Subarachnoid hemorrhage–induced upregulation of the 5-HT_{1B} receptor in cerebral arteries in rats

JACOB HANSEN-SCHWARTZ, M.D., PH.D., NATALIE LØVLAND HOEL, B.S., CANG-BAO XU, M.D., PH.D., NIELS-AAGE SVENGAARD, M.D., PH.D., AND LARS EDVINSSON, M.D., PH.D.

Department of Clinical Experimental Research, Glostrup Hospital, University of Copenhagen, Glostrup, Denmark; and Department of Internal Medicine, Lund University Hospital, Lund, Sweden

Object. Cerebral vasospasm following subarachnoid hemorrhage (SAH) leads to reduced blood flow in the brain. Inspired by organ culture–induced changes in the receptor phenotype of cerebral arteries, the authors investigated possible changes in the 5-hydroxytryptamine (HT) receptor phenotype after experimental SAH.

Methods. Experimental SAH was induced in rats by using an autologous prechiasmatic injection of arterial blood. Two days later, the middle cerebral artery (MCA), posterior communicating artery (PCoA), and basilar artery (BA) were harvested and examined functionally with the aid of a sensitive in vitro pharmacological method and molecularly by performing quantitative real-time reverse transcription–polymerase chain reaction (PCR).

In the MCA and BA the 5-HT_{1B} receptor was upregulated, as determined through both functional and molecular analysis. In response to selective 5-HT receptor agonists both the negative logarithm of the 50% effective concentration was increased (one log unit in the MCA and one half unit in the BA), as was the agonist’s potency (increased by 50% in the MCA and doubled in the BA). In addition, the authors found an approximately fourfold increase in the number of copies of messenger RNA coding for the 5-HT_{1B} receptor as determined by quantitative real-time PCR. In the PCoA no upregulation of the 5-HT_{1B} receptor was observed.

Conclusions. Changes in the receptor phenotype in favor of contractile receptors may well represent the end stage in a sequence of events leading from SAH to the actual development of cerebral vasospasm. Insight into the mechanism of upregulation may provide new targets for developing specific treatment against cerebral vasospasm.

KEY WORDS • subarachnoid hemorrhage • cerebral vasospasm • serotonin • 5-hydroxytryptamine • polymerase chain reaction • rat

Cerebral vasospasm occurring after SAH was first demonstrated angiographically by Ecker and Riemenschneider in 1951; it is a syndrome that through the pathological narrowing of the cerebral arteries compromises blood flow to the brain parenchyma. Although clinically well described, the actual changes in the cerebral arteries are only poorly understood. It may of course be logically deduced that the origin of cerebral vasospasm is the rupture of a cerebral artery with the extravasation of blood into the subarachnoid space; without it, no vasospasm is observed. The sequence of events thus initiated results in a greater or lesser degree of vasospasm; for some patients this vasospasm manifests itself through cerebral ischemia and subsequent neurological deficit. Current knowledge of cerebral vasospasm may be summed up in three main hypotheses to explain the sequence of events. The extravasated blood may itself exert a local toxic effect on cerebral arteries through the generation of superoxide radicals, it may trigger neurogenic mechanisms activating the hypothalamus, or it may generate an inflammatory response around the cerebral arteries. Nevertheless, the absence of a clinical effect does not per se exclude the involvement of 5-HT, but does perhaps point to a multifactorial pathogenesis.

The interest in 5-HT as a key player has, in the past decade, lost impetus mainly due to the lack of clinical efficacy of 5-HT antagonists in treating cerebral vasospasm. Nevertheless, the absence of a clinical effect does not per se exclude the involvement of 5-HT, but does perhaps point to a multifactorial pathogenesis.

Organ culture of entire segments of the BA from rats induces an upregulation of contractile receptors, including the contractile 5-HT_{1B} receptor, making the arterial segment considerably more sensitive to 5-HT. Because this intrinsic ability of an artery to upregulate contractile receptors may be of pathophysiological significance, we set out to investigate possible upregulation after induction of experimental SAH in rats.
Materials and Methods

All animal procedures were performed strictly within national laws and guidelines and approved by the Danish Animal Experimentation Inspectorate.

Subarachnoid Hemorrhage Model in Rats

Male Sprague–Dawley rats (350–400 g) were anesthetized using 5% halothane. The rats were subsequently intubated and kept on artificial respiration with 30% O₂ and 70% N₂O. A state of anesthesia was maintained with 0.5 to 1% halothane. The rats were prepared according to the method described by Prunell and colleagues.28,29

After 30 minutes of equilibration, adjusting the level of anesthesia to establish an MABP in the range 80 to 100 mm Hg, 250 μl of blood was withdrawn from the tail catheter and injected intracranially at a pressure equal to the MABP. Subsequently, the rat was kept in a state of anesthesia for another 60 minutes to allow for recovery from the cerebral insult, after which catheters were removed and incisions closed. The rat was then revived and extubated.

During the observation period the rat was monitored; if it appeared to be in distress, a subcutaneous injection of Temgesic 0.2 mg/kg was administered and repeated if necessary. In addition, the rats were hydrated using a 40-ml injection of subcutaneous isotonic sodium chloride, which was administered at the end of the operation and on Day 1 postsurgery. If an animal displayed signs of severe distress, it was killed prematurely.

A series of sham-operated rats were also prepared. These went through the same procedure described earlier, except that no blood was injected.

Harvest of Cerebral Arteries

After 2 days of observation (during which the rats showed no signs of focal neurological deficit), the rats were anesthetized using CO₂, killed by decapitation, and their brains were removed (in Fig. 1, the macroscopic signs of induced SAH are shown). With the aid of a microscope, the MCA, PCoA, and BA were carefully dissected free from the brain, cleared of connective tissue, and cut into 1-mm-long cylindrical segments.

In Vitro Pharmacological Method

The cylindrical segments were mounted on two metal wires 40 μm in diameter on a myograph; a temperature-controlled buffer solution (37°C) of the following composition was used (in millimoles): NaCl 119, NaHCO₃ 15, KCl 4.6, MgCl₂ 1.2, NaH₂PO₄ 1.2, CaCl₂ 1.5, and glucose 5.5. The buffer was continuously aerated with O₂ enriched with 5% CO₂, resulting in a pH of 7.4. The vessels were stretched to an initial resting tone of 2 mN and then allowed to stabilize at this tone for 1 hour. Viability was tested by exposing the vessel segments to an isotonic solution containing 60 mM K⁺, which was obtained by a partial change of NaCl for KCl in the aforementioned buffer. The contraction induced by K⁺ was used as reference for subsequent contractile experiments.

The presence of an intact functional endothelium was tested by precontracting the vessel by using 5-HT (10⁻⁵ M) and subsequently exposing it to acetylcholine (10⁻⁴ M). A relaxant response of more than 70% of the precontracted tension was considered to be indicative of a functional endothelium. The vessels were allowed to rest for 20 minutes before commencing the experiments.

Concentration–response curves were constructed with 5-CT, a specific 5-HT₆ receptor agonist, in the concentration range from 10⁻¹⁰ M to 10⁻⁴. To characterize responses, the specific 5-HT₆ antagonist GR 5562 is used at a concentration of 10⁻⁶ M.²⁻⁵,²⁶

Molecular Biological Techniques

To quantify mRNA for the 5-HT₆ and 5-HT₇ receptors, reverse transcription–PCR with real-time detection was performed as previously described.²⁴,³⁰

Cellular RNA was extracted from the vessels by using the TRIzol agent after mechanical homogenization. The reverse transcriptase synthesis of cDNA was performed using 1 μg total RNA in a 20-μl reaction volume. The reaction mixture was incubated at 25°C for 10 minutes and 42°C for 30 minutes, heated to 99°C for 5 minutes, and afterward cooled to 4°C.

Quantitative real-time PCR was performed using a sequence detection system, with the cDNA synthesized as described above but in a 50-μl reaction volume. A blank control containing only water was included in all experiments.

Primers had the following sequences: 5-HT₆ receptor forward, 5'-TCCGGGTCTCCTGTGTACGT-3' and reverse, 5'-GGCGTC-TGAGACTCGCACTT-3'; 5-HT₇ receptor forward, 5'-CACCTT-GCTTTACCGGAAAG-3' and reverse, 5'-AGCCGTGATAAGCTGTACGT-3'.

Messenger RNA for EF-1 was used as a reference because it is expressed constantly and independently of cell type (in a pilot study we found no difference in expression compared with β-actin [data not shown]). The EF-1 primers were designed as follows: EF-1 forward, 5'-GACAGCCTCGATGCTG-3' and reverse, 5'-TGATGACACCCCAAGGACTG-3'.

The real-time PCR was performed according to the following profile: the first cycle at 50°C for 2 minutes and 95°C for 10 minutes, and subsequent cycles (40) at 95°C for 15 seconds and 60°C for 1 minute.

To verify that each primer pair generated only one PCR product at the expected size, the real-time PCR products were separated electrophoretically. The expected sizes of the amplification products for the two receptors were 51 base pairs. In addition, standard curves for each primer were derived to verify a uniform amplification process throughout.

Calculation and Statistics

In Vitro Pharmacological Method. Data are expressed as the means ± SEM. Contractile responses in each segment are expressed as a percentage of the contraction induced in that segment by 60 mM
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of K". In a given experiment $E_{\text{max}}$ denotes the maximal contractile response elicited, and $\text{pEC}_{50}$ the negative logarithm of the concentration that elicits one half of the maximal response. For biphasic responses, $E_{\text{max}(1)}$ and $\text{pEC}_{50(1)}$ are used to describe the high-affinity phase and $E_{\text{max}(2)}$ and $\text{pEC}_{50(2)}$ to describe the low-affinity phase.

**Real-Time PCR.** The number of cycles for a given sequence to be amplified to a predefined detection threshold is denoted by $C_T$. The amount of cDNA for a given primer is expressed as a percentage of the amount of cDNA for EF-1, as given by the equation $X = (1 + E)^{\Delta C_T}$, where $E$ is the replication efficiency of the PCR reaction, and $\Delta C_T$ is the difference in the number of cycles to reach the detection limit between the reaction to amplify the cDNA in question and the reaction to amplify cDNA for EF-1.

Measurements were obtained three times, and differences were tested for statistical significance with the Student t-test (p = 0.05).

**Sources of Supplies and Equipment**

Halothane was obtained from Halocarbon Labs (River Edge, NJ); Temgesic (buprenorphine hydrochloride) from Reckitt & Colman, Hull, UK; and 5-CT, 5-HT, and acetylcholine from Sigma (Copenhagen, Denmark). The GR 55652 was a gift kindly provided by Dr. H. Connor (GlaxoSmithKline, Stevenage, UK).

For the molecular biological analyses we obtained TRIzol from Invitrogen (Taastrup, Denmark). Primers were custom synthesized by GibcoBRL Custom Primers (Invitrogen, Paisley, UK). All other reverse transcription–PCR reagents were obtained from Applied Biosystems (Stockholm, Sweden). All chemicals were obtained from Merck Eurolab (Albertslund, Denmark). To check blood gas levels during anesthesia we used a radiometer (ABL 520) from Radiometer (Copenhagen, Denmark). For in vitro pharmacological studies vessel myographs from Danish Myotechnology (Aarhus, Denmark) was used, and for recording experiments an analog digital converter (PowerLab, Oxford, UK) and the associated software program (Chart) from ADInstruments (Oxford, UK) were used. Quantitative real-time PCR was performed in a sequence detection system (GeneAmp SYBR Green, model 5700; Applied Biosystems, Foster City, CA). Specific primers for the rat 5-HT1B and 5-HT1D receptors were designed using commercially available software (Primer Express, version 2.0; Applied Biosystems).

**Results**

**In Vitro Pharmacological Aspects**

The in vitro response to 5-CT was clearly upregulated in the MCA and the BA in rats with SAH (Fig. 2a and b). The biphasic response to 5-CT indicates the presence of two receptors; in an earlier study on cerebral arteries in rats, authors demonstrated that the first phase was generated by the 5-HT1B receptor response and the second by a degenerate 5-HT2A receptor response. In the present study this was confirmed using GR 55652, a selective 5-HT1B receptor antagonist, shifting the high-affinity phase to the right and removing the 5-HT1B component of the low-affinity phase (data not shown). In the MCA and BA the first phase of the contractile response was significantly upregulated (p < 0.05; Student t-test), indicating an enhanced 5-HT1B receptor response. During the first phase in the MCA, the $\text{pEC}_{50}$ increased from 7.6 ± 0.2 (mean ± SEM) to 8.5 ± 0.1 and the $E_{\text{max}}$ increased from 14 ± 3% (relative to the contraction induced in the vessel by 60 mM K") to 21 ± 5%. There was no significant difference in the values during the second phase of the contraction; that is, $\text{pEC}_{50}$ increased slightly from 5.3 ± 0.2 to 5.2 ± 0.1, and $E_{\text{max}}$ from 48 ± 8% to 51 ± 8%. In the BA, a similar upregulation was noted. In rats with SAH, $\text{pEC}_{50}$ in the 5-HT1B phase was 8.2 ± 0.2 compared with 7.7 ± 0.1 in control rats, and $E_{\text{max}}$ was 27 ± 7% and 11 ± 3%, respectively. During the 5-HT2A phase of the contraction in the BA, values were similar in comparing the rats with SAH and the control rats: $\text{pEC}_{50}$ 5.5 ± 0.2 and 5.1 ± 0.1, respectively; and $E_{\text{max}}$ 62 ± 9% and 59 ± 8%, respectively. In the PCoA (Fig. 2c), part of the circle of Willis, no change in the 5-HT1B phase was induced by SAH. In the 5-HT2A phase, however, we noted a significantly (p < 0.05) downregulated response in the rats with SAH; that is, $E_{\text{max}}$ amounted to only 47 ± 8%, approximately one half of the value observed in control rats (86 ± 15%). Interestingly, the...
concentration–contraction curve for the PCoA in rats with SAH was not affected by the addition of GR 55562, thus indicating that the induced contraction was not dependent on the 5-HT1B receptor.

Quantitative Real-Time PCR

To evaluate whether the upregulated response was generated through an increased transcription of the gene coding for the 5-HT1B receptors, functional data were substantiated by measuring the number of copies of 5-HT1B receptor mRNA in the arteries. The standard curve was straight for all primers used, yielding slopes between 3.1 and 3.2 and indicating replication efficiencies close to 1 (a replication efficiency of unity would yield a slope of 3.3). The standard curves regressed with correlations between 0.98 and 0.99, indicating a uniform PCR product throughout that was independent of the cDNA concentration (Fig. 3). Results of electrophoresis demonstrated only one product for each primer pair at the expected size (data not shown).

In the MCA and the BA the number of copies of mRNA coding for the 5-HT1B receptor were 3.8 \( \pm \) 0.8 and 3.9 \( \pm \) 1.2 times greater in the rats with SAH compared with the control rats, respectively (p < 0.05, Student t-test). In the PCoA there was no significant difference between rats with SAH and control rats (Fig. 4). Messenger RNA for the 5-HT1D receptor could not be detected in significant amounts (data not shown).

Discussion

In this study we have shown that blood injected into the subarachnoid space of the rat clearly leads to altered pharmacological properties of the cerebral arteries; more specifically, the sensitivity to the ubiquitously present amine 5-HT is increased in the MCA and BA. The implication of this finding is that the upregulated arteries in rats with SAH contract in response to much lower concentrations of 5-HT than do vessels in control animals.

In the present series the PCoA did not undergo phenotyp-
Upregulation of the 5-HT\textsubscript{1B} receptor after experimental SAH

SAH\textsuperscript{1,2,4,23} reports on an increased concentration of 5-HT in the CSF in patients with vasospasm\textsuperscript{3}, and studies demonstrating that perineurymal CSF was capable of causing contraction of human intracranial arteries.\textsuperscript{36} It has previously been shown, like the results reported here, that SAH induces an increased sensitivity of cerebral arteries toward 5-HT.\textsuperscript{2,25} The increased sensitivity, in view of the lack of clinical effect of 5-HT\textsubscript{1B} antagonists, could be explained as a passive secondary phenomenon to the products of fibrin degradation,\textsuperscript{10} to hemoglobin and serum,\textsuperscript{37} and to hemolysate;\textsuperscript{11} however, the novelty of the present study lies in our use of more specific antagonists and more advanced molecular biological techniques. With these methods we have clearly shown which receptor is upregulated (the 5-HT\textsubscript{1B} receptor) and that this upregulation is due to a synthesis of mRNA, which in turn is a result of increased gene transcription. Data from the present study thus complete or strengthen findings obtained some time ago.

Upregulation of Other Receptors and its Possible Mechanism

Nevertheless, this does not change the fact that 5-HT antagonists have proven to be ineffective in relieving clinical vasospasm,\textsuperscript{39,40} and hence care should be exercised in interpreting the data. In another study we have shown that endothelin receptors are also upregulated.\textsuperscript{13} We therefore believe that the true inference of this study lies within documenting a change in the vascular receptor phenotype as a result of SAH perhaps not hitherto appreciated. This new phenotype invariably makes the artery much more sensitive to humoral factors already present and may represent an end point in a cascade of events starting with the extravasation of blood in the subarachnoid space. Using in vitro organ culture of entire arterial segments, upregulation of the 5-HT\textsubscript{1B} receptor was also observed.\textsuperscript{18} In a subsequent study conducted to examine the role of protein kinase A and PKC in this upregulation, it was found that inhibition of protein kinase A led to enhanced upregulation of the 5-HT\textsubscript{1B} receptor, whereas inhibition of PKC with staurosporine led to an inhibition of organ culture–induced receptor changes.\textsuperscript{14} Staurosporine is not exclusively specific for PKC, but results indicate an involvement of these protein kinases, from which we infer the presence of a signal transduction pathway mediating the changes in receptor phenotype. It is thus reasonable to conclude that a signal transduction pathway must also exist in the case of SAH-induced changes in the receptor phenotype. The question then becomes, What is the nature of the signal involved in SAH?

As previously stated, it is obvious that the extravasation of blood at the time of the SAH is the starting point. Various theories exist to explain the course thenceforward. One theory is the generation of free radicals (notably superoxides) resulting from the decay of hemoglobin.\textsuperscript{12,25,38} Superoxides peroxidize membrane phospholipids, causing an intracellular increase in the concentration of diacylglycerol and inositol phosphates. While inositol phosphates cause an intracellular release of Ca\textsuperscript{2+} (itself causing contraction), diacylglycerol activates PKC and makes a link to receptor upregulation possible. Another theory, based on experience in experimental lesioning of the trigeminal pathway, which has been shown to be protective against vasospasm, implies a neural reflex pathway.\textsuperscript{33,34} Trigeminal afferents stimulated by subarachnoid blood (or breakdown products of blood) could relay interoceptive sensory information through the ascending noradrenergic pathways through the A2-solitary nuclei complex in the medulla oblongata to the hypothalamus, which is suggested to coordinate autonomic and endocrine responses.\textsuperscript{31} Vasospasm could then be induced by either the production of a humoral factor in the CSF or trigeminal or sympathetic efferents, to the liberation of vasoactive factors perivascularly. At least some of the sympathetic efferent transmitters are known to be able to activate PKC, for example, neuropeptide Y.\textsuperscript{9} A third theory involves the perivascular immunogenic response after SAH.\textsuperscript{24,32,41} In a study in which researchers described the influence of cytokines on the upregulation of endothelin receptor, they also found that interleukin-1 and tumor necrosis factor–α enhanced the organ culture–induced upregulation of endothelin receptor B in cerebral arteries in rats.\textsuperscript{25} It is therefore not unreasonable to suggest an influence on 5-HT\textsubscript{1B} receptor upregulation as well.

Conclusions

The as yet lamentable results of various pharmacological trials in treating clinical vasospasm may be an indication that searching for a single pharmacological factor that changes in response to SAH is fruitless. Thus, we propose the use of a multivariate model in which several receptor systems are upregulated, including the 5-HT receptor changes described in the present study. The challenge will then be the identification of a unifying factor that causes these changes, and perhaps such a factor may lend itself to manipulation to reverse vasospasm. Protein kinase C may be a candidate for such a factor.

References


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