Norepinephrine transport by the extraneuronal monoamine transporter in human bronchial arterial smooth muscle cells

Gabor Horvath, Zoltan Sutto, Aliza Torbati, Gregory E. Conner, Matthias Salathe, and Adam Wanner

Norepinephrine (NE) released from airway sympathetic nerve endings, mediates vasoconstriction in the tracheobronchial circulation (4). NE acts largely via postjunctional α1-adrenoceptors on bronchial vascular smooth muscle cells (SMC). The NE concentration and duration at its receptor determine the extent of neurogenic vasoconstriction. Because NE is metabolically stable in the extracellular space (i.e., the metabolizing enzymes are localized intracellularly) and because NE cannot freely diffuse through membranes, the NE concentration at postjunctional α1-adrenoceptors is largely dependent on the rates of release, neuronal reuptake [uptake1, or cocaine-sensitive uptake (36)], and extraneuronal uptake [uptake2, or steroid-sensitive uptake (7)]. In contrast to other tissues where uptake1 predominates (7), NE removal from the extracellular space in the airways by uptake2 is five times greater than removal by uptake1 (31), possibly because of the relatively sparse sympathetic innervation (6). Inhibition of uptake2 in the airways is therefore expected to increase overall sympathetic tone and cause local vasoconstriction, in keeping with observations of uptake2 inhibition in other vascular beds (9, 19, 21, 27).

We previously showed that human tracheobronchial blood flow decreases in response to α1-adrenergic stimulation in vivo (2) and that inhaled glucocorticosteroids (GSSs) cause an acute, α1-adrenoceptor-mediated bronchial vasoconstriction (20, 26). This GSS-mediated vasoconstriction occurred too rapidly to be due to the "classical," transcriptional steroid effect (1, 23), suggesting that GSSs exert this vasoconstrictive effect via a nongenomic action (39). In search of such a nongenomic action, we previously showed that GSSs inhibit NE uptake by rabbit aortic SMC in a membrane-dependent fashion and that rabbit aortic SMC express mRNA of the GS-sensitive, extraneuronal monoamine transporter [EMT, or organic cation transporter 3 (OCT-3)] (12, 15). Preliminary observations in human bronchial arterial SMC showed that these cells also exhibit GS-sensitive NE uptake and express EMT mRNA (15). These findings suggested that inhaled GSSs could cause bronchial vasoconstriction by acutely interfering with NE uptake into bronchial vascular SMC, but the process remained to be carefully characterized in human cells.
The present investigation was designed to expand our preliminary human data by carefully characterizing NE uptake into freshly isolated human bronchial arterial SMC and determining mRNA expression profiles of different known NE transporters in these cells. Furthermore, we studied whether the inhibitory effect on NE uptake by corticosterone is also applicable to GSs used in the treatment of airway diseases. Finally, using immunocytochemistry, we examined the presence of a specific plasma membrane binding site for corticosterone in human bronchial arterial SMC.

MATERIALS AND METHODS

Materials. All media and agents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Human bronchial arterial SMC isolation. Donor lungs, rejected for transplantation, were obtained through the University of Miami Life Alliance Organ Recovery Agency with approval from the local Institutional Review Board. Donors had no history of lung diseases and were not preselected on the basis of their age or gender. Lungs obtained from at least three different donors were used for each experiment. Major branches of bronchial arteries from the main bronchi (~0.5–1 mm diameter) were excised using a dissecting stereomicroscope under sterile conditions. To confirm that the dissected structure was, in fact, an artery (thick muscular wall and narrow lumen), a small portion of each vessel was fixed in 4% formaldehyde buffered with PBS, processed according to regular procedures for histology, and stained with hematoxylin and eosin. The rest of the vessel was dissected from adhering fat and connective tissue and opened longitudinally. Endothelial cells were removed by scraping the inside surface. From this muscle preparation, strips were cut transversely and immediately used for RNA extraction (see below) or cell isolation. SMC were isolated as described previously (15, 16), with some modifications. Briefly, muscle strips were transferred to a constantly oxygenated incubation solution (137 mM NaCl, 4.17 mM NaHCO3, 0.34 mM Na2HPO4, 5.37 mM KCl, 0.44 mM KH2PO4, 7 mM glucose, 0.15 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, 0.02% BSA, pH 7.4) containing papain (1.5 mg/ml) and 2 mM DPT and incubated at 37°C for 30 min with shaking. Then the muscle strips were transferred to a constantly oxygenated incubation solution containing collagenase type F (1.5 mg/ml) and hyaluronidase type I-S (1 mg/ml) and incubated at 37°C for an additional 20 min with shaking. At the end of the digestion period, individual SMC were obtained by gentle tituration, followed by filtration through a 500-µm sieve. Finally, cells were collected by centrifugation at 1,000 g for 3 min and resuspended in fresh (enzyme free) incubation solution. The viability of freshly isolated SMC after enzymatic dispersion was always >95% as tested by trypan blue exclusion. The SMC suspension was deposited onto human placental collagen (type VI)-coated glass coverslips. Cells were allowed to settle for 60 min at 37°C before NE uptake experiments.

Cell culture techniques. For immunocytochemical detection of a corticosterone binding site, bronchial arterial SMC were maintained for 3 days in DMEM (Life Technologies, Rockville, MD) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) within a humidified atmosphere containing 5% CO2 at 37°C. This was necessary because acutely dissociated cells did not sufficiently adhere to the coverslips to withstand the staining process, and they were lost during the immunocytochemical procedure. The 3-day-cultured cells still revealed corticosterone-dependent NE uptake similar to the freshly isolated cells.

To provide a positive control sample for RT-PCR optimization of EMT mRNA, human renal carcinoma-derived Caki-1 cells were purchased from the American Type Culture Collection (Manassas, VA). Caki-1 cells were cultured in McCoy’s 5A medium (American Type Culture Collection) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere containing 5% CO2 at 37°C. Media were changed every other day.

Airway epithelial cell air-liquid interface cultures were prepared as described previously (22).

RT-PCR analysis of mRNA expression of NE transporters. NE transporter expression was determined in bronchial arterial smooth muscle and compared with expression in Caki-1 cells, airway epithelium, and brain, liver, and kidney tissues. Total RNA was extracted from freshly isolated bronchial arterial smooth muscle as well as cultured Caki-1 and airway epithelial cells using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA). RNA samples were treated with DNase (DNase I Amplification Grade, Life Technologies), precipitated with ethanol, and quantified spectrophotometrically at 260 nm. Good quality of isolated RNA (28S-to-18S rRNA ratio > 1.75) was confirmed using an RNA 6000 LabChip Kit (Agilent Technologies, Palo Alto, CA) and a bioanalyzer (model 2100, Agilent Technologies) provided by the University of Miami DNA Microarray Facility. Total RNA samples from human brain, liver, and kidney were purchased from Ambion (Austin, TX). RNA (1 µg per sample) was used for first-strand cDNA synthesis with Superscript II RT (Life Technologies) using oligo(dT)16 primers. For PCR amplification, oligonucleotide primers were designed on the basis of the published sequences of neuronal epinephrine transporter (NET; GenBank NM_0001043), organic cation transporter 1 and 2 (OCT-1 and OCT-2; GenBank NM_003057 and GenBank NM_003058), extraneuronal monoamine transporter (EMT; GenBank NM_021977), and GAPDH (GenBank NM_002046) cDNAs (Table 1). PCR amplifications were done using Taq DNA polymerase (Life Technologies) using opti-

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NET, neuronal epinephrine transporter; OCT-1 and OCT-2, organic cation transporter-1 and -2; EMT, extraneuronal monoamine transporter; Tm, annealing temperature; NE, norepinephrine.
mized annealing temperatures and cycle numbers for each primer pair (Table 1). RT-PCR products were electrophoresed on ethidium bromide-stained 2% SeaKem agarose (BMA, Rockland, ME) gels. Control reactions were performed in the absence of RT to verify that the amplified products were from mRNA, and not from genomic DNA contamination. In the absence of RT, no PCR products were observed. To confirm specific amplification, RT-PCR products were purified on a silica spin column (Qiaquick PCR Purification Kit, Qiagen) and sequenced by the University of Miami DNA Core Laboratory. Sequences were compared with the published cDNA sequences by PileUp (Wisconsin Package, GCG, Madison, WI).

**NE uptake experiments.** For NE uptake studies, coverslips with SMC were placed in 12-well cell culture clusters (Corning, Corning, NY), and the cells were exposed to incubation solution containing NE with or without different NE transporter inhibitors (see below) in a humidified atmosphere containing 5% CO₂ at 37°C. To inhibit the intracellular NE-metabolizing enzymes, 500 μM pargyline (a monoamine oxidase inhibitor) (14) and 1 μM Ro-41-0860 (a catecholaminemethyltransferase inhibitor) (30) were added to the incubation solution 30 min before the NE uptake experiments. The following inhibitors were used: 1 μM desipramine, which inhibits only NET (Kᵣ = 4 nM) (3), 1 μM corticosterone, which inhibits OCT-1, OCT-2, and EMT (IC₅₀ = 21.7, 34.2, and 0.29 μM, respectively) (3, 25, 33), or 1 μM O-methylisoprenaline (Boehringer Ingelheim), which inhibits OCT-2 (Kᵣ = 580 μM) and EMT (IC₅₀ = 4.38 μM), but not OCT-1 (10, 33). GSs, such as corticosterone, budesonide, and methylprednisolone, were dissolved in ethanol and freshly diluted into the incubation solution just before use. The final concentration of ethanol was 0.1%, a concentration with no significant effect on NE uptake measurements as compared with a vehicle control. After they were air-dried for 5 min, the specimen was covered with 1% glyoxylic acid monohydrate, pH 7.4) at room temperature. After NE uptake experiments, coverslips with SMC were then washed with SPG solution (0.2 M sucrose, 236 mM KH₂PO₄, 1% glyoxylic acid monohydrate, pH 7.4) at room temperature. After being washed with PBS and mounted in permanent aqueous mounting medium (Gel/Mount, Biomedia, Foster City, CA), cells were visualized on the Nikon Eclipse E600FN microscope described above, and TRITC fluorescence was imaged using an appropriate excitation-emission filter set.

**Fluorometric NE uptake assay.** At the end of the incubation period, SMC were washed with ice-cold incubation solution. Intracellular NE was visualized using a sucrose-potasium phosphate-glyoxylic acid (SPG) method described for tissue slices (37) and adapted by us for use in isolated vascular SMC (15, 16). Briefly, coverslips with SMC were washed with SPG solution (0.2 M sucrose, 236 mM KH₂PO₄, 1% glyoxylic acid monohydrate, pH 7.4) at room temperature. After they were air-dried for 5 min, the specimen was covered with a drop of light mineral oil. Then the sample was sealed with a coverslip and placed in an oven at 95°C for 2.5 min. To quantify fluorescence, a microscope (Eclipse E600FN, Nikon, Melville, NY) with a Lambda DG-4 excitation system (Sutter Instruments, Novato, CA), a cooled charge coupled device camera (Coolnap HQ, Roper Scientific), and ISee software (ISee Imaging Systems, Raleigh, NC) were used. Cells were imaged at ×600 magnification with differential interference contrast microscopy, and individual cells were identified as regions of interest. For quantification 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 charge coupled device camera was always set to a predefined gain, which was held constant throughout the experiments. SMC fluorescence was measured by selecting five well-separated regions on each coverslip (5–10 cells per region). Each single cell’s mean fluorescence intensity value (Fᵢ), expressed in arbitrary units, was normalized for background fluorescence by subtracting the mean Fᵢ of SMC from the same tissue that had not been exposed to NE. Average NE uptake of each experimental group was calculated using the mean normalized Fᵢ of all cells. Because we have shown that the intracellular fluorescence is nearly linear to the intracellular NE concentration over a wide concentration range (15), we chose to report here the fluorescence in arbitrary units and only convert the values to NE concentration for the Kᵢ determination of NE uptake.

**Immunohistochemical detection of a plasma membrane binding site for corticosterone in bronchial arterial SMC.** Cells maintained on collagen-coated coverslips for 3 days were washed three times with PBS and incubated with 1 μM BSA, 1 μM corticosterone-21-hemisuccinate-BSA, or 1 μM corticosterone-21-hemisuccinate-BSA + 100 μM corticosterone for 5 min at 37°C. Then the cells were fixed with 4% paraformaldehyde buffered with PBS for 30 min at room temperature. Fixed cells were washed with PBS containing 200 μg/ml goat IgG (blocking buffer) and incubated with blocking buffer for 60 min at room temperature. The cells were washed again with blocking buffer and then incubated with rabbit anti-BSA IgG primary antibody (10 μg/ml; Molecular Probes, Eugene, OR) for 60 min at room temperature. After they were washed again with blocking buffer, the cells were incubated with a tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG secondary antibody (20 μg/ml) for 60 min at room temperature. After being washed with PBS and mounted in permanent aqueous mounting medium (Gel/Mount, Biomedia, Foster City, CA), cells were visualized on the Nikon Eclipse E600FN microscope described above, and TRITC fluorescence was imaged using an appropriate excitation-emission filter set.

**Data analysis and statistical methods.** Uptake experiments were carried out in triplicate with measurements on 25–50 cells each. Values are means ± SE. For time-course analysis of NE uptake, the data were fit with nonlinear regression methods using Prism version 3.0a (GraphPad Software, San Diego, CA) with the following equations: A(t) = kᵢ/kᵢout *[1 − e⁻^(−kᵢt)], where A(t) is uptake of NE at time t, kᵢ, and kᵢout are rate constants for inward and outward transport, respectively, and t is incubation time. The variables for the fit were kᵢ and kᵢout and, thus, were determined during the fitting procedure.

Kᵢ was determined with a nonlinear regression fit (Prism) using the following equation: y = (V_max * x)/(Kᵢout + x), where y is uptake of x amount of NE. Again V_max was a variable of the fitting procedure.

For IC₅₀ calculations, the data were fit to a multisite inhibition model (Prism) using the following equation: y = inhibition_max + (inhibition_min − inhibition_max)/(1 + IC₅₀⁻¹⁻¹⁺⁻¹), where y is the effect of x amount of the inhibitor.

Statistical significance was determined with an unpaired Student’s t-test for comparison of two groups and ANOVA followed by the post hoc Tukey-Kramer honestly significant difference test for multiple groups. P < 0.05 was considered significant.

**RESULTS**

**NE transporter mRNA expression.** Because nonneuronal cells may express various and/or multiple transport systems for NE, RT-PCR was used as described in MATERIALS AND METHODS to examine the presence of neuronal [NET (29)] and nonneuronal [OCT-1 (11), OCT-2 (28), and EMT] transporter mRNAs. On the basis of the published expression data, human brain, liver, kidney, and Caki-1 cell mRNA samples were used to optimize the RT-PCR for NET, OCT-1, OCT-2, and...
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EMT mRNAs, respectively. Gel electrophoresis of the RT-PCR products for these samples showed bands of expected sizes (Fig. 1). Gel purification and sequence analysis confirmed that these amplicons were fragments of the corresponding NE transporter cDNAs. The isolated fragments contained only exon sequences (from ≥3 different exons for each), confirming the amplification of mRNA, rather than genomic sequences. Interestingly, brain and kidney expressed mRNA for every neuronal and nonneuronal NE transporter, whereas Caki-1 cells expressed only mRNA for every nonneuronal NE transporter. In addition to the previously reported mRNA expression of EMT (15), only OCT-1 mRNA was detectable in human bronchial arterial smooth muscle. The possibility of EMT and OCT-1 mRNA amplifications from cells other than bronchial arterial smooth muscle (e.g., endothelial cells and neurons) is unlikely because these cells are expressing NET mRNA (29), which was not detected (Fig. 1). Cultured airway epithelial cells expressed neuronal and nonneuronal (OCT-1 and EMT) transporters for NE.

**NE uptake characteristics in bronchial arterial SMC.** Because rabbit aortic and human bronchial arterial SMC showed steroid-sensitive NE uptake in our prior study (15) and because our new RT-PCR data indicated that EMT and OCT-1 mRNAs are expressed in human bronchial arterial SMC, NE uptake was measured in these cells in the absence or presence of 10 μM corticosterone. This concentration of corticosterone was chosen to inhibit EMT, but not other OCTs, in a significant amount (13; see also below). The amount of uptake inhibited by 10 μM corticosterone was defined as EMT-mediated.

To show first that NE uptake into human bronchial arterial SMC is a time-dependent phenomenon, cells were incubated in 50 μM NE with and without 10 μM corticosterone for 5, 15, 30, 45, and 90 min. NE uptake was detectable after 5 min and increased in a time-dependent fashion for 45 min (Fig. 2A). The concentration dependence was evaluated by incubating the cells in 25, 50, 250, and 1,000 μM NE with and without 10 μM corticosterone for 5 min. EMT-mediated uptake appeared to saturate (Fig. 2B). The calculated $K_m$ for NE uptake was 240 μM. This is close to the $K_m$ of 245 μM calculated for NE uptake into rabbit aortic SMC (15), a process likely mediated by EMT (16, 33).

The NE uptake inhibition by 10 μM corticosterone as well as the calculated $K_m$ for NE uptake suggested that EMT played the major part in NE uptake into human bronchial arterial SMC. To support this hypothesis, the pharmacological inhibitor profile of NE uptake was further investigated. In these experiments, SMC were exposed to 50 μM NE for 5 min without or with 1 μM desipramine, which inhibits only NET ($K_i = 4$ nM) (3), 1 μM corticosterone, which inhibits OCT-1, OCT-2, and EMT ($IC_{50} = 21.7, 34.2, \text{and } 0.29$ μM, respectively) (33), or 1 μM O-methylisoprenaline, which inhibits OCT-2 ($K_i = 580$ μM) and EMT ($IC_{50} = 4.38$ μM), but not OCT-1 (13, 33). Control experiments showed that these inhibitors did not affect the fluorometric assay at

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**Fig. 1.** RT-PCR analysis of norepinephrine (NE) transporter mRNA expression. With use of primers and conditions listed in Table 1, RT-PCR products were electrophoresed on ethidium bromide-stained 2% agarose gels. Gel purification and DNA sequencing were used to confirm specific amplification of neuronal epinephrine transporter (NET), organic cationic transporters 1 and 2 (OCT-1 and OCT-2), extraneuronal monoamine transporter (EMT), and GAPDH cDNAs. Starting RNA was from human brain, liver, kidney, Caki-1 cells, bronchial arterial smooth muscle (BASM), and airway epithelial cells (AEC) cultured at the air-liquid interface.

**Fig. 2.** Time and concentration dependence of NE uptake by human bronchial arterial smooth muscle cells (SMC). A: cells were exposed to 50 μM NE-containing incubation medium for 0–90 min. EMT-mediated NE uptake; ○, non-EMT-mediated NE uptake. EMT-mediated uptake was defined as amount of uptake inhibited by 10 μM corticosterone. B: EMT-mediated NE uptake rates (v) calculated from the 5-min time point and plotted against NE concentrations. F, fluorescence (in arbitrary units). Lines were fitted to experimental data using nonlinear regression methods. Values are means ± SE for triplicate experiments with 25–50 cells each.
the concentrations used. (It was necessary to use O-
methylisoprenaline below its reported IC$_{50}$ for EMT,
because its autofluorescence interfered with the assay
at higher concentrations.) Desipramine did not de-
crease NE uptake significantly (the decrease was only
4.2 ± 5.7% in triplicate experiments with 25–50 cells
each, $P > 0.05$ vs. control), whereas corticosterone and
O-methylisoprenaline inhibited NE uptake by 63.2 ±
6.9 and 57.8 ± 2.9%, respectively (in triplicate experi-
ments with 25–50 cells each, $P < 0.05$ vs. control for
both; Fig. 3). Together with NE transport kinetics data
(see above), these inhibitor characteristics suggest that
the majority of NE uptake is mediated by EMT.

**Inhibition of NE uptake by different GSs.** Because
inhaled and systemic drug administration can expose
the bronchial arterial smooth muscle to GSs, we inves-
tigated the effects of budesonide and methylpred-
nisolone on NE uptake by bronchial arterial SMC.
Similar to corticosterone, budesonide and methylpred-
nisolone inhibited NE uptake after 5 min of incubation
(Fig. 4). The inhibitory potency was ranked in the
following order: corticosterone > budesonide > meth-
ylprednisolone. These compounds inhibited EMT-me-
diated uptake (i.e., with only the amount of uptake that
is inhibited by 10 μM corticosterone taken into ac-
count) with apparent IC$_{50}$ values of 0.2 μM (close to the
reported IC$_{50}$ of 0.29 μM for EMT in other cells), 0.9
μM, and 5.6 μM, respectively.

To investigate whether the combination of budes-
onide and methylprednisolone inhibited NE uptake
differently from each single inhibitor, bronchial arte-
erial SMC were exposed to these compounds and 50 μM
NE for 5 min in the presence of 1 μM corticosterone
(which, by itself, almost completely inhibits EMT-me-
diated uptake). Because 10 μM budesonide and 30 μM
methylprednisolone alone caused the same degree of
NE uptake inhibition, these concentrations were used
for the experiments. In the presence of 1 μM cortico-
sterone and 10 μM budesonide or 30 μM methylpred-
nisolone, NE uptake was inhibited by 61.2 ± 8.2 and
64.8 ± 5.6%, respectively (in duplicate experiments,
$P > 0.05$ vs. corticosterone alone for both). Because
budesonide and methylprednisolone did not further
increase the inhibition induced by corticosterone, these
data suggest that these compounds also act on EMT.

**EMT inhibition by corticosterone is a nongenomic
effect.** Because NE uptake into rabbit aortic SMC is
mediated by a nongenomic action of GSs, independent
of transcription, protein synthesis, and membrane pen-
etration of the drug (15), we repeated these experi-
ments with human bronchial arterial SMC. In addi-
tion, using freshly dissociated human bronchial arte-
rial SMC, we examined reversibility of the
corticosterone effect and its dependence on the classi-
cal, intracellular GS receptor.

To investigate reversibility, human bronchial arte-
rial SMC were exposed to 1 μM corticosterone for 5
min, which blocked NE uptake by ~65% in our exper-
iments (see above). Then the cells were transferred to
a corticosterone-free medium containing 50 μM NE for
uptake measurements. There was no significant differ-
ence in NE uptake between SMC pretreated with
corticosterone but washed and control cells not exposed
to corticosterone (17.1 ± 2.2 vs. 21.1 ± 0.9 arbitrary units
in triplicate experiments with 25–50 cells each, $P$
> 0.05; Fig. 5A). Thus the inhibition of NE uptake by
corticosterone is reversible after removal of the GS.

To show that the classic genomic pathway (i.e., bind-
ing to cytoplasmic receptors followed by changes in
transcription and protein synthesis of target genes)
was not involved in corticosterone’s rapid action, 10
μM RU-486 (a cytoplasmic GS receptor antagonist),
100 μM actinomycin D (a transcription inhibitor), or 10
μM cycloheximide (a protein synthesis inhibitor) was
added to the incubation medium 30 min before the
addition of 50 μM NE and 1 μM corticosterone. Control
experiments showed that RU-486, actinomycin D, or
cycloheximide by themselves did not inhibit NE uptake
at the concentrations used. Corticosterone inhibited

![Fig. 3. Effects of NE transporter inhibitors on NE uptake by human bronchial arterial SMC. Cells were exposed to 50 μM NE for 5 min without (control) or with 1 μM desipramine (DESI) to inhibit NET, 1 μM corticosterone (CORT) to inhibit EMT, or 1 μM O-methyliso-
prenaline (OMI) to inhibit EMT. Values are means ± SE for triplicate experiments with 25–50 cells each. *$P < 0.05$ vs. control.](image-url)
Fig. 5. Corticosterone inhibits NE uptake into human bronchial arterial SMC via a nongenomic mechanism. A: cells exposed to 50 μM NE for 5 min without (control) or with 1 μM corticosterone. Additionally, cells were pretreated with 1 μM corticosterone for 5 min but exposed to NE in corticosterone-free solutions (CORT: NE). B: cells exposed to 50 μM NE without (control) or with 10 μM RU-486 (RU) + 1 μM corticosterone, 100 μM actinomycin D (ACT) + 1 μM corticosterone, or 10 μM cycloheximide (CYC) + 1 μM corticosterone. C: cells exposed to 50 μM NE without (control) or with 1 μM corticosterone-BSA conjugate (CORT:BSA). Values are means ± SE for triplicate experiments with 25–50 cells each. *P < 0.05 vs. control.

NE uptake by 66.7 ± 12.4, 62.1 ± 6.8, and 69.4 ± 11.6% in the presence of RU-486, actinomycin D, or cycloheximide, respectively (in triplicate experiments with 25–50 cells each, P < 0.05 vs. control for all, P > 0.05 vs. corticosterone treated; Fig. 5B). Thus the NE uptake inhibition by corticosterone does not depend on the cytoplasmic GS receptor or on changes in transcription or translation.

To investigate whether corticosterone acts at the plasma membrane, corticosterone was prevented from entering the cell by conjugation to the membrane-impermeant carrier protein BSA (40). Corticosterone-21-hemisuccinate-BSA (1 μM) decreased NE uptake into SMC by 57.7 ± 16.3% (in triplicate experiments with 25–50 cells each, P < 0.05 vs. control, P > 0.05 vs. corticosterone treated; Fig. 5C). Because membrane-impermeant corticosterone also inhibited NE uptake into human bronchial arterial SMC, we concluded that the acute effects of GSs are likely mediated through a GS binding site at the plasma membrane.

Plasma membrane binding site for corticosterone in human bronchial arterial SMC. Inasmuch as the activity of the membrane-impermeant corticosterone-BSA conjugate suggested that corticosterone acted at a site located at or in the plasma membrane, we looked for further evidence of a membrane-binding site for corticosterone in human bronchial arterial SMC. To immunocytochemically visualize the binding of membrane-impermeant corticosterone-BSA conjugate to the plasma membrane, rabbit IgG antibodies against BSA were used in combination with TRITC-labeled goat anti-rabbit IgG secondary antibodies. Fluorescent labeling was absent in control cells that were incubated in 1 μM BSA-containing medium for 5 min (Fig. 6, A and B), whereas cells incubated in 1 μM corticosterone-21-hemisuccinate-BSA for 5 min were specifically labeled (Fig. 6, C and D). Inasmuch as the conjugated corticosterone was necessary for BSA binding, this experiment demonstrated a binding site for corticosterone in the cell membrane. Competition with 100 μM corticosterone completely inhibited binding of corticosterone-21-hemisuccinate-BSA to the cells (Fig. 6, E and F), indicating that corticosterone binding to the plasma membrane was specific.

DISCUSSION

In the present study, we showed that human bronchial arterial SMC express mRNAs for two transmembrane NE transporters: EMT and OCT-1. Our uptake measurements demonstrated that NE uptake is mainly EMT mediated and acutely inhibited by various GSs, including budesonide and methylprednisolone, which are commonly used in clinical practice. The inhibitory action of GSs is rapidly reversible and seems to be mediated by a specific GS binding site in the plasma membrane.

After the original description of uptake2 (18), it became clear that three transmembrane transporters for catecholamines are expressed at various extraneuronal locations, either individually or simultaneously: OCT-1 (11), OCT-2 (28), and EMT [i.e., the classic uptake2 transporter (12)]. These transporters have a characteristic regional distribution that may reflect their specialized roles. OCT-1 appears to be confined to the liver, kidney, and intestine, OCT-2 is mainly expressed in the kidney and brain, and EMT has a broad tissue distribution (7). In blood vessels, OCT-1 mRNA is expressed at 15-fold lower levels than in the kidney, and OCT-2 mRNA is undetectable (35), whereas EMT mRNA expression is the highest (16, 35). In human bronchial arterial smooth muscle, we found RT-PCR amplicons for EMT and OCT-1 mRNAs.
developed and OCT-1 in NE uptake into these cells, a recently
tors (13). To investigate the physiological roles of EMT
quences homology, they can be pharmacologically dis-

Fig. 6. Immunochemical demonstration of a specific plasma mem-
brane binding site for corticosterone in human bronchial arterial
SMC. Cells were incubated with 1 μM BSA (A and B), 1 μM corti-
costerone-BSA (C and D), or 1 μM corticosterone-BSA + 100 μM
corticosterone (E and F). Rabbit anti-BSA primary and a tetramethyl-
rylodamine isothiocyanate-labeled goat anti-rabbit IgG secondary
antibody were used for immunocytochemistry. A, C, and E: differen-
tial interference contrast microscopy; B, D, and F: fluorescence
(tetramethylrhodamine isothiocyanate) microscopy. Original magni-
ification ×600.

Although EMT and OCT-1 show a high degree of
sequence homology, they can be pharmacologically dis-
tinguished on the basis of their sensitivities to inhibi-
tors (13). To investigate the physiological roles of EMT
and OCT-1 in NE uptake into these cells, a recently
developed fluorometric assay was used. In contrast to
other uptake measurement methods using tissue sam-

cortosterone and possibly other GSs inhibit NE
uptake by human bronchial arterial SMC through a
nongenomic mechanism as demonstrated by cortico-
sterone’s rapid action, insensitivity to changes in tran-
scription or protein synthesis, and lack of need to
penetrate the plasma membrane. In addition to these
findings, shown previously by us in rabbit aortic SMC
(15), we demonstrated here that corticosterone’s action
is acutely reversible and not mediated by the classic
cytoplasmic GS receptors. However, the mechanism by
which GSs acutely inhibit NE uptake is still unknown.
Because many rapid steroid actions influence plasma
membrane ion channels [e.g., K⁺ channels and, thus,
membrane potential (38)] and intracellular Ca²⁺-me-
diated signaling mechanisms (5), which have recently
been shown to play a key role in EMT’s functional
regulation (24), GSs might inhibit EMT through these
mechanisms. Alternatively, GSs might act directly on
the transporter.

A specific plasma membrane binding site for cortico-
sterone was also demonstrated in bronchial arterial
SMC by immunocytochemical labeling of the mem-
brane-impermeant corticosterone-BSA conjugate. Al-
though hydrophobic steroids are thought to pass easily
across a plasma membrane into a cell and interact with
cytosolic receptors, various rapid GS actions have re-
cently been attributed to plasma membrane-bound re-
ceptors (39). The existence of GS binding sites has been
reported previously, but not in vascular SMC. Their

gest a role for OCT-1, our functional studies, together
with the reported GS sensitivities of NE transporters
(13), indicate that EMT is the primary target for GSs in
bronchial arterial smooth muscle. Uptake via OCT-1 is
unlikely, inasmuch as ≥1 μM corticosterone did not
decrease NE uptake into the cells further, as expected
if OCT-1 were to play a role, given the IC₅₀ of its
inhibition by corticosterone (21.7 μM; Fig. 4). Because
papain digestion is unlikely to differentially digest
these related transport molecules, we do not believe
that the enzymatic treatment step influenced the re-

The remaining NE uptake after blocking EMT could
be due to nonspecific uptake, possibly related to the
measurement technique or, less likely, due to binding
of NE to receptors on the cell surface. Similar nonspe-
cific uptake has been observed in experiments on EMT
using the synthetic substrate 1-methyl-4-phenylpyr-
ridium (17).

There is a considerable variability in the ability of
different steroid hormones to inhibit EMT-mediated
uptake of catecholamines (32, 33). Among GSs, corti-
costerone is the most potent, with an IC₅₀ of ~130 nM
in Caki-1 cells (33). In our experiments performed on
human bronchial arterial SMC, corticosterone inhib-
ited NE uptake with an almost identical IC₅₀ (i.e., 0.2
μM). Although EMT inhibition requires relatively high
GS amounts, such concentrations can be encountered
after systemic GS drug administration and even in
stress conditions. The concentrations of inhaled GSs in
the airway wall may also be in the inhibitory range of
EMT (8).

Corticosterone and possibly other GSs inhibit NE
uptake by human bronchial arterial SMC through a
nongenomic mechanism as demonstrated by cortico-
sterone’s rapid action, insensitivity to changes in tran-
scription or protein synthesis, and lack of need to
penetrate the plasma membrane. In addition to these
findings, shown previously by us in rabbit aortic SMC
(15), we demonstrated here that corticosterone’s action
is acutely reversible and not mediated by the classic
cytoplasmic GS receptors. However, the mechanism by
which GSs acutely inhibit NE uptake is still unknown.
Because many rapid steroid actions influence plasma
membrane ion channels [e.g., K⁺ channels and, thus,
membrane potential (38)] and intracellular Ca²⁺-me-
diated signaling mechanisms (5), which have recently
been shown to play a key role in EMT’s functional
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cently been attributed to plasma membrane-bound re-
ceptors (39). The existence of GS binding sites has been
reported previously, but not in vascular SMC. Their
molecular identity as well as their role in inhibiting NE uptake remain to be clarified.

Although the detailed cellular mechanisms of GSs in vascular tissues remain largely unknown, they apparently include transcriptional regulation of gene expression (e.g., increased α-adrenoreceptor number and NO synthase inhibition) and nongenomic responses (e.g., uptake2 inhibition). NE uptake mechanisms have attracted increased interest recently, because several studies suggest that impaired NE reuptake could be responsible for increased sympathetic tone seen in essential hypertension (6) and postural tachycardia (34).

Because extraneuronal NE uptake is quantitatively larger than neuronal reuptake in the airways, it is likely that EMT has a role in regulating local concentrations of NE as well as other transported and intracellularly metabolized compounds. Our data suggest that the interaction of GSs with local NE metabolism is physiologically important to regulate the noradrenergic vasomotor tone in the tracheobronchial circulation and possibly other vascular beds. This mechanism can certainly explain the acute bronchial vasoconstrictive action of inhaled GSs.

DISCLOSURES

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