bronchial arteries and whether NE uptake into freshly isolated human bronchial arterial SMCs occurs and is sensitive to steroids. These experiments were carried out on conduit-type arteries of the bronchial circulation because smaller vessels (arterioles and small arteries) could not be obtained. To further characterize the action of steroids on NE uptake, we used rabbit aortic SMCs as a substitute for the limited supply of bronchial arterial SMCs.

Materials and Methods

Materials

All materials were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Human Bronchial Arteries

Donor lungs found unsuitable for transplantation were obtained through the University of Miami Organ Procurement Program with approval from the local Institutional Review Board. Major branches of bronchial arteries (approximately 2 mm diameter) from the left main bronchus were dissected and the adventitia was removed. To confirm that the dissected structure was in fact an artery, a portion of each vessel was fixed in 4% phosphate-buffered formaldehyde and processed according to regular procedures for histology. Sections were stained with hematoxylin and eosin. The endothelium was removed from the rest of the dissected vessels with a small brush. Then, the pieces (approximately 20 mm long) were used immediately for RNA extraction or cell isolation.

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Address correspondence to: Adam Wanner, M.D., Div. of Pulmonary and Critical Care Medicine, University of Miami School of Medicine, P.O. Box 016960 (R-47), Miami, FL 33101. E-mail: awanner@miami.edu

Abbreviations: complementary DNA, cDNA; catecholamine-O-methyltransferase, COMT; differential-interference-contrast, DIC; extraneuronal monoamine transporter, EMT; mean fluorescence intensity value, F_N; glucocorticosteroid, GS; Michaelis constant, K_m; monoamine oxidase, MAO; messenger RNA, mRNA; norepinephrine, NE; organic cation transporter, OCT; reverse transcriptase/polymerase chain reaction, RT-PCR; standard error of the mean, SEM; smooth muscle cell, SMC; sucrose–potassium phosphate–glyoxylic acid, SPG.

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Rabbit Aorta

Adult female New Zealand albino rabbits (3 to 4 kg) were killed by an overdose of pentobarbital sodium according to protocols approved by the University's Institutional Animal Care and Use Committee. The thoracic aorta was excised, adhering fat and connective tissue were removed, and the vessel was opened longitudinally. Endothelial cells were removed by carefully scraping the inside surface of the vessel, and 1-mm-wide strips of the smooth-muscle layer were separated from the adventitia. A total of 15 to 20 strips (approximately 1 × 10-mm pieces) were cut transversely from the muscle preparation and were used immediately for RNA extraction and cell isolation.

Reverse Transcriptase/Polymerase Chain Reaction

Total RNA from human and rabbit vascular smooth muscle was extracted using the Ultraspec RNA Isolation System (Biotecx Laboratories, Houston, TX), treated with deoxyribonuclease (DNase I Amplification Grade; Life Technologies, Rockville, MD), precipitated with ethanol, and quantitated spectrophotometrically at 260 nm. RNA (0.5 μg) was used for first-strand complementary DNA (cDNA) synthesis with Superscript II RT (Life Technologies). Oligonucleotide primers for polymerase chain reactions (PCRs) were designed on the basis of regions of sequence similarity between human, rat, and mouse EMT cDNAs. PCRs (35 cycles of 30 s at 94°C, 45 s at 61°C, and 1 min at 72°C, followed by final elongation of 10 min at 72°C) were done with 5′-CTG GGT GGT CCC TGA GTC TCC-3′ (forward) and 5′-TCC CAG GCG CAT GAC AAG TCC-3′ (reverse) primers using Taq DNA polymerase (Life Technologies). The predicted size of the reverse transcriptase (RT)–PCR products was 265 base pairs. Control reactions were performed in the absence of RT.

DNA Sequencing and Analysis

RT-PCR products were electrophoresed on ethidium bromide–stained 2% SeaKem agarose (BMA, Rockland, ME) gels, purified on a silica spin column (QiAquick PCR Purification Kit; Qiagen, Valencia, CA), and sequenced by the University of Miami DNA Core Laboratory. Sequences were compared with the published EMT cDNA sequences (human: GenBank accession #AJ001417; rat: GenBank accession #AF055286; mouse: GenBank accession #AF078750) by PileUp (Wisconsin Package; GCG, Madison, WI).

SMC Isolation

SMC isolations from human and rabbit vascular smooth muscle were carried out on the basis of the method of Clapp and Gurney (23) with some modifications. To dissociate cells, muscle strips were transferred to constantly oxygenated incubation solution (137 mM NaCl, 10 mM $NaHCO_3$, 0.2 mM NaH_2PO_4, 5.4 mM KCl, 0.5 mM KH_2PO_4, 6 mM glucose, 0.15 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-ethane sulfonic acid, and 0.02% bovine serum albumin [BSA], pH 7.0) containing papain (4 mg/ml) and dithiothreitol (2 mM), and were incubated at 37°C for 60 min with shaking. At the end of the dissociation period, individual SMCs were obtained by gentle titration followed by filtration through a wire sieve (500 μm pore size). To eliminate papain from the solution, cells were collected by centrifugation at 150 × *g* for 3 min and resuspended in fresh incubation solution. The viability of freshly isolated SMCs after enzymatic dispersion was greater than 95%, as determined by trypan blue exclusion.

NE Uptake Experiments

Freshly prepared SMCs were equilibrated in incubation solution for 30 min at 37°C. For NE uptake experiments, cells were exposed to incubation solution with or without defined NE concentrations and with or without different inhibitors at 37°C. To investigate the effects of pargyline, Ro-41-0960, cycloheximide, and actinomycin D, these drugs were added into the incubation solution 30 min before the NE uptake experiments. Steroid hormones, cycloheximide, and actinomycin D were dissolved in ethanol and freshly diluted into the incubation solution just before use. The final concentration of ethanol never exceeded 0.04%, a concentration with no significant effects on any of the measurements. At the end of the incubation period, SMCs were centrifuged at 150 × *g* for 3 min, followed by resuspension in 0.3 ml ice-cold, high KCl (20 mM)–containing incubation solution. The SMC suspension was then deposited onto a poly-L-lysine–coated slide and the cells were allowed to settle for 10 min at 4°C before estimating the intracellular NE concentration.

Intracellular NE was visualized using a sucrose–potassium phosphate–glyoxylic acid (SPG) method described for tissue slices (24) and adapted by us for use in isolated cells. This method is based on the cyclization of ethylamine side chains of monoamines by highly reactive aldehydes (glyoxylic acid) in the presence of heat to produce 2-carboxymethyl-dihydroisoquinoline compounds that fluoresce under suitable illumination (25–27). Slides with SMCs processed as indicated earlier were dipped into SPG solution (0.2 M sucrose, 0.236 M KH_2PO_4, and 1% glyoxylic acid monohydrate, titrated to pH 7.4 with 1 N NaOH) at room temperature for 3 s. Excess fluid was removed and the slide was placed under a flow of cool air. After 5 min of air-drying, the specimen was covered with a drop of light mineral oil and the slide was sealed with a coverslip and put into an oven at 95°C for 2.5 min. To observe and quantitate fluorescence, a Nikon Eclipse E600FN microscope (Nikon, Melville, NY) with a Lambda DG-4 excitation system (Sutter Instruments, Novato, CA), a cooled CCD camera (Quantix; Photometrics, Tucson, AZ), and Isee software from Inovision, Inc. (Durham, NC), were used. Cells were imaged at original magnification ×600 (5 to 25 cells/field) with differential-interference-contrast (DIC) microscopy, and individual cells were identified as regions of interest. For quantitation of the SPG fluorescence (or intracellular NE concentration) in these cells (or regions of interest), a 10-nm-wide filter centered on 405 nm was used for excitation and the emission was measured at > 455 nm using a long-pass filter (emission maximum: 480 nm), integrating the signal for 1 s. The cooled CCD camera was always set to a predefined gain (held constant throughout the experiments). If possible, the fluorescence of all SMCs on a slide (approximately 50) was measured, which could usually be accomplished by selecting six different, well-separated regions on each slide. Each single cell's mean fluorescence intensity value (F_N), expressed in arbitrary units, was corrected for background fluorescence by subtracting the mean F_N of SMCs from the same tissue not exposed to NE. Average NE uptake of each experimental group was calculated using the mean normalized F_N of all cells. On each experimental day, a control experiment with 20 min of incubation with 50 μM NE was carried out to confirm an intact NE uptake mechanism (positive controls).

Statistics

Results are expressed as means ± standard error of the mean (SEM) with *N* and *n* representing numbers of cells and experiments, respectively. Statistical significance determined with analysis of variance was followed by a pairwise comparison with the Tukey–Kramer honestly significant difference *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

EMT Expression in Human Bronchial Arterial and Rabbit Aortic SMCs

To examine whether EMT mRNA is expressed in human bronchial arterial and rabbit aortic SMCs, RT-PCR reac-

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           h/exon-5 →
Rabbit  CTGGGTGGTCCCTGAGTCTCCCCGCTGGCTGATCACTCGGAAGGAAGGAGATAAAGCCTTGAAGATTCTC  70
Human   ........................T........T.........A.............A..AC....C..G
Rat     ....................................C......C.G.....G.........C....C..G
Mouse   ....................................C......C.......G.........C....C..G

                                                           h/exon-6 →
Rabbit  AGGAAGATTGCTAAATGCAACGGGAAGTACCTCTCGCCCAATTACTCGGAGATCACTGTTACAGATGAGG  140
Human   ..ACGC........G.....T.....A........AT.A........A......................
Rat     ...CGTG.G.....G.....T.....AC........T.A........A........A...........A.
Mouse   ...CGCG.G.....G.....T..A..AC.......AT.A........A........A...........A.

Rabbit  AGGTTAGTAATCCGTCCTTTTTAGATCTGGTGAGAACTCCCCAAATGAGGAAGTGCACCCTTATACTTAT  210
Human   .A...........A......................................A.....A.....T.....
Rat     ....C.....C..A....G......C..T.....G.......................A..C..T..C..
Mouse   .A..C.....C..A....G......C..T.......................A.....G..C..C.....

                 h/exon-7 →
Rabbit  GTTTGCTTGGTTCACCAGTGCGGTGGTGTACCAAGGACTTGTCATGCGCCTGGGA                265
Human   ...............A..C..A........T........................
Rat     ...............A.....C........T........................
Mouse   ...............G..C..C.................................
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Figure 1. Partial cDNA sequence of rabbit EMT and its comparison with the corresponding cDNA fragments of human, rat, and mouse EMTs (differences are shown). Nucleotide similarity scores are 89, 86, and 86%, respectively. Splice junctions of the human exons, as determined from the published structure of the human EMT gene (SLC22A3), are marked (h/exon 5, 6, and 7).

tions were initiated as described in MATERIALS AND METHODS. Gel electrophoresis of the RT-PCR products showed bands of expected size. After gel-purification, both sense and antisense strands were sequenced. Sequence analysis of the product from human bronchial artery confirmed that it was a fragment of the published human EMT, perfectly matching positions 882 to 1,146 of the full-length cDNA. The isolated fragment contained sequences from several exons (4, 5, 6, and 7) of the human EMT gene (SLC22A3) (28) that further confirmed the amplification of mRNA rather than genomic sequences. Sequence analysis of the amplified rabbit product showed that it was similar to the human, rat, and mouse EMT cDNAs (Figure 1): nucleotide and amino-acid identity scores were at least 86 and 90%, respectively. This partial rabbit EMT cDNA sequence has been deposited in GenBank under the accession number AF294824.

Properties of NE Uptake by Rabbit Aortic SMCs

The supply of human lungs was limited, as was the yield of bronchial arterial SMCs from a single vessel. We therefore decided to use rabbit aortic SMCs to develop our NE uptake measurement technique and to get initial data for its sensitivity to steroids. This approach seemed justified because rabbit aortic SMCs also expressed mRNA for EMT and because bronchial arteries, which arise from the aorta, are part of the systemic circulation. Later, we confirmed these results in human bronchial arterial SMCs.

After enzymatic dispersion of rabbit aortic SMCs as described in MATERIALS AND METHODS, the majority of the cells had an elongated shape (Figure 2A). Freshly isolated cells remained elongated in incubation solution for at least 1 h (Figure 2B). Cells assumed a round shape after membrane depolarization with 20 mM KCl (Figure 2C) or adrenergic stimulation with 5 to 250 μM of NE (Figure 2D), indicative of SMC contraction as described by others (29). Measurements were taken only from contracted cells that all had similar shapes. Thus, the increase in fluorescence measured when cells exposed to increasing NE concentrations was not due to changes in cell shape. Cells exposed to NE and processed with SPG showed clear intracellular fluorescence with no indication of a fluorescent rim around the cells at any focal plane (Figure 3). The absence of such a rim suggests that the fluorescence was not due to NE binding to its surface receptor but in fact due to uptake of NE into the cell. When NE was poured onto slides and processed with SPG, a diffuse fluorescence was revealed. Thus, detection of fluorescence inside the cells with no surrounding background indicates that significant leakage of NE from the cells was unlikely to occur during SPG processing.

To assess whether the measured fluorescence correlated with the actual intracellular NE concentration, permeabilized cells were equilibrated with defined NE concentrations. Because freshly dissociated SMCs insufficiently attached to the carrier slide for this procedure and were lost during permeabilization, cultured ovine airway epithelial cells (growing on collagen-coated glass coverslips) were used for these experiments (30). The cells were fixed and permeabilized with ice-cold methanol (used at −20°C twice for 5 min each time), then were equilibrated with different NE concentrations (10 to 1,000 μM) for 20 min. The fluorescence of these cells showed a nearly linear relation to the NE concentration in the 10- to 1,000-μM range (Figure 4).

To show that NE uptake by rabbit aortic SMCs is a time-dependent phenomenon (as expected for an uptake mechanism), cells were incubated in 50 μM NE for 5, 10, 20, 40, and 60 min. NE accumulation was detectable after 5 min of NE exposure and increased over time with a time course expected for a transporter (11, 12, 20, 31) (Figure 5, *left panel*).

The concentration dependence of NE uptake was measured by exposing the rabbit aortic SMCs to different NE concentrations (5, 25, 50, 100, and 250 μM). After a 20-min incubation, NE uptake was detectable even at the lowest NE concentration. There was a concentration-dependent

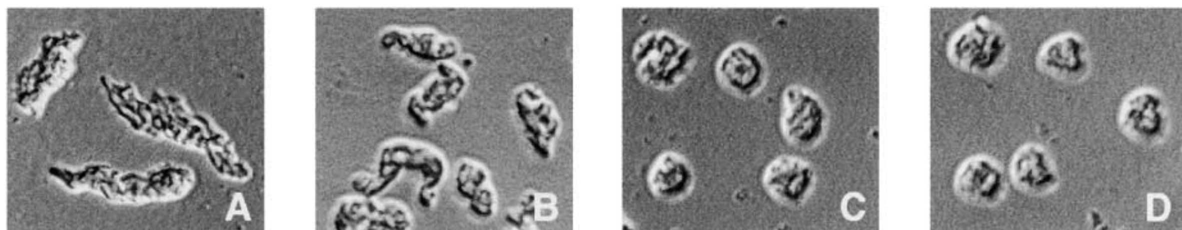

Figure 2. Representative shapes of SMCs dissociated with papain (4 mg/ml) from rabbit aorta. Cells are (*A*) freshly isolated, (*B*) exposed to incubation solution for 20 min, (*C*) exposed to 20 mM KCl for 20 min, and (*D*) exposed to 50 μM NE for 20 min. Original magnification: ×400, DIC microscopy.

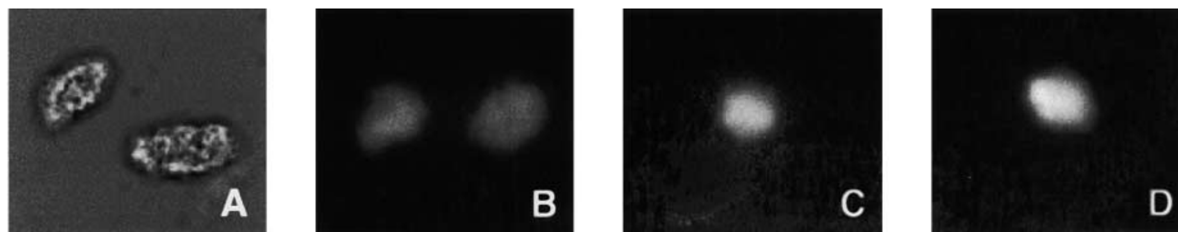

Figure 3. NE uptake into SMCs isolated from rabbit aorta. Cells were processed by the SPG method. (*A*) DIC and (*B–D*) fluorescence microscopy. Cells were exposed to incubation solution (*A* and *B*) without NE or containing (*C*) 25 or (*D*) 50 μM NE for 20 min before processing. Original magnification: ×600.

increase in NE uptake rates without a clear maximum (Figure 5, *middle panel*). In fact, using rate estimates of NE uptake (assuming a linear relationship between fluorescence and NE concentration as validated in airway epithelial cells), we estimated the Michaelis constant (K_m) for this uptake mechanism to be around 245 μM NE (Figure 5, *right panel*). This K_m is in the range of the published K_m values for NE uptake (224 to 290 μM) in different nonneuronal cell types (25, 32, 33). Thus, our kinetic analysis as well as the time course of NE uptake provide further evidence that the detected NE fluorescence was intracellularly located, because binding of NE to its receptor occurs quickly, would not be changing in this manner over time, and would not be expected to result in a K_m of this magnitude (the reported dissociation constant of NE binding to α-adrenergic receptors in aorta is 60 nM [34]).

To examine whether intracellular NE-metabolizing enzymes had any influence on the measured fluorescence, cells were exposed to 50 μM NE for 20 min in the presence of 500 μM pargyline (MAO inhibitor) or 1 μM Ro-41-0960 (COMT inhibitor). These concentrations have been shown to be effective in other cell types (20, 35). In comparison with rabbit aortic SMCs exposed solely to 50 μM NE (N = 50 cells), both pargyline (N = 54 cells) and Ro 41-0960 (N = 62 cells) increased the average fluorescence values by only 1.31 ± 3.46 (7.1 ± 18% of controls) and 2.41 ± 2.69 (12.9 ± 14.4% of controls), respectively ($P >$ 0.05 for both). This may be due to the fact that these enzymes were overwhelmed by these concentrations of NE as reported before (4).

Steroid Inhibition of NE Uptake by Rabbit Aortic SMCs

To see whether steroids have an effect on NE uptake, rabbit aortic SMCs were exposed to 50 μM NE for 20 min in the presence or absence of different steroids. The NE concentration was chosen at 50 μM because MAO and COMT inhibitors did not significantly change the measured intracellular NE accumulation at this concentration (*see* earlier text). Steroid hormones were used at 1 μM, the concentration that produces 50% inhibition of effect of corticosterone for NE uptake into rabbit aorta (31). After 20 min of coincubation with 50 μM NE, corticosterone, hydrocortisone, and 17β-estradiol inhibited NE uptake into SMCs by 68.8 ± 14.4, 39.9 ± 7.7, and 54.3 ± 13.7%, respectively (means ± SEM for n = 3 experiments; all $P <$ 0.05 versus NE-exposed controls) (Figure 6, *left panel*).

The ability of steroids to inhibit NE uptake into SMCs within 20 min suggests a nongenomic mechanism of action. To provide additional evidence for this, the experiments were repeated with corticosterone rendered membrane-impermeant by conjugation to albumin (corticosterone-21-hemisuccinate-BSA [C-BSA]). After 20 min of coincubation with 50 μM NE, 1 μM C-BSA decreased NE uptake into SMCs by 62.3 ± 19.3% (mean ± SEM for n = 3 experiments; $P < 0.05$ versus NE-exposed controls; $P > 0.05$ versus NE plus corticosterone-exposed controls). To provide further evidence that transcription and protein synthesis were not required for corticosterone's action, experiments were repeated with 10 μM cycloheximide or 100 μM actinomycin D, concentrations shown to be effective in isolated arteries (36). Corticosterone inhibited NE uptake by 71.4 ± 6.8% in the presence of cycloheximide and 74.2 ± 1.6% in the presence of actinomycin D (means ± SEM for n = 3 exper-

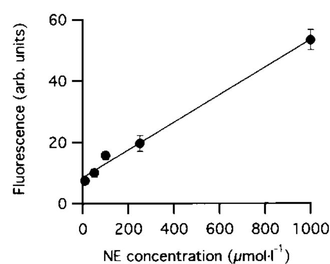

Figure 4. Relationship of normalized fluorescence levels to intracellular NE concentrations in ovine epithelial cells. Cells were permeabilized at −20°C with 100% methanol (twice, 5 min each time) and equilibrated in 10 to 1,000 μM NE for 20 min. A nearly linear relation was found between known NE concentrations and fluorescence levels. The line was fit to the data using linear regression. Shown are means ± SEM for N = 50 cells at each point.

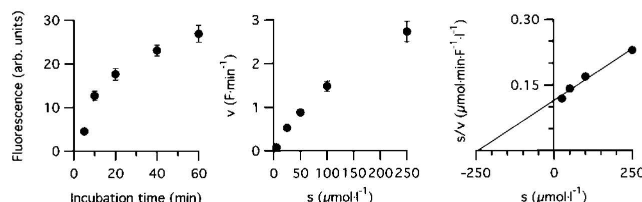

Figure 5. Time course and concentration dependence of NE uptake into rabbit aortic SMCs. *Left panel:* Uptake into cells exposed to 50 μM NE for different time periods. Shown are means ± SEM for N = 67–75 cells at each point. *Middle panel:* Uptake rates (v) were calculated from the 20-min time point and plotted against NE concentrations (s). F is fluorescence in arbitrary units. Shown are means ± SEM for N = 38–67 cells at each point. *Right panel:* A plot of s/v against s (Woolf–Hanes) that was used to estimate the apparent K_m value of NE uptake (245 μM; excluding the lowest NE concentration measured).

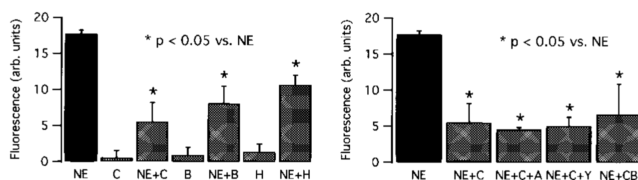

Figure 6. Steroid sensitivity of NE uptake. *Left panel:* Effects of 1 μM corticosterone (C), 1 μM 17β-estradiol (B), and 1 μM hydrocortisone (H) on NE uptake into rabbit aortic SMCs. Steroid hormones inhibited NE uptake after 20 min of coincubation with 50 μM NE. Shown are means ± SEM for $n = 3$ experiments. *Right panel:* Steroids work through a nongenomic mechanism; 1 μM corticosterone (C), 1 μM corticosterone plus 100 μM actinomycin D (A), 1 μM corticosterone plus 10 μM cycloheximide (Y), and 1 μM corticosterone conjugated to albumin (CB) to make it membrane-impermeant, all inhibited NE uptake into rabbit aortic SMCs. Shown are means ± SEM for $n = 3$ experiments.

iments; all $P > 0.05$ versus NE- and corticosterone-treated controls) (Figure 6, *right panel*). Therefore, the data presented here suggest that NE uptake by SMCs can be blocked by steroids acting through a nongenomic effect directly at the plasma membrane.

NE Uptake by Human Bronchial Arterial SMCs

The supply of human lungs and hence the yield of human bronchial arterial SMCs was limited for these studies. Therefore, a small number of experiments was performed. The cells were exposed to 50 μM NE with and without 1 μM corticosterone for 20 min. The bronchial arterial SMCs took up NE as expected and revealed fluorescence levels of 18.8 ± 0.8 arbitrary units (means ± SEM for $n = 2$ experiments); a value not significantly different from rabbit aortic SMCs, with 17.8 ± 0.7 arbitrary units (means ± SEM for $n = 3$ experiments). Corticosterone significantly inhibited NE uptake, reducing the fluorescence levels to 10.12 ± 1.23 arbitrary units (mean ± SEM for $n = 2$ experiments; $P < 0.05$ versus NE-exposed controls). The inhibition was less in human bronchial arterial SMCs than in rabbit cells (46.17 ± 6.5 versus 68.8 ± 14.4%, respectively). These data suggest that human bronchial arterial SMCs also express NE transporter that is functionally blocked by steroids.

Discussion

In the present study we showed that human bronchial arteries express mRNA of a steroid-sensitive membrane transporter for NE, namely EMT, and that SMCs freshly isolated from these vessels can take up NE in a steroid-sensitive fashion. Steroid sensitivity of NE uptake was further investigated in rabbit aortic SMCs that also expressed mRNA of EMT. The steroid effect occurred rapidly, was not altered by protein synthesis and transcription inhibitors, and could be reproduced with membrane-impermeant corticosterone. Together with our previous *in vivo* measurements (17, 37), these data suggest therefore that a steroid-sensitive NE uptake is likely to have an important role in the termination of noradrenergic signaling in the human bronchial circulation.

A newly developed single-cell assay was used to measure NE uptake by freshly isolated human bronchial arterial and rabbit aortic SMCs. Although vascular tissue preparations have been extensively used for such measurements before, tissues have the disadvantage of diffusion limitations through the extracellular space, leading to substrate distribution inequalities (38). Uptake rates have also been shown to differ depending on whether the catecholamine was applied to the intimal or adventitial side of the vessels (20). Using enzymatically dispersed, poly-L-lysine–immobilized cells and the modified SPG histofluorescence technique (24, 39), we eliminated these variables. Further, the method described here allowed experiments with highly limited numbers of SMCs (< 100 cells) that were freshly isolated from small vessels (e.g., human bronchial arteries). We validated our single-cell NE uptake assay using SMCs isolated from rabbit aorta, a more accessible systemic artery, by comparing the obtained results with the readily available data of previous studies. We showed that: (*1*) NE uptake was time-dependent, with a time course expected for an uptake mechanism as shown by others (11, 12, 20, 31); (*2*) NE uptake rate was concentration-dependent, with an estimated K_m value in the range of those previously published (25, 32, 33); and (*3*) a near-linear correlation exists between NE concentrations and measured fluorescence. The experimental NE concentrations (5 to 250 μM) used to measure NE uptake and the steroid inhibition in human bronchial arterial and rabbit aortic SMCs were in the estimated range of the neuromuscular junctional NE concentrations (i.e., approximately 200 to 300 μM initially, and ⩽ 10 μM finally) (40), thereby providing physiologically relevant information about the role of NE uptake in sympathetic neurotransmission.

The rapid inhibition of NE uptake into human bronchial arterial and rabbit aortic SMCs suggests a nongenomic mechanism of action. This would not require changes in gene expression (for review, *see* 41–43) and more readily explains the rapid inhibition of NE uptake. Our additional data (insensitivity to transcription and protein synthesis inhibitors, activity of the macromolecule-coupled steroid) confirm unambiguously that steroids inhibit NE uptake by rabbit aortic SMCs via a plasma membrane effect. Other acute/rapid nongenomic steroid actions have been linked to a change in membrane potential resulting from activation of maxi-K channels (44), the inhibition of L-Ca^{2+} channels (45), an increase in the membrane binding of calmodulin (46), and changes in the lipid composition of the plasma membrane (36). The exact link between nongenomic steroid action and the inhibition of NE uptake by SMCs needs further elucidation.

We showed the expression of mRNA for a membrane transporter, namely EMT, in both human bronchial arteries and rabbit aorta. RNA samples for RT-PCR were extracted from the media of these vessels, therefore the possibility of EMT mRNA amplification from cells other than SMCs (e.g., fibroblasts, nerves) is possible but unlikely. Inasmuch as EMT is known to transport NE (11–14), it may be the primary transporter responsible for the NE uptake by SMCs in both human bronchial arteries and rabbit aorta. EMT expression further confirms the functional measurements of NE uptake. Although two other membrane transporters, OCT1 and OCT2, have been shown to mediate NE uptake processes by non-neuronal cells (for review,

see 47–49), our data show that NE uptake by human bronchial arterial and rabbit aortic SMCs occurs through either OCT2 or EMT because steroids blocked NE uptake by these cells at concentrations not expected to block OCT1 (50). Because OCT2 has shown to be primarily restricted to kidney (8, 10) with detectable expression in the spleen, placenta, intestine, and brain (10, 51), the uptake mechanism in SMCs is most likely due to EMT. Further, because OCT1 and OCT2 have not been found in the lung and EMT is expressed in human bronchial arteries, we think that EMT is the steroid target in these vessels.

In summary, our data show that GSs interfere with NE uptake by SMCs freshly isolated from human bronchial arteries. This could consequently increase NE concentration at α-adrenergic receptor sites of the bronchial vascular smooth muscle. The rapid inhibitory effect of GSs, which was demonstrated on large conduit vessels, may also potentiate the α-adrenergic responsiveness in resistance vessels of the bronchial vasculature and decrease blood flow. Therefore, although other, alternative mechanisms may be involved (52, 53), we suggest that the inhibitory effect of GSs on NE uptake could explain or contribute to the α_1-adrenergic antagonist–blockable, rapid, and transient bronchial vasoconstriction measured after inhaled GS *in vivo*.

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