Characterization of Beta Adrenergic Receptors in Human Cerebral Arteries and Alteration of the Receptors After Subarachnoid Hemorrhage

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SUMMARY The nature of beta adrenergic receptors in human cerebral arteries was characterized and alteration of these receptors after subarachnoid hemorrhage was examined using a radioligand binding assay. The specific \(^3\)H-dihydralprenolol, a beta adrenergic antagonist, binding to human cerebral arteries was saturable and of high affinity \((K_D = 12.3 \text{ nM})\) with a Bmax of 790 fmol/mg protein. Ki values and Hill coefficients of adrenergic agents for \(^3\)H-dihydralprenolol were as follows: propranolol, 4.1 \(\times 10^{-8}\) M, 1.01; isoproterenol, 1.7 \(\times 10^{-8}\) M, 0.80; epinephrine, 8.3 \(\times 10^{-8}\) M, 0.48; norepinephrine, 2.3 \(\times 10^{-7}\) M, 0.45; metoprolol, 6.8 \(\times 10^{-7}\) M and 7.9 \(\times 10^{-7}\) M, 0.62; butoxamine, 2.2 \(\times 10^{-6}\) M and 2.1 \(\times 10^{-6}\) M, 0.43. The analysis of inhibition of specific \(^3\)H-dihydralprenolol binding by these adrenergic agents suggests that human cerebral arteries contain a high density of beta adrenergic receptors and that the receptors are classified into two types, namely beta 1 and beta 2 adrenergic receptors. The calculated beta 1/beta 2 ratio from Hofstee plots was approximately 4/6.

ISOLOTT CEREBRAL ARTERIES of human\(^1,2\) and other species\(^3\), contract in the presence of adrenergic agonists, in a dose dependent manner. When the contraction is blocked by alpha adrenergic antagonists, isoproterenol induces relaxation, in a dose dependent manner in human,\(^1,4\) cat,\(^3\) and dog\(^4\) cerebral arteries. This relaxation was blocked by propranolol. These data suggested the existence of not only alpha adrenergic receptors but also beta adrenergic receptors in the cerebral arteries. Beta adrenergic receptors have been classified into beta 1 and beta 2, according to affinity to their agonists and antagonists, in various tissues.\(^5\) Pharmacological studies suggested that beta 1 adrenergic receptors mediated relaxations in human\(^1\) and cat\(^3\) cerebral arteries, whereas in other peripheral vessels, beta 2 adrenergic receptors seemed to mediate relaxations.\(^6\) It was reported that blood flow in the caudate nucleus of rabbit brain was increased by isoproterenol and that the increase was blocked by a selective beta 1 adrenergic antagonist, practolol.\(^7\) On the contrary, biochemical studies suggested the predominant existence of beta 2 adrenergic receptors in the cat cerebral microvessels.\(^8\) Thus subtypes of beta adrenergic receptors on the cerebral arteries were not clearly characterized.

Vasospasm of cerebral arteries in the case of subarachnoid hemorrhage (SAH) frequently presents severe clinical problems, as a result of cerebral ischemia. The pathogenesis of vasospasm is still poorly understood. The level of circulatory catecholamines often increases after SAH.\(^9,10\) In addition, the contractile response of human cerebral arteries to norepinephrine

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is larger than the responses in other species. Thus, neurogenic factors in controlling brain circulation would play an important role in pathological conditions such as SAH. We reported evidence of alteration of alpha adrenergic receptors after SAH.

In this study, beta adrenergic receptors in human cerebral arteries were characterized using \(^3\)H-dihydropyridine (DHA), and alterations in these receptors after SAH were analyzed.

Materials and Methods

Collection of Arteries

Cerebral arteries (mainly basilar, circle of Willis and middle cerebral arteries) were carefully removed at autopsy between 1 and 2 hours after death. These arteries were then washed in saline and placed in a freezer (—80°C). Clinical profiles of patients are summarized in Table 1.

Membrane Preparation

Membrane was prepared as described in the previous report. The arteries were minced with scissors and homogenized in 10 volumes of ice-cold 50 mM sodium phosphate buffer (pH 7.4) with a glass homogenizer. The homogenates were filtered through two layers of gauze, re-homogenized at setting 10 on a Polytron with 20s burst, centrifuged at 1,000 X g for 10 min and supernatant carefully removed and centrifuged at 100,000 X g for 60 min. The resulting pellet was resuspended in 50 mM sodium phosphate buffer (pH 7.4). Protein concentration was determined by the method of Lowry et al.

Binding Assay

\(^3\)H-DHA bindings were performed by incubating aliquots of the cerebral artery homogenates at a temperature of 37°C for 30 min in 250 μl of sodium phosphate buffer, containing \(^3\)H-DHA, in the absence or presence of high concentrations of propranolol (10 μM). The binding in the presence of 10 μM propranolol was termed “nonspecific” and was subtracted from that obtained in the absence of propranolol “total binding” to obtain the binding termed “specific binding”.

The assay was terminated by the addition of 3 ml of the ice-cold buffer and the rapid filtration through Whatman GF/B glass fiber filters, under suction. After washing twice with 3 ml of the buffer, the filters were dried in an oven, transferred to counting vials and 8 ml of scintillation fluid was added. Radioactivity was counted in a Packard Tri-Carb scintillation spectrometer (Model 3255).

To obtain the displacement curve, homogenates of the artery were added to the tubes containing the beta adrenergic agents at various concentrations, and 15 nM \(^3\)H-DHA.

Drugs Used

\(^3\)H-DHA (specific activity, 34.1 Ci/mmoles) was purchased from New England Nuclear, Boston, MA, USA, stored at —20°C in ethanol and protected from light. Immediately prior to use, appropriate amounts of stock solutions were diluted with distilled water so that the ethanol concentrations in the final assay system did not exceed 0.5%. Acebutolol (May & Baker, England), butoxamine (Burroughs Wellcome Co., USA), Metoprolol (AB Hassle, Sweden) and Salbutamol (Schering, USA) were obtained from the manufacturers. All other chemicals were of reagent grade or of the purest grade commercially available.

Computer Analysis of the Data

Scatchard analysis was performed according to Bennett. Hofstee plots were curvilinear, suggesting the presence of multiple binding sites. Data on bindings were analyzed by an nonlinear best-squared fit of the amount of specific \(^3\)H-DHA binding, as a function of the free \(^3\)H-DHA in the assay, using a FUJITSU MICRO 7 computer. Data points were fitted to a two-independent binding site model (Eq. 1) described by Olsen et al.

\[
B = \frac{B_{max1} \times S}{K_{D1} + S} + \frac{B_{max2} \times S}{K_{D2} + S}
\]

where B is the total amount of ligand binding at a free ligand concentration S (15 nM). Bmax1 and Bmax2 are the Bmax (total number of binding sites) values for two sites having dissociation constants K_{D1} and K_{D2}, respectively.

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**Table 1: Clinical Data on 10 Patients**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age and sex</th>
<th>Cause of death</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>74 M</td>
<td>Lung cancer</td>
<td>Operation on Day 0 Died on Day 10 of progressive neurological deficit</td>
</tr>
<tr>
<td>2</td>
<td>74 M</td>
<td>Pontine hemorrhage</td>
<td>No operation Died on Day 2 of acute neurological deficit</td>
</tr>
<tr>
<td>3</td>
<td>77 F</td>
<td>Cecum cancer Intracerebral hemorrhage</td>
<td>No operation Died on Day 5 of acute neurological deficit</td>
</tr>
<tr>
<td>4</td>
<td>30 M</td>
<td>Intracerebral hemorrhage</td>
<td>No operation Died on Day 14 of progressive delayed ischemic deficit</td>
</tr>
<tr>
<td>5</td>
<td>75 M</td>
<td>Cerebral infarction Pneumonia</td>
<td>Operation on Day 0 Died on Day 14 of progressive delayed ischemic deficit</td>
</tr>
<tr>
<td>6</td>
<td>77 F</td>
<td>Cerebral infarction</td>
<td>Operation on Day 0 Died on Day 10 of progressive neurological deficit</td>
</tr>
<tr>
<td>7</td>
<td>54 M</td>
<td>SAH (left-MCA)</td>
<td>Operation on Day 0 Died on Day 10 of progressive neurological deficit</td>
</tr>
<tr>
<td>8</td>
<td>42 M</td>
<td>SAH (ACoM)</td>
<td>No operation Died on Day 2 of acute neurological deficit</td>
</tr>
<tr>
<td>9</td>
<td>71 F</td>
<td>SAH (ACoM)</td>
<td>No operation Died on Day 5 of acute neurological deficit</td>
</tr>
<tr>
<td>10</td>
<td>50 M</td>
<td>SAH (ACoM)</td>
<td>Operation on Day 0 Died on Day 14 of progressive delayed ischemic deficit</td>
</tr>
</tbody>
</table>

SAH of four patients were caused by ruptured cerebral aneurysms. The locations of the aneurysms are indicated.

MCA = middle cerebral artery; ACoM = anterior communicating artery.
The results given in the text are the means ± S.E.M. of N experiments. The data were analyzed by Student’s t-test for unpaired comparison and values of \( p < 0.05 \) were accepted as statistically significant.

### Results

#### Saturability of Specific \( ^3\)H-DHA Binding

Specific binding of increasing concentration of \( ^3\)H-DHA was saturable (fig. 1, closed circles). Scatchard analysis indicated a single class of binding sites with an apparent equilibrium dissociation constant (\( K_d \)) and maximum binding capacity (\( B_{max} \)) (fig. 2, closed circles). \( K_d \) and \( B_{max} \) were 12.3 nM and 790 fmol/mg protein, respectively.

#### Inhibition of Specific \( ^3\)H-DHA Binding

The specificity of \( ^3\)H-DHA binding was studied using beta adrenergic agonists and antagonists. The \( K_i \) values and Hill coefficients \((n_H)\) for the inhibition of \( ^3\)H-DHA binding by various beta adrenergic agents are shown in table 2. Inhibition curves of \( ^3\)H-DHA binding by beta adrenergic agents except salbutamol and acebutolol are shown in figure 3. Among agonists, isoproterenol was most potent with \( K_i \) of \( 1.7 \times 10^{-6} \)M and \( n_H \) of 0.8. The order of potency isoproterenol > epinephrine > norepinephrine is consistent with the stimulation of beta adrenergic receptors containing beta 2 subtype. Salbutamol, a relatively beta 2 specific agonist, gave a shallow inhibition curve with \( n_H \) of 0.44.

The most potent antagonist in inhibiting \( ^3\)H-DHA binding was propranolol with \( K_i \) of \( 4.1 \times 10^{-6} \)M and \( n_H \) of 1.01. The inhibition curve of propranolol was steep and without biphasic characteristics. Relatively beta 1 specific antagonists, acebutolol and metoprolol, and relatively beta 2 specific antagonist, butoxamine, inhibited the \( ^3\)H-DHA binding biphasically, suggesting 2-classes of binding sites in beta adrenergic receptors. Hofstee plots of metoprolol and butoxamine (fig. 4, closed circles) indicated a high affinity and a low affinity component. For metoprolol, computer determined high affinity (\( K_i = 6.8 \times 10^{-6} \)M; beta 1 receptor sites) and low affinity (\( K_i = 7.9 \times 10^{-6} \)M; beta 2 receptor sites) sites represented 40 ± 2% and 60 ± 2%

<table>
<thead>
<tr>
<th>Drugs</th>
<th>( K_i ) (M)</th>
<th>( n_H )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agonists</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-) Isoproterenol</td>
<td>1.7 ± 0.7 \times 10^{-6}</td>
<td>0.80 ± 0.18</td>
</tr>
<tr>
<td>(-) Epinephrine</td>
<td>8.3 ± 6.9 \times 10^{-6}</td>
<td>0.48 ± 0.15</td>
</tr>
<tr>
<td>(-) Norepinephrine</td>
<td>2.3 ± 1.3 \times 10^{-5}</td>
<td>0.45 ± 0.12</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>5.3 ± 4.1 \times 10^{-6}</td>
<td>0.44 ± 0.16</td>
</tr>
<tr>
<td>Antagonists</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-) Propranolol</td>
<td>4.1 ± 0.7 \times 10^{-8}</td>
<td>1.01 ± 0.07</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>6.8 ± 0.9 \times 10^{-8}</td>
<td>0.62 ± 0.15</td>
</tr>
<tr>
<td>Acebutolol</td>
<td>7.9 ± 0.8 \times 10^{-6}</td>
<td>0.40 ± 0.32</td>
</tr>
<tr>
<td>4.9 ± 2.7 \times 10^{-8}</td>
<td>1.1 ± 0.7 \times 10^{-6}</td>
<td>0.40 ± 0.32</td>
</tr>
<tr>
<td>2.2 ± 0.3 \times 10^{-8}</td>
<td>2.1 ± 1.0 \times 10^{-6}</td>
<td>0.43 ± 0.17</td>
</tr>
<tr>
<td>Butoxamine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The values are mean ± SEM of three separate experiments in the control group.

\( K_i \) for agonists was determined from the equation \( K_i = IC_{50}/(1 + S/K_d) \), where \( S \) is the concentration of \( ^3\)H-DHA used in the assay (15 nM).

\( K_i \) for antagonists was determined from nonlinear data analysis program as described in the text.
FIGURE 3. The displacement of specific $^3$H-DHA binding to human cerebral arteries by adrenergic agonists and antagonists. Each point represents the mean of three separate experiments in the control group (case 1, 2, 3). (●); propranolol, (■); metoprolol, (○); isoproterenol, (□); epinephrine, (▲); norepinephrine.

± 2% of $^3$H-DHA binding, respectively. For butoxamine, computer determined high affinity ($K_i = 2.2 \times 10^{-4}$M; beta 2 receptor sites) and low affinity ($K_i = 2.1 \times 10^{-4}$M; beta 1 receptor sites) sites represented 61 ± 4% and 39 ± 4% of $^3$H-DHA binding sites, respectively. Considering the Bmax value of $^3$H-DHA binding site (790 fmol/mg protein), Bmax of beta 1 receptor sites and Bmax of beta 2 receptor sites were calculated 316 ± 16 fmol/mg protein and 474 ± 16 fmol/mg protein, respectively, from the inhibition curve of metoprolol and 308 ± 36 fmol/mg protein and 482 ± 36 fmol/mg protein, respectively, from the inhibition curve of butoxamine (fig. 5).

Saturability of Specific $^3$H-DHA Binding after SAH

Specific binding of $^3$H-DHA to arterial homogenates after SAH was also saturable (fig. 1, open circles). Scatchard analysis of the saturation curve indicated a single class of binding sites with a similar $K_D$ and larger Bmax compared with the control group (fig. 2, open circles). $K_D$ and Bmax were 13.9 nM and 1140 fmol/mg protein, respectively. Specific $^3$H-DHA binding at the free $^3$H-DHA concentration of 12 nM (near $K_D$ value) was 382 ± 28 fmol/mg protein in the control group ($N = 6$) and 519 ± 36 fmol/mg protein in the SAH group ($N = 4$), respectively. The difference between the control and SAH groups was statistically significant.

Inhibition of Specific $^3$H-DHA Binding after SAH

Hofstee plots of metoprolol and butoxamine on the arterial homogenates after SAH also indicated 2 components in specific $^3$H-DHA binding sites (fig. 4, open circles). The ratio of high affinity and low affinity sites was altered after SAH. For metoprolol, computer determined high affinity and low affinity sites represented 62 ± 5% and 38 ± 5% of $^3$H-DHA binding sites, respectively. For butoxamine, computer determined high affinity and low affinity sites represented 29 ± 7% and 71 ± 7% of $^3$H-DHA binding sites, respectively. Considering the Bmax value of $^3$H-DHA binding site of SAH group (1140 fmol/mg protein), Bmax of beta 1 receptor sites and Bmax of beta 2 receptor sites were calculated 708 ± 57 fmol/mg protein and 482 ± 57 fmol/mg protein, respectively, from the inhibition curve of metoprolol and 810 ± 80 fmol/mg protein and 330 ± 80 fmol/mg protein, respectively, from the inhibition curves of butoxamine (fig. 5). Differences between Bmax values of beta 1 receptor sites of the control group and SAH group were significant. But the differences between Bmax values of beta 2 receptor sites were not significant (fig. 5).
Discussion

Using ³H-DHA, a beta adrenergic antagonist, we obtained evidence for the presence of beta adrenergic receptors in human cerebral arteries. Specific ³H-DHA binding to human cerebral arteries was saturable, of high affinity and reversible. Scatchard and Hill plot analyses of the data indicated that ³H-DHA binding sites in human cerebral arteries are of a single population. Kᵦ value of ³H-DHA binding in this study is similar to the values in dog myocardium and in frog erythrocyte. These data suggested that there is a high density of beta adrenergic receptors in human cerebral arteries.

Since ³H-DHA has similar affinities to beta 1 and beta 2 adrenergic receptors, Scatchard analysis of ³H-DHA binding determines the total concentration of beta adrenergic receptors in a tissue. The inhibition of specific ³H-DHA binding by propranolol and isoproterenol, which bind nonselectively to beta 1 and beta 2 adrenergic receptors yield linear Hofstee plots with a Hill coefficient of approximately 1.0. On the other hand, the inhibition of specific ³H-DHA binding by agents which have relatively selective affinities to beta 1 and beta 2 receptors show two types of binding sites, fitting to beta 1 and beta 2 adrenergic receptors. The ratio of beta 1:beta 2 adrenergic receptors calculated by Hofstee plots was approximately 40:60 in human cerebral arteries.

Pharmacodynamic studies revealed that beta 1 adrenergic receptors mediate vasodilatory effect of isoproterenol in human pial arteries. The postsynaptic beta adrenergic receptors in the cerebral arteries may be mainly the beta 1 subtype. In other tissues, the postsynaptic beta adrenergic receptors were subclassified into beta 2 type. Although the population of the receptors in presynaptic and postsynaptic sites cannot be determined in binding studies, it is probable that beta 1 adrenergic receptors locate mainly in the postsynaptic sites and beta 2 adrenergic receptors mainly in the presynaptic sites.

We analyzed alterations in beta adrenergic receptors in the cerebral arteries after SAH. Kᵦ value of ³H-DHA binding sites after SAH was not significantly different from values in the control group, whereas the density of ³H-DHA binding sites was increased after SAH. In addition, the ratio of beta 1/beta 2 adrenergic receptors was reversed after SAH. The ratio was 62/38 from the inhibition curve by metoprolol and 71/29 from that by butoxamine. This means that beta 1 adrenergic receptors increase after SAH without significant changes in the number of beta 2 adrenergic receptors. Morphological changes were noted on human cerebral arteries after SAH and in monkey cerebral arteries after experimental SAH. These changes are mainly degenerative ones, including myonecrosis of the media and intimal thickening. When the smooth muscle layer of blood vessels became necrotic and decreased in volume, the receptor sites of the vessel would be relatively increased in appearance, as the receptor density was measured per mg protein of tissue. These morphological changes would explain the increase in ³H-DHA binding sites after SAH. However, the reversal of the ratio of beta 1/beta 2 adrenergic receptors cannot be explained on such basis. It has been reported that cat cerebral arteries became sensitive to catecholamines after sympathetic denervation and that such supersensitivity was induced by experimental SAH. In a previous study, we demonstrated the increase in number of alpha adrenergic receptors in human cerebral arteries after SAH. Increase of beta 1 adrenergic receptors after SAH in the present study is in line with the previous study. Preganglionic denervation sometimes induced an increase in the number of muscarinic cholinergic receptors in the superior cervical ganglia in cats. The increase of beta 1 adrenergic receptors may also be a denervation effect in the postsynaptic beta adrenergic receptors after SAH. Since beta 1 adrenergic receptors mediate relaxation cerebral arteries and they become sensitive after SAH, the administration of selective beta 1 adrenergic agonists may possibly prevent the occurrence of delayed vasospasms after SAH.

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Vasodilator Proteins: Role in Delayed Cerebral Vasospasm

RICHARD P. WHITE, PH.D.

SUMMARY Blood proteins could play a critical role in the pathogenesis of cerebral vasospasm in subarachnoid hemorrhage (SAH) as agonists and as antagonists of vasoconstriction. The present study was designed primarily to quantify the inhibition produced by antithrombin III of the phasic responses elicited by cumulative doses of KC1, serotonin (5-HT), undine triphosphate (UTP), and thrombin in isolated canine cerebral arteries. Antithrombin III (1 unit/ml and 3 units/ml) given 2 min beforehand inhibited all agonists. The inhibition of the contraction of arteries was not dependent on a functional endothelium nor due to stimulation of the electrogenic sodium pump. Alpha2-macroglobulin (0.1 mg/ml and 0.4 mg/ml) inhibited the contractile responses to high K+ 5-HT, and thrombin. Kallikrein (1 and 4 units/ml) did not inhibit UTP but inhibited high K+ and 5-HT through an effect on the endothelium. The results demonstrate that anticoagulant proteins are very effective nonspecific inhibitors of the vasoconstriction, whereas the serine protease kallikrein selectively blocks thrombin. The remarkable potency of antithrombin III suggests that it may protect cerebral arteries from exhibiting vasospasm in SAH.

A COMMON FEATURE OF SUBARACHNOID HEMORRHAGE (SAH) is the occurrence of cerebral vasospasm. The incidence of severe vasospasm in SAH is related to the volume of intracisternal blood, 1 and the manifestations of brain ischemia in turn are related to the magnitude of the vasospasm. 2 One characteristic of cerebral vasospasm that has eluded an explanation is the delayed nature of the phenomenon. The median time for its occurrence is about 7 days after the SAH. 3 Another enigma is that not all patients with ruptured aneurysms develop vasospasm. 4, 5

The disposition of proteins after SAH may be responsible for the delayed vasospasm. Some investigators have hypothesized that oxyhemoglobin and other proteins from hemolyzed erythrocytes are responsible for or contribute to the vasospasm. 6, 7 Fibrin degradation products (FDP) may also play a role because these are reported to produce a contractile response as well as enhance the contractions produced by serotonin or oxyhemoglobin. 8, 9 The serine proteases trypsin, thrombin and plasmin likewise produce vasoconstriction of isolated cerebral arteries. 10 Since fibrinolytic activity peaks many days after the extravasation of blood, 11 plasmin could play a critical role in pathogenesis of vasospasm as a vasoconstrictor and as a source of FDP. In contrast, antithrombin III is a potent

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