Uptake of Norepinephrine in an Isolated Artery From Normotensive Humans

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Previous studies have suggested that catecholamine uptake may play a role in vascular responsiveness in hypertension. The current study was undertaken to characterize amine uptake and effects of its inhibition in an isolated human artery from normotensive subjects and to provide a basis for future study in hypertensive individuals. Accumulation of tritiated norepinephrine into the artery of nonhypertensive subjects was time-dependent and 16.9-fold more than the incubation media concentration (1 μM) of amine after 60 minutes. There was a lesser accumulation of tritiated normetanephrine (3.1-fold), and it was not increased over time. Increasing the concentration of norepinephrine to 30 μM did not significantly change the proportional accumulation. Inhibition of neuronal uptake with cocaine (10 μM) reduced the average accumulation of both concentrations of tritiated norepinephrine to 3.9-fold (p < 0.001). Inhibition of extraneuronal uptake with corticosterone alone (10 μM) had no significant effect on average accumulation of norepinephrine, and where combined with cocaine, there was no further effect of corticosterone. Neither cocaine nor corticosterone had any effects on accumulation of normetanephrine. In spite of elimination of approximately 75% of the uptake of norepinephrine, cocaine had very little potentiating effect on mechanical responses to exogenous norepinephrine and neurally released transmitter. Thus, norepinephrine uptake in human cystic artery is characteristic of neuronal uptake, but cocaine treatment has only a very modest potentiating effect on responsiveness to endogenous norepinephrine and no significant effect on responsiveness to exogenous amine. (Hypertension 1991;18:348-354)

Methods

Subjects

Patients admitted to the Foothills Hospital for elective cholecystectomy for lithiasis were approached, and informed consent was obtained. A total of 177 human subjects were studied over a period of 6 years (January 1984 to April 1990). This project and its consent form were reviewed and approved by the Joint Ethics Committee of the University of Calgary and the Foothills Hospital. After consent was obtained, a clinical history was elicited via a written questionnaire, and subjects were excluded when this revealed any current medical condition other than cholelithiasis. Subjects taking any medication with known cardiovascular effects were also excluded. We have previously reported a lack of effect of drugs used for premedication and anesthesia on responses of rat tail arteries studied in the manner described for the arteries used in the present study. The following laboratory tests were performed: serum K+, serum creatinine, and urinalysis. Subjects were excluded when there was any abnormality in these laboratory investigations. Finally, the pathologic report on the gallbladder was reviewed, and subjects were excluded when any abnormality other than cholelithiasis was noted. Three lying and standing blood pressures were measured on the first hospital day and averaged.

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Subjects were considered normotensive at the time of study when all these recorded diastolic blood pressures were less than 90 mm Hg. Subjects with any diastolic blood pressure measurements 90 mm Hg or greater were excluded from the present analysis.

Preparation of Arteries

Immediately on removal of the gallbladder, the extramural artery was removed and placed in modified Krebs-Henseleit bicarbonate solution (in mM): NaCl 115.3, KCl 4.6, CaCl2 1.8, MgSO4 1.1, NaHCO3 22.1, glucose 7.8, ascorbic acid 0.11, and disodium EDTA 0.027 and gassed with 5% CO2 in oxygen. The artery was transported to the laboratory where excess fat and connective tissue were trimmed, taking care not to damage the outer tunica adventitia or inner tunica intima (endothelium). The outside diameter of this artery is 1.3±0.1 mm.8 Helical strips were cut under a dissecting microscope at an angle of approximately 75° to the longitudinal axis of the artery. Strips of artery (weight, 4.3±1.9 mg; unstretched length, 15.0±3.6 mm) were suspended between two parallel platinum wire electrodes in a 10 ml muscle bath containing Krebs-Henseleit solution at 36±1°C and gassed with 5% CO2 in oxygen. The tension required to produce optimum resting length was determined for each strip as previously described11 and was then applied to the strip. Tension changes were recorded isometrically. Although one to three arterial strips were obtained from each subject, usually only one strip was used for individual experiments, and when more than one was used, the results were averaged (i.e., n=subjects).

Electrical Stimulation

Transmural stimulation was started immediately after the length–tension determination. The techniques for electrical stimulation of postganglionic adrenergic nerve endings in the vessel wall have been described in detail elsewhere.12 Briefly, biphasic pulses with a total pulse width of 1.6 msec were used. Stimuli were administered from two Model S48 stimulators (Grass Instrument Co., Quincy, Mass.) via two Grass Model SIU5 stimulus isolation units. The output waveform was then amplified by a custom-built amplifier (University of Calgary Technical Services, Calgary, Canada). The voltages between the anode and cathode in the bath fluid and across a 1 Ω resistance connected in series were monitored on a Type 205 oscilloscope (Tektronix, Beaverton, Ore.). The current delivered to the bath was individually adjusted to 1.0 A for each electrode. This current setting has previously been demonstrated to be supramaximal, and biphasic stimulation does not accelerate the spontaneous oxidation of norepinephrine known to occur in alkaline physiological salt solutions.13 After a 30-second train of stimulation at one frequency followed by 90 seconds rest, the next frequency was tested without changing the bath solution. Frequencies tested were 4, 8, 12, and 16 Hz. Frequency–response curves were done before and after inhibition of either neuronal or extraneuronal amine uptake (see below). Control tissues had frequency–response curves done twice without exposure to amine uptake inhibitors. Contractions are expressed as a percent of the norepinephrine-induced maximum response obtained during the baseline concentration–response curve.

Concentration–Response Curves

Concentration–response curves were done in a cumulative fashion and half to full log increments were used in final bath concentration of agonist. Norepinephrine concentration–response curves were done before and after inhibition of either neuronal or extraneuronal amine uptake. Control tissues had norepinephrine concentration–response curves done twice without exposure to amine uptake inhibitors. Contractions are expressed as a percent of the norepinephrine-induced maximum response obtained during the baseline concentration–response curve.

Measurement of Neuronal and Extraneuronal Uptake

Arterial strips from an individual patient were prepared as described above and divided into equal portions of approximately 5 mg each. When sufficient artery was available, up to eight equal portions were studied, and each portion was used in a different experiment. One portion served as a control for norepinephrine uptake and another for normetanephrine uptake, using the concentrations and incubation times indicated below. The remaining portions were preincubated for 15 minutes with cocaine (10 μM inhibits neuronal uptake14,15), corticosterone (10 μM inhibits extraneuronal uptake15,16), or both inhibitors. Arteries were incubated in Krebs-Henseleit solution gassed with 5% CO2 in oxygen and kept at 36±1°C. Tritium labeled norepinephrine (l-ring-2,5-6-[3H]-norepinephrine; specific activity, 49.1 Ci/mmol; New England Nuclear, Boston, Mass.) or normetanephrine (DL-7-[3H]-normetanephrine; specific activity, 7.6 Ci/mmol; New England Nuclear) was then added to both control and treated tissues. Two concentrations (1 and 30 μM) of amine were used and two incubation times (15 and 60 minutes) were tested. In some tissues, sorbitol (p-[U-14C]-sorbitol); specific activity, 274 mCi/mmol; Amersham, Arlington Heights, Ill.) was concurrently added to the bath in a concentration of either 1 or 30 μM. At the end of the incubation period, the tissue was removed from the incubation fluid, briefly blotted, and weighed. Arteries were then homogenized in ice-cold 0.1N perchloric acid (0.05 ml/mg tissue) using a ground glass homogenizer. Five milliliters Aquasol II Solution (New England Nuclear) was added, and homogenization was repeated. This mixture was centrifuged at 4°C for 10 minutes using 14,500g. The pellet was discarded, and the supernatant was used for scintillation counting. A 0.1-ml aliquot of incubation media was also added to 5 ml Aquasol II Solution. Radioactivity of tissue, 3H and 14C, and incubation media were measured separately.
by liquid scintillation techniques (model Mark III, SearleAnalyticInc., Chicago, I11.) by using specific window settings for each isotope and by correcting for quenching by the external standard method. Total $^3\text{H}$ or $^{14}\text{C}$ uptake is expressed as the T/M ratio where T is disintegrations per minute per gram tissue and M is disintegrations per minute per milliliter incubation media. The degree of uptake inhibition was calculated as a percentage by the following formula:

$$\left[ \text{control}[^{3}\text{H}]-\text{NE}-\text{T/M ratio} - \text{inhibitor}[^{3}\text{H}]-\text{NE}-\text{T/M ratio} \right] \div \left[ \text{control}[^{3}\text{H}]-\text{NE}-\text{T/M ratio} \right] \times 100$$

In this formula, $^{14}\text{C}$-T/M ratio was the average of several experiments (see below); 0.6 for 1 $\mu$M and 1.3 for 30 $\mu$M. Sorbitol is used in these experiments to allow for adjustment of the portion of amine uptake that may be due to diffusion into the extracellular space.15 The proportion of total tritium uptake represented by $[^{3}\text{H}]$-norepinephrine was estimated by high-performance liquid chromatography (HPLC) techniques. Tissue was homogenized in 0.1N perchloric acid as described above and then centrifuged at 4°C for 10 minutes using 1,100g. The pellet was discarded and the supernatant was alkaline-sedi to pH 8.5 by adding 1.6 ml of 1M TRIS, 60 $\mu$l of 5 mM sodium bisulfate and 500 $\mu$l H$_2$O for a final volume of 3.4 ml. A 2.2-ml aliquot of bath fluid was alkaline-sedi in the same manner. Twenty milligrams alumina was added to each sample, and after gentle shaking for 15 minutes, the mixture was filtered in millipore microfiltration tubes, the alumina was washed three times with distilled H$_2$O, and the effluent was discarded. The alumina was resuspended in 200 $\mu$l of 0.1N perchloric acid, and after filtration, 20 $\mu$l of the acidic effluent was injected onto a plasma catecholamine resolve C18 column (Waters, Mississauga, Canada). Waters catecholamine solvent mixture (sodium acetate 50, citric acid 20, sodium 1-octane-sulfonate 3.75, di-N-butylamine 1, NaEDTA 135 mM in H$_2$O/methanol [95:5], pH 4.3) was used as the moving phase. Fractions of effluent were collected at 15- second intervals for the first 10 minutes, then 30-second intervals for 10 minutes, and those fractions containing norepinephrine were identified from standard solutions and an electron capture detector. Total $^3\text{H}$ in each fraction was determined by liquid scintillation techniques described above.

**Data**

Unless otherwise stated, data are presented as mean±SD. Responses to electrical stimulation and norepinephrine in the same strip before and after inhibitor treatment were compared using the paired t test. Comparison of unpaired data was by unpaired t test. Dichotomous data were compared by Fisher's exact test. Linear trends for relations of subjects' blood pressure, age, and weight to amine uptake were sought by multiple regression analysis to account for known relations between three independent variables (blood pressure, age, and weight). The analysis was done on all subjects and on men and women separately. The null hypothesis was rejected when $p<0.05$. Statistical analysis used programs in the Medical Research-Time Oriented Database (MR-TOD).17

**Drugs**

Norepinephrine bitartrate and corticosterone were supplied by Sigma Chemical Company, St. Louis, Mo. Cocaine hydrochloride was supplied by Allen and Hanbury Division of Glaxo Inc., Toronto, Canada. All drugs were dissolved in water, and final bath concentrations are expressed as moles per liter.

**Results**

**Cocaine- and Corticosterone-Sensitive Amine Uptake**

The effect of time on accumulation of tritium in cystic artery incubated with $[^{3}\text{H}]$-normetanephrine (1 $\mu$M) was studied in the arteries of 82 subjects. There were 59 women and 23 men who were 39±12 years of age. As can be seen in Figure 1, the accumulation of $^3\text{H}$ in the artery after incubation with $[^{3}\text{H}]-$norepinephrine increases from a T/M ratio of 5.3±3.4 at 15 minutes to 16.9±13.8 at ($p<0.001$) 60 minutes of incubation. The accumulation of $^3\text{H}$ in the artery after incubation with $[^{3}\text{H}]$-normetanephrine remains constant at a T/M of approximately 3 (3.4±1.3 versus 3.1±0.9; $p=\text{NS}$).

The effect of substrate concentration on accumulation of radioisotope in cystic artery incubated with $[^{3}\text{H}]$-norepinephrine, $[^{3}\text{H}]$-normetanephrine, and $^{14}\text{C}$-sorbitol for 60 minutes was studied in the arteries of 99 subjects (Figure 2). There were 68 women and 31 men who were 40±11 years of age. The overall accumulation of tritium increased when the concentration of $[^{3}\text{H}]$-norepinephrine was in-
increased from 1 μM to 30 μM, but the T/M ratio remained constant (16.9±13.8 versus 13.4±9.0; p=0.206). There was, however, a decrease in T/M ratio for [3H]-normetanephrine (3.1±0.9 versus 0.9±0.3; p<0.001) and an increase in T/M ratio for [14C]-sorbitol (0.6±0.1 versus 1.3±0.3; p<0.001) when their concentrations were increased from 1 μM to 30 μM.

The effects of cocaine and corticosterone and their combination on accumulation of tritium in arteries incubated for 60 minutes with [3H]-norepinephrine and [3H]-normetanephrine at concentrations of 1 and 30 μM were studied in arteries of 95 subjects (Figure 3). There were 66 women and 29 men who were 39±11 years of age. After 60 minutes incubation with [3H]-norepinephrine, cocaine treatment reduced the average T/M ratio for tritium from 16.9±13.8 to 3.9±1.8 (1 μM; upper left panel, p<0.001) and from 13.4±9.0 to 3.5±1.1 (30 μM; lower left panel, p<0.001). These T/M ratios represent 72±23% and 77±13% inhibition of norepinephrine uptake into the artery. In contrast, the average T/M ratio after incubation with [3H]-normetanephrine, cocaine treatment reduced the average T/M ratio for tritium from 16.9±13.8 to 3.9±1.8 (1 μM; upper left panel, p<0.001) and from 13.4±9.0 to 3.5±1.1 (30 μM; lower left panel, p<0.001). These T/M ratios represent 72±23% and 77±13% inhibition of norepinephrine uptake into the artery. In contrast, the average T/M ratio after incubation with [3H]-normetanephrine, cocaine treatment reduced the average T/M ratio for tritium from 16.9±13.8 to 3.9±1.8 (1 μM; upper left panel, p<0.001) and from 13.4±9.0 to 3.5±1.1 (30 μM; lower left panel, p<0.001). These T/M ratios represent 72±23% and 77±13% inhibition of norepinephrine uptake into the artery. In contrast, the average T/M ratio after incubation with [3H]-normetanephrine, cocaine treatment reduced the average T/M ratio for tritium from 16.9±13.8 to 3.9±1.8 (1 μM; upper left panel, p<0.001) and from 13.4±9.0 to 3.5±1.1 (30 μM; lower left panel, p<0.001). These T/M ratios represent 72±23% and 77±13% inhibition of norepinephrine uptake into the artery. In contrast, the average T/M ratio after incubation with [3H]-normetanephrine, cocaine treatment reduced the average T/M ratio for tritium from 16.9±13.8 to 3.9±1.8 (1 μM; upper left panel, p<0.001) and from 13.4±9.0 to 3.5±1.1 (30 μM; lower left panel, p<0.001). These T/M ratios represent 72±23% and 77±13% inhibition of norepinephrine uptake into the artery. In contrast, the average T/M ratio after incubation with [3H]-nor}
to corticosterone. The maximum response to norepinephrine during the baseline (pretreatment) concentration–response curve was 2.6±1.3 g developed tension. Finally, 45 arterial strips from 33 women and 12 men who were 41±15 years of age served as untreated controls. The maximum response to norepinephrine during the baseline (first) concentration–response curve was 2.8±1.6 g developed tension.

The norepinephrine concentration–response curves obtained at baseline and after treatment are illustrated in Figure 4. There were no differences in norepinephrine-induced mechanical responses at baseline in the three groups of tissues, and similarly, there were no differences among the three groups after treatment, although there was a slight but nonsignificant trend for corticosterone treatment to increase responses to electrically induced contractions. The frequency–response curves to electrical stimulation obtained at baseline and after treatment are illustrated in Figure 5. There were no differences in mechanical responses to electrical stimulation at baseline in the three groups of tissues, and similarly, there were no differences among the three groups after treatment, although there was a slight but nonsignificant trend for corticosterone treatment to increase responses to electrically induced contractions. The maximal responses at 16 Hz stimulation were only 12–15% of the maximum response to exogenous norepinephrine. There were no differences in mechanical responses to electrical stimulation at baseline testing of the three groups. However, there was a time-dependent, significant decrease in mechanical responses to electrical stimulation in the control arteries and those treated with corticosterone. Although cocaine treatment did not increase responses to electrical stimulation, it did attenuate the decreased responsiveness observed in the control and corticosterone-treated arteries. Thus, corticosterone had no effect on either norepinephrine-induced or electrically induced responses. Cocaine on average did not increase responses, but it did retard the time-dependent decrease in responsiveness in the case of electrically induced contractions.

Specificity of Norepinephrine Uptake

Storage conditions of stock solutions and samples of radiolabeled norepinephrine are critical in such experiments, and there are data to suggest that samples should be stored frozen at −80°C. HPLC was used in the present experiments to verify that tritium remained exclusively associated with the norepinephrine peak determined by electron capture. Incubation of our [3H]-norepinephrine stock solutions in the physiological salt solution (pH 7.4) at 37°C for 1 hour did not substantially alter the stability of the radiolabel, which remained almost exclusively associated with the norepinephrine peak with an estimated 15% or less loss of specificity (not shown). In Figure 6, sample chromatograms are shown demonstrating that the arterial tissue accumulates tritium almost exclusively in a single HPLC peak corresponding to the position of norepinephrine (Figure 6, left panel). In this particular example, cocaine treatment almost completely eliminated norepinephrine uptake (Figure 6, middle panel) and cocaine and corticosterone together (Figure 5, right panel) did eliminate it. No significant peaks of radioactivity other than for norepinephrine itself were found in the incubation media after 1 hour of incubation with tissue (not shown).

Relation of Norepinephrine Uptake to Subject's Blood Pressure

As is already known, a significant relation was found between age and blood pressure and weight and blood pressure. For example, age was related to
systolic blood pressure with $r=0.377$ and $p<0.001$, and weight was related to systolic blood pressure with $r=0.472$ and $p<0.001$. Therefore, multivariate analysis was used to examine for relations between norepinephrine uptake and blood pressure independent of age and weight. There was no relation between norepinephrine uptake in cystic artery and blood pressure over the normotensive range of pressures examined. For example, the relation between mean arterial blood pressure and control norepinephrine T/M ratio had an $r=0.035$ and $p=0.756$, and the relation between mean arterial blood pressure and cocaine treatment norepinephrine T/M ratio had an $r=0.059$ and $p=0.0603$. The lack of any such relation was noted for men and women.

**Discussion**

**Cocaine- and Corticosterone-Sensitive Amine Uptake**

Uptake of catecholamine in the human cystic artery is most characteristic of that termed Uptake\textsubscript{2} or neuronal uptake.\textsuperscript{14,15,19} Specifically, approximately 75\% of the uptake of norepinephrine is inhibited by cocaine; very little normetanephrine is accumulated in comparison with norepinephrine. The uptake of norepinephrine by this process did not appear to be significantly saturated over the concentration range examined (1–30 $\mu$M) because at both of these concentrations the T/M ratios were equivalent (Figure 2). In other tissues and species, the higher concentration used may have been expected to result in evidence of a second, extraneuronal uptake process,\textsuperscript{15–17,19,20} but on average, none was seen in this human artery. The time course of norepinephrine accumulation (Figure 1) seems roughly similar to that reported for blood vessels of other species\textsuperscript{14} where steady-state saturation occurs within 1 hour, but the present data are incomplete for demonstration that uptake is saturable within 1 hour in cystic artery.

The fact that a plateau for time-dependent accumulation of [H]-norepinephrine was not identified may be part of the reason the high variance was noted for estimate of Uptake, in cystic artery. However, most of the variance is due to a few outlier subjects, and we have previously noted\textsuperscript{21} marked interindividual variation in the properties of cystic artery. Catecholamine uptake characteristic for that termed Uptake\textsubscript{2} or extraneuronal uptake\textsuperscript{15–17,19,20} was not demonstrated since on average T/M ratios were unaltered by corticosterone treatment and uptake inhibition by cocaine was not augmented by combined treatment with cocaine and corticosterone (Figure 3). The latter was true at both concentrations of norepinephrine used. Furthermore, there was only modest accumulation of normetanephrine, which was equal in magnitude after 15 and 60 minutes of incubation and the accumulation of the latter amine did not increase when the incubation concentration was increased from 1 to 30 $\mu$M. Normetanephrine is thought to be preferentially accumulated by extraneuronal uptake.\textsuperscript{19}

**Effect of Cocaine and Corticosterone Treatment on Arterial Mechanical Responses**

In spite of clear evidence for a substantial effect of cocaine on norepinephrine uptake, cocaine had only very modest effects on development of mechanical responses to exogenous norepinephrine and nerve stimulation. In fact, cocaine or corticosterone treatment had no significant effect on responsiveness to exogenous norepinephrine (Figure 4). Cocaine treatment did have a minor potentiating effect on mechanical responses evoked by transmural electrical stimulation of postganglionic sympathetic neurons leading to mechanical responses due to the release of neuronal norepinephrine (Figure 5). Inhibition of neuronal uptake by cocaine generally has minor effects on responses to exogenous norepinephrine in blood vessels of other species, particularly when those blood vessels have a network of sympathetic neuroeffector complexes that is small in relation to the muscular layer.\textsuperscript{22–24} Thus, in the cystic artery as in blood vessels of other species, an effect of cocaine, an inhibitor of neuronal amine uptake, is most characteristic of that termed Uptake\textsubscript{2} or neuronal uptake.
extrajunctional α-adrenergic receptors are of the α₁ subtype.9 Thus, the effect of cocaine in human cystic artery is not specific for a particular subtype of α-adrenergic receptor, although it does seem most prominent for responses to neuronal norepinephrine. Measurement of development of mechanical responses to exogenous norepinephrine and nerve stimulation, however, may not be the best assessment of effects of an agent like cocaine or of the importance of the neuronal uptake process itself. Relaxation after contraction by norepinephrine may be a better index of the effects of inhibition of a process that is responsible for dissipation of the amine.22–24

Effects of cocaine on norepinephrine responses have been reported to be much more evident in arteries of the spontaneously hypertensive rat1–2 and, albeit only modest in size, in small resistance arteries of human hypertensive subjects.3 Unfortunately, in the present experiments we were unable to obtain arteries of a sufficient number of hypertensive subjects to compare them with arteries of normotensive subjects. However, the present data is consistent with the previous observation of Aalkjaer et al4 that cocaine has little effect on development of mechanical responses induced by norepinephrine in human subcutaneous small arteries from normotensive subjects.

Specificity of Norepinephrine Uptake

As has been previously reported,18 it was noted that storage conditions such as temperature and duration can result in loss of the specificity of the radiolabel, and careful attention to these details is important. The present experiments indicate that uptake of radiolabel in this artery on incubation with tritiated norepinephrine is exclusively due to the accumulation of the parent amine. No significant peaks of radioactivity attributable to metabolites were noted in the tissue (Figure 6). In addition, metabolism of norepinephrine and release of tritiated metabolites into the bathing media were not detected by the techniques used in this study. There are at least two factors potentially contributing to the latter observation. First, the artery is relatively poorly innervated and the present experiments suggest that most catecholamine metabolism in this artery would be intraneuronal. Second, there would be a marked dilutional effect from incubation of approximately 5 mg artery in 10 ml incubation fluid.

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