Aminergic Histofluorescence and Contractile Responses to Transmural Electrical Field Stimulation and Norepinephrine of Human Middle Cerebral Arteries Obtained Promptly After Death

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The responses of cerebral arteries to catecholamines and sympathetic nerve stimulation show wide variation between animal species. We examined the catecholaminergic histofluorescence and the contractile responses elicited by transmural electrical field stimulation and norepinephrine (NE) in proximal segments of human middle cerebral artery (MCA) obtained during autopsy. Twenty-four percent of the specimens were obtained within 2 hours and 76% within 4 hours of death. A moderately dense catecholaminergic histofluorescence was seen in all segments of human MCA using the glyoxylic acid technique, counterstained with pontamine sky blue. However, only seven of 35 (20%) MCA segments tested showed tetrodotoxin-blocked transmural electrical field stimulation contractions, and all of these were harvested within 4 hours of death. The responses were mostly seen in the most proximal MCA segments and, at 32 Hz, only achieved 6±1% of the maximal tissue contraction. NE caused two distinct responses in human MCA segments. At low concentrations, it acts via an α-like adrenoceptor to cause contractions 20 ±3% of the maximal tissue response. The NE ED₅₀ for the three successive segments were not different from each other; the value for the most-proximal segment was 7.9±0.2×10⁻⁷ M. At concentrations above 10⁻⁵ M, this catecholamine acts on low-affinity sites resistant to α-adrenergic antagonists causing contractions that at 10⁻³ M reach 52±5% of the maximal tissue response. Our results suggest that it is important when studying human blood vessels to harvest them as soon as possible after death, that the smooth muscle response to sympathetic activation is small, frequently absent, and that the postsynaptic sympathetic mechanism includes not only α-adrenoceptors but low-affinity sites as well. (Circulation Research 1989;65:316-324)

A dense, catecholaminergic innervation within the adventitia of cerebral arteries has been consistently identified using a histofluorescent technique in many species, including man.¹⁻⁵ However, there seems to be a considerable species variation in the type and subtype of adrenoceptors on the vascular smooth muscle cells in cerebral arteries.⁶ For example, segments of rabbit large cerebral arteries show responses mediated by α₁-like adrenoceptors while responses of equivalent segments from the adult pig depend exclusively on β-adrenoceptors.⁷⁻⁹ The norepinephrine (NE)-induced contractions of dog cerebral arteries are blocked by yohimbine but not by prazosin, suggesting the presence of α₂-adrenoceptors, while the receptors in the monkey and human, like the rabbit, exhibit α₁-adrenoceptor-like properties.¹⁰ Thus, generalization cannot be made from single animal studies, and extrapolation to the human is unreliable. Demonstrations of catecholamine innervation by histofluorescence in human cerebral arteries are scant,⁴⁻⁵,¹¹ and the adrenergic responses of these arteries are mentioned in only a few reports. Early studies simply showed the relative insensitivity of human cerebral compared with systemic arteries to exogenous NE.¹²⁻¹⁴ Later investigations surveyed human cerebrovascular responses to a number of pharmacological agents,¹⁵,¹⁶ but often the same iden-
tified vascular segment was not used in the experimental series, and arteries were usually obtained 8–60 hours after death.

The present study was undertaken to evaluate some of the catecholaminergic mechanisms present in three standardized segments of human proximal middle cerebral arteries (MCAs) harvested never more than 6 hours, and in the majority of instances much more promptly, after death. Catecholamine histofluorescence and the contractile response of isolated segments in vitro to transmural electrical field stimulation (TEFS) and exogenous NE were investigated.

Only 20% of the MCA segments exhibited neural-mediated contractions, and these were small. The NE responses were the composite of small responses initiated by lower doses via α-adrenoreceptors and of larger contractions originating from low-affinity sites not influenced by α-adrenoreceptor antagonists. These results provide quantitative information regarding the adrenergic neuroeffector mechanism of human MCAs obtained under as ideal conditions as practicable, allowing the artery to be placed in the perspective of similar studies on cerebral arteries of other species. The human adrenergic neural vascular mechanism has recently been reviewed.17

A preliminary communication of this paper has been previously made.18

Materials and Methods

When autopsy permission had been obtained after a hospital death, the brain was removed as soon as possible using standard autopsy procedures.19 The MCA was dissected free of surrounding arachnoid from its origin at the internal carotid to beyond a tertiary branching point as it coursed through the Sylvian fissure. Care was taken in handling the vascular segment during removal. It was immediately placed in Krebs' solution (millimolar concentration: Na+ 144.2, K+ 4.9, Ca2+ 1.3, Mg2+ 1.2, Cl− 126.7, HCO3− 25.0, SO4− 1.19, glucose 11.1, CaEDTA 0.023, ascorbic acid 0.11) at room temperature gassed with 95% O2-5% CO2, maintaining pH at 7.4 for transport to the laboratory.

Three-millimeter segments of MCA were obtained: 1) from the most proximal segment (M1), 2) from just past its first branch (M2), and 3) from just past the second branching point (M3). Two segments were taken from each site. Only segments free of frank atherosclerosis and without any adherent blood clots were studied.

One segment from each area was mounted for in vitro study of isometric contraction in a double-jacketed tissue bath.20 Ring segments were cannulated with two 30-gauge wires under a dissecting microscope. One wire was bent into a "U" shape to fix the segment to a stationary bar in the bath. The other wire was attached to a tension transducer (model FT03, Grass Instruments, Quincy, Massachusetts) mounted on a base moved by a micrometer. This allowed the segment to be stretched. Changes in isometric force were recorded on a Sun Valley, Sun Valley, California). Platinum wire electrodes (0.3 mm diameter, 3 mm long) were placed on either side of the suspended vascular segments. The electrodes were connected to a Grass stimulator and subsequently used for transmural electrical field stimulation. Each vascular segment was equilibrated for 90 minutes in Krebs' solution maintained at 37°C and gassed continuously with 95% O2-5% CO2. The bathing solution was changed every 15 minutes. Vessels were then stretched in a step-wise manner to optimum preload of 2.0 g for M1, 1.5 g for M2, and 1 g for M3 segments. These preloads were determined from active tension-length curves in preliminary experiments for this project.

After 30 minutes of equilibration at a stable preload in which Krebs' solution was replaced twice, a trial period of TEFS was carried out in the presence of propranolol (10−6 M) and atropine (10−6 M) to eliminate possible β-adrenergic or cholinergic effects. Fifteen-second trains of square-wave pulses of 0.3-msec duration were delivered to the electrodes at 16 Hz. At 10-minute intervals, stimulation was made at 4, 8, 10, 15, and 20 V. If no constrictor response was recorded, TEFS trains of 2 minutes' duration using the same pulse parameters with step-wise increases in voltage were applied. If again no response was seen, the segments were tested for responses to exogenously applied NE. If segments contracted to TEFS, tetrodotoxin (TTX; 10−7 M) was added to the bathing solution, and the stimulation sequence was repeated 30 minutes later until contraction was seen. The protocol was designed to find the highest TEFS voltage causing a contractile response that was completely eliminated by adding TTX. If all TEFS responses to the initial voltage series were completely eliminated by TTX, then a new stimulation trial was carried out that involved higher stimulation voltages. After washing the segments (Krebs' solution changed every 15 minutes three times), the highest TTX-blocked TEFS voltage was applied at different frequencies (2, 4, 8, 16, 20, and 32 Hz) in a random sequence. From this, the frequency-response relation for TEFS was obtained with stimulation parameters that were completely eliminated by TTX. If contractions were reduced but not completely eliminated by TTX at all voltages eliciting a contraction, the segments were washed as above, and the TEFS sequence of increasing voltage was repeated. Subtraction of these responses was considered the TTX-sensitive component of contraction, presumably that associated with neural stimulation.

Contractions to the cumulative addition of NE (10−5–10−3 M) were recorded from all segments 15 minutes after the addition of propranolol (10−6 M) and deoxycorticosterone acetate (10−6 M) to eliminate β-adrenoreceptor and uptake (extraneuronal) mechanisms during dose-response measurements. We were unable to use desmethylimipramine...
(10^{-7} \text{ M}) to inhibit uptake, (neuronal) since it caused a significant reduction rather than the anticipated increase in the NE response. This probably represented the $\alpha$-adrenoceptor antagonist properties of this agent. In three cases, NE dose-response sequences were repeated in the presence of phenolamine (10^{-7} \text{ M}). In another three instances, dose-response sequences were repeated after exposure to phenoxybenzamine 3x10^{-8} \text{ M} for 15 minutes followed by washing for 1 hour. After the NE dose-response curve sequence was complete, the arteries were washed, and the maximal contraction of the segment (tissue maximum) was elicited by cumulative addition of NE (10^{-4}), prostaglandin F_2\alpha (10^{-5} \text{ M}), serotonin (10^{-4} \text{ M}), and potassium chloride (100 \text{ mM}) to the bath.

The other MCA segments obtained were prepared for examination of catecholaminergic histofluorescence of perivascular nerves. Segments processed by the glyoxylic acid method showed a sporadic, moderately dense, perivascular fluorescent plexus characteristic of catecholaminergic-containing nerves. However, the intensity of fluorescence was low and not easily distinguished from background autofluorescence. Neuronal fluorescence was intensified by incubating vascular segments in NE (10^{-7} \text{ M}) and pargyline (10^{-8} \text{ M}) in Krebs' solution for 20 minutes and then exposing them to pontamine sky blue (0.5% wt/vol) and glyoxylic acid (2% wt/vol) mixed in phosphate buffer (0.1 M; pH adjusted to 7.0 with 10N sodium hydroxide, notated NaOH) for 30 minutes at room temperature. Segments were then rinsed twice with phosphate buffer, and the circular segments were cut open and spread on glass slides with the endothelial surface down. Slides were air dried and then placed in a convection oven at 100°C for 4 minutes. Tissues were immediately covered with oil. Observations of histofluorescence were made as soon as possible with a Nikon fluorescent microscope.

Medical records were reviewed on all cases. Special attention was given to age, sex, medications, illnesses (past and present), blood gasses, and serum chemistries as indicators of the "viability" of vascular segments obtained and the reason for any variation that may be observed during the experimental protocol.

Statistics

All results are reported as mean±SEM. Significance was accepted using the Student's $t$ test, when $p<0.05$.

Results

Human MCAs were obtained from 21 autopsies less than 6 hours after death. Twenty-four percent were harvested less than 2 hours after death, and 76% were obtained less than 4 hours after death. The immediate causes of death are listed in Table 1. Ages ranged from 27 to 90 years; the average age was 64. No specimens were accepted from patients who had a clinical history of diabetes, dysautonomia, or other diseases that might involve the efferent postganglionic sympathetic neurone, or who had had prolonged drug therapy considered likely to affect specifically the sympathetic-related responses that were the object of this study.

Not all segments contracted to the agonists employed. Despite rapid vessel recovery, four of 21 (19%) cases showed no contractions. However, three of these were obtained 5–6 hours after death. The fourth group of segments were from a 69-year-old woman who died after prolonged respiratory insufficiency.

All other vascular segments exhibited contractile responses, but adequate data allowing quantitation of responses to TEFS and NE cumulative dose responses could not be obtained in six segments (29%). Spontaneous NE-induced oscillating or rhythmic contractions occurred in four instances (see Figures 1A and 1B), precluding a quantitative study. In two other instances, arteries did not constrict to TEFS or NE but did respond to prostaglandin F_2\alpha.

From eight cases, vascular segments that showed graded maintained contractions when exposed to cumulative NE concentrations were obtained. Five cases produced vascular segments that showed "TTX-sensitive" TEFS contractions (see Table 1). All cases showing NE or TTX-sensitive TEFS contractions were obtained less than 4 hours after death.

Catecholamine Histofluorescence

The histofluorescence of catecholaminergic nerves was difficult to demonstrate in human cerebral arteries using the standard glyoxylic acid technique. After counterstaining with pontamine sky blue, a broad multifascicular plexus, typical of preterminal axons, was seen in the outer adventitial layer. However, no bright focal collections usually indicative of terminal axons were seen (Figure 2, top). Although quantitative assessment was not made, there was no obvious difference in the appearance or magnitude of this fluorescence between M1, M2, M3, and M4.
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**Figure 1.** Part of experimental traces of segments of human middle cerebral arteries showing spontaneous rhythmic activity. A: Spontaneous and B: after addition of norepinephrine ($10^{-7}$ M).

and M3 segments. When segments were incubated with NE ($10^{-2}$ M) and pargyline ($10^{-2}$ M), fine bright fluorescent axonal bundles typical of terminal systems were observed (Figure 2, bottom). These terminal varicosities were best seen in smaller, thinner segments of M3 but were also present in M2 and M1 segments. On the basis of absence of its characteristic fluorescence, no serotonin-containing neurons were observed.

**Transmural Electrical Field Stimulation**

TTX-sensitive contractions could be elicited by TEFS in seven arterial segments from five cases (see Table 2). In three segments, stimulation parameters were found that caused contractions that were completely eliminated by TTX ($10^{-7}$ M) and that returned after washing. In two segments, we could only determine stimulation parameters that produced contractions that were reduced by TTX ($10^{-3}$ M) but that recovered after washing. In eight cases, TEFS contractions were not reduced by TTX. These were typically seen with high voltages at 16 Hz. The number of M1 segments exhibiting TTX-sensitive TEFS was greater than the number of M2 or M3 segments showing this response ($p<0.01$). These TTX-sensitive contractions were seen in 50% of the M1 segments but only in 8% of the M2 and 12% of the M3 segments (Table 2). Segments exhibiting TTX-sensitive contractions tended to be from younger patients, although this conclusion did not reach statistical significance. The TTX-sensitive group was not different from the TTX-insensitive group with respect to illness or time after death although the series is probably too small to allow a conclusion. To quantitate the "neural" contraction, we pooled the greatest contraction that was subsequently shown to be TTX-sensitive elicited during the standard experimental protocol, when expressed as a percent of the maximal tissue response, for all responding segments (Figure 3). The parameters used to elicit these contractions were generally 10 V at 32 Hz. The mean TTX-sensitive component of TEFS contraction was 6.0±1.3% ($n=7$) of the tissue maximum contraction.

In a majority of the segments, TEFS was also applied when tone was raised. Using a voltage previously shown to be just short of breakthrough for contractions in the presence of tetrodotoxin (see "Materials and Methods"), no relaxation was ever observed.
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![Graphs showing norepinephrine (NE) dose-response curves from human middle cerebral artery.](http://circres.ahajournals.org/)

**FIGURE 4.** Norepinephrine (NE) dose-response curves from human middle cerebral artery. Plotted as percent of the maximal NE contraction (A), percent of the maximal contraction for the segment (B), percent of the contraction to $10^{-4}$ M NE (C), and percent of the maximal NE contraction in M2 segment in the presence of phentolamine $10^{-6}$ M (D).

- $\sigma$, most-proximal segment ($M_1$);
- $\bullet$, segment just past the first branch ($M_2$);
- $\triangle$, segment from just past the second branching point ($M_3$).

Number of arterial segments studied: $M_1$, five; $M_2$, eight; $M_3$, five (A, B, and C, respectively); $M_2$, three (D).

In contrast to some other human arteries, attempts to activate vascular smooth muscle cells of the human cerebral artery by electrical stimulation of their perivascular neural elements has proven to be difficult. There are two early reports of successful neuronal stimulation leading to contraction. Shibata et al.\(^27\) found TEFS contractions blocked by TTX or phentolamine in two of four MCA segments tested. However, their stimulation parameters were extremely high (80 V, 100 Hz), pulse duration was long (10 msec), and only very small phasic contractions were produced. Edvinsson et al.\(^11\) reported TEFS contractions of human adult pial arteries but show only one frequency-response curve. These authors do not describe this result in detail, and their rate of success and consistency of responses was not mentioned. Others have reported an inability to elicit TEFS contraction in human arteries, both from the circle of Willis\(^16\) and from the pial surface.\(^28\) We find the TEFS to be inconsistent, and when it does occur, it is less than 10% of the maximal contraction for the arterial segment. This seemingly poor response is not the consequence of the experimental set-up. We have been able to obtain consistently a neural-mediated TEFS contraction in human superficial temporal arteries removed at surgery that were 26% of the maximal NE response for the segment (J. Duckworth, unpublished data). Furthermore, the same experimental procedure has provided consistent responses in many different arteries from many species (for example, see Bevan and Bevan\(^29\) and Lee et al.\(^30\)).

The lack of TEFS response may not necessarily be true of all cerebral arteries and may reflect a regional variation in the cerebral arteries. Bevan and Bevan\(^29\) found that only the proximal rabbit basilar artery responded to TEFS. Likewise, we found a TTX-sensitive component of TEFS in the proximal MCA (i.e., M1) much more prominent than in more distal branches of the same artery. The overall weak effects of neural-mediated contraction may be due to postmortem change but may also reflect the high threshold of these arteries to NE associated with the low affinity of the $\alpha$-adrenoceptors, the relative low density of these receptors,\(^31\) the presence of low-affinity sites that mediate NE contraction, and the wide adrenergic synapse that has been reported for cerebral arteries, at least from the cat.\(^32\)
The electrically mediated contraction of animal cerebral vessels shows other unusual characteristics. Lee et al. found that TEFS contractions of rabbit basilar artery were increased after \( \alpha \)-adrenoreceptor blockade with phenoxybenzamine and phenolamine. Araki et al. found no denervation supersensitivity of rabbit or cat cerebral arteries, even though aminergic histofluorescence and TEFS contractions were eliminated by sympathectomy. A generally acceptable explanation of these features has not yet been put forward.

The NE-mediated constriction of human cerebral arteries also shows some unusual features. On a par with the contractions to TEFS, NE causes only a weak response of human cerebral vessels. In this study, only 20% of the tissue maximum is reached when \( 10^{-4} \) M NE is present. This is in good agreement with other reports. At \( 10^{-5} \) M NE, Rose and Moulds found that large human cerebral vessels contracted 27.42% of that elicited by potassium chloride (80 mM). In this regard, they differed significantly from human digital arteries where this value was 136.77%. The contraction of human cerebral arteries to less than \( 10^{-4} \) M NE is mediated through an \( \alpha \)-like adrenoreceptor since it is eliminated by phenolamine and also phenoxybenzamine, a result similar to that found in the rabbit basilar artery.

Additional development of force occurred when NE exceeded \( 10^{-4} \) M. In the presence of \( 10^{-5} \) M NE, 52% of the tissue maximum was reached, and this additional contraction was both phenolamine and phenoxybenzamine resistant. This effect has been previously demonstrated in cerebrovascular studies of animals, particularly the rabbit, but not man. In the rabbit, such contractions occur with a variety of adrenoreceptor agonists and, at \( 10^{-7} \) M NE, are approximately 60% of the maximal tissue contraction for the segment. They are not blocked by \( \alpha \)-adrenoreceptor, histamine, or serotonin antagonists. They may be similar to the \( \gamma \)-adrenoreceptor reported by Hirst and Neild. Such sites of drug action have been given the alternative name of extraceptors. The role of these receptors, if any, in adrenergic transmission is problematical. Postsynaptic NE concentrations may be in excess of \( 10^{-5} \) M and would be sufficient to activate such sites, but whether this would result in effective transmission is not known.

The ED\( _{50} \) for the first part of the MCA NE dose-response curve, which up to \( 10^{-4} \) M is blocked by phenolamine and phenoxybenzamine, is \( 1 \times 10^{-9} \) M. This is similar to published data for the human basilar and MCA segments. Other reports are quite different and range from \( 9.8 \times 10^{-9} \) M to \( 2.05 \times 10^{-7} \) M. These discrepancies may be due to several factors. First, previous studies of human cerebral arteries did not always use an exactly identified segment. Second, previous studies have used arterial segments obtained long after death (i.e., greater than 8 hours). Toda et al. has suggested that relative NE activity in human cerebral arteries remains intact up to 20 hours after death, but these authors do not detail the NE dose-response curve. Thirdly, records of cerebral vascular tissue are often difficult to analyze. Spontaneous oscillating contractions like those shown in Figure 1 have plagued researchers, and pronounced interindividual and intra-individual variation of human cerebral artery segments has been found. When the same arterial segment is analyzed as soon after death as possible, these difficulties may be minimized, since, in our study, variation in sensitivity was small.

Finally, another important factor that complicates the measurement of catecholaminergic sensitivity is the presence of both normal and low-affinity sites. NE ED\( _{50} \) is obviously influenced by the choice of the response that is considered maximum. We suggest that NE acts in human and animal vascular tissue at two sites: 1) a low-dose, moderate-affinity site inhibited by adrenoreceptor antagonists and 2) a high-dose, low-affinity site insensitive to these drugs. NE ED\( _{50} \) has significance only when one response component is measured at a time. The limitation of the size of the first phase, to some 20% of the maximum tissue response, may reflect a low receptor number; at least this seems to be the case for the rabbit.

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**KEY WORDS**: human artery, middle cerebral innervation, norepinephrine, a-adrenoceptor, extracerebral
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