Bilirubin Levels in Subarachnoid Clot and Effects on Canine Arterial Smooth Muscle Cells

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Background and Purpose: Previous studies have suggested that bilirubin is a potential contributor to cerebral vasospasm. The purpose of this investigation was to determine whether bilirubin accures in subarachnoid clot, whether its vasoconstrictive effect could involve a direct action on arterial smooth muscle cells, and, if so, whether bilirubin affects their Ca\(^{2+}\) uptake.

Methods: Subarachnoid clots were analyzed for bilirubin using high-performance liquid chromatography. The length and \(^{45}\)Ca\(^{2+}\) uptake of vascular smooth muscle cells enzymatically dissociated from canine carotid arteries were measured before and after exposure to bilirubin solution. Additional experiments were conducted on cultured smooth muscle cells from canine basilar artery and on ATP-depleted cardiac myocytes.

Results: Mean\(\pm\)SE bilirubin concentration in experimental clot was 263\(\pm\)35.7 \(\mu\)mol/L. Vascular smooth muscle cells exposed to bilirubin showed progressive shortening \((P<.01)\) and an increased uptake of \(^{45}\)Ca\(^{2+}\) \((P<.001)\). Contraction was prevented by Ca\(^{2+}\)-free media but not by verapamil. Experiments with heart myocytes showed that bilirubin caused an increased uptake of \(^{45}\)Ca\(^{2+}\) but not of \(^{125}\)I-sucrose.

Conclusions: The results indicate that bilirubin accures in subarachnoid clot, that it exerts a direct vasoconstrictive effect on arterial smooth muscle cells, and that this effect is associated with an increased uptake of Ca\(^{2+}\). Studies on heart myocytes suggest that the Ca\(^{2+}\) uptake induced by bilirubin could be due to a selective increase in membrane permeability to Ca\(^{2+}\). (Stroke 1993;24:1241-1245)

Key Words: bilirubin • calcium • vasoconstriction • vasospasm • dogs

A number of substances have been proposed to play a role in the development of cerebral vasospasm after subarachnoid hemorrhage. The weight of evidence suggests that the primary agents are derived from the hematoma itself\(^1\) and from the red blood cell in particular.\(^2\)\(^-\)\(^3\) In addition to extensive documentation of the vasoactivity of oxyhemoglobin,\(^1\)\(^-\)\(^7\) several reports have indicated that bilirubin, a hemoglobin breakdown product, is capable of producing constriction of cerebral arteries, both in vivo and in vitro.\(^8\)\(^-\)\(^9\) One part of this study was undertaken to determine the levels of bilirubin in subarachnoid clot. The remainder of the study focused on the mechanisms of bilirubin-induced vasoconstriction. In principle, bilirubin could act (1) directly on the smooth muscle cells; (2) indirectly, by eliciting the release of vasoactive substances from nerve varicosities in the adventitia; and/or (3) also indirectly, through the release of similar types of substances from endothelial cells. In this report we investigated the first possibility by studying the effects of bilirubin on the morphology and the calcium ion uptake of arterial smooth muscle cells. The effects of Ca\(^{2+}\)-free media and of a calcium channel blocker were also investigated. The results of these studies led us to analyze further the manner in which bilirubin

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affects membrane permeability, using isolated rat heart cells as a model system.

Materials and Methods

Eight adult mongrel dogs were anesthetized with halothane, and a right frontal craniotomy was performed under sterile techniques. An incision was made in the dura anterior to the Sylvian fissure, and the right frontal lobe was gently elevated to expose the internal carotid artery at its bifurcation. Five milliliters of fresh, autologous arterial blood was gradually deposited to create an adherent clot over the exposed segments of internal and middle cerebral arteries. The dura and scalp tissues were then closed with sutures. After the animal had awakened, it was returned to its cage and maintained under close observation for the following 48 hours. Seven days after clot deposition, the animal was reanesthetized, the cranial wound was reopened, and the clotted material was harvested and immediately refrigerated at 4°C. Specimens of clotted material removed from two patients at the time of craniotomy for clipping of a ruptured cerebral artery aneurysm were likewise refrigerated.

For analysis of bilirubin concentrations, the clots were homogenized in 5.0 mL of 0.1 mol/L ammonium acetate, pH 4.0. Bilirubin was twice extracted with 20 mL of chloroform/methanol (2:1, vol/vol) by the technique of Folch et al.\(^10\) The two chloroform extractions were pooled, rotovaporated to dryness, and redissolved in 0.5 mL dimethylsulfoxide/chloroform (1:4, vol/vol).
Bilirubin was subsequently quantitated using high-performance liquid chromatography methodology, as previously described.\textsuperscript{11} Bilirubin concentrations were first expressed in terms of micrograms bilirubin per grams clot. Considering grams clot as grams fluid, the factor used to convert the value for micrograms bilirubin per grams clot to micromolar bilirubin was 1.71.

Smooth muscle cells were enzymatically dissociated, following the technique of Wadsworth et al,\textsuperscript{12} from carotid arteries of adult mongrel dogs. Cells were suspended in buffer containing (mM/L): NaCl 118, KCl 4.8, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) 25, KH\textsubscript{2}PO\textsubscript{4} 1.2, CaCl\textsubscript{2} 0.5, glucose 11, pH adjusted to 7.4 with NaOH, at 37°C. Cell concentration was 1 mg protein per milliliter. After isolation, 65% to 75% of the cells were spindle shaped, and more than 90% demonstrated Trypan blue exclusion. Ca\textsuperscript{2+}, free medium contained 2 mM/L ethylene glycol-bis(\beta-aminoethyl ether) N,N,N’,N’-tetraacetic acid and no CaCl\textsubscript{2}.

Cardiac myocytes were isolated as previously described\textsuperscript{13} and resuspended in the same buffer as above. These cells were ATP depleted by incubation with 4 \textmu M/L rotenone and 2 \textmu M/L carbonyl cyanide p-trifluoromethoxyphenylhydrazone\textsuperscript{14} for 10 minutes to eliminate the effects of energy-dependent calcium pumps.

For analysis of morphological changes, smooth muscle cells from eight carotid arteries were fixed in 2% glutaraldehyde after exposure to bilirubin solution for various time periods. Samples of these cells were placed on microscope slides, and the length of 25 consecutive cells was measured using a calibrated grid.

Calcium uptake measurements were made using a dual-label (\textsuperscript{45}Ca\textsuperscript{2+}, tritiated water) technique.\textsuperscript{14} After exposure to test solutions, myocytes were centrifuged through 500 \textmu L 1 bromododecane/1,10 dichlorodecane (1:1), the pellets were placed in scintillation media, and radioactivity was measured in a scintillation counter. Calcium uptake was expressed as percent permeation (R\textsubscript{P}/R\textsubscript{S}\times 1000), where R\textsubscript{P} is the pellet ratio, and R\textsubscript{S} the supernatant ratio, of \textsuperscript{65}Ca\textsuperscript{2+} to tritium.\textsuperscript{15} A23187 was used at a concentration of 5 \textmu M/L. Sucrose uptake studies were conducted using 2 \textmu Ci/mL [\textsuperscript{3}H]sucrose with 1 mM/L sucrose carrier.

For the aforementioned experiments, bilirubin solutions were prepared by dissolving crystalline bilirubin (Sigma Chemical Co, St Louis, Mo) in 0.01N NaOH, which was then diluted with distilled water to achieve the desired final concentration. Twenty-five microliters of this solution was added to 1 mL of cellular suspensions. The same amount of NaOH solution without bilirubin was used for control measurements. Experiments were carried out under dark conditions to minimize photoisomerization and oxidation of bilirubin.

All procedures for the isolation and primary culture of basilar artery smooth muscle cells were performed under aseptic conditions. The basilar artery was resected from adult mongrel dogs under general anesthesia. The artery was cleaned of connective tissue and adventitia in M199 containing 25 mM/L HEPES, 100 U/mL penicillin, and 100 \textmu g/mL streptomycin. The arteries were opened longitudinally, and the intimal surface was gently scraped with a scalpel blade to remove the endothelium. The arteries were then minced into 1-mm squares and digested in M199 containing 25 mM/L HEPES, collagenase class II (600 U/mL), elastase type III (50 U/mL), soybean trypsin inhibitor (0.1%), bovine serum albumin (1.5%), amino acid standard (1.5%), ATP (4.0 mM/L), and penicillin and streptomycin as above, at 37°C for 1 hour in a shaker bath. After spontaneous settling of the digested debris, the supernatant cell suspension was carefully collected with a pipette and centrifuged at 1000 rpm for 3 minutes. The cell pellet was resuspended in M199 supplemented with 10% fetal calf serum, 25 mM/L HEPES, and the aforementioned antibiotics and then seeded in plastic culture flasks. These cells were reacted with mouse monoclonal anti-\alpha-actin smooth muscle cell antibody (Sigma), then with biotinylated horse antirabbit immunoglobulin antibody, and finally with avidin DH and biotinylated horseradish peroxidase H. 3,3’Diaminobenzidine was used as the chromophore. These cells stained positively for \alpha-actin, whereas similar cells in which the initial step with anti-\alpha-actin antibody had been omitted did not. After reaching confluency the cells were seeded at an initial density of 2 \times 10\textsuperscript{4} cells per dish. The medium was changed to serum-free medium 2 days after seeding. Bilirubin first dissolved in 0.1 N NaOH, and serum albumin (Sigma) were then added to achieve a final concentration of 50 \textmu M/L bilirubin and a molar ratio of bilirubin to albumin of 1.5:1.0. The same amount of NaOH and of albumin without bilirubin was added to control dishes. Dishes of cells were fixed with 2.5% glutaraldehyde in situ at 0, 24, and 48 hours. The length of 50 consecutive cells from each dish of cells was measured as described previously.

**Results**

The mean±SE concentration of bilirubin in clotted material removed 7 days after deposition was 263±35.7 \mu M/L. The concentrations of bilirubin in subarachnoid clot removed from two patient subjects, one 8 days and the other 10 days after hemorrhage, were 172 \mu M/L and 149 \mu M/L, respectively.

The effects of bilirubin on acutely dissociated and on cultured arterial smooth muscle cell length are shown in Figs 1, 2, and 3. The degree of change was related to both the concentration of bilirubin and the duration of exposure. Additional experiments on acutely dissociated cells showed that, as measured at 30 minutes, the contraction of cells exposed to 25 \mu M/L bilirubin (54.3±0.92 \mu M) was not reduced by the presence of 1 \mu M/L verapamil (55.8±0.99 \mu M). However, no contraction of cells exposed to 25 \mu M/L bilirubin in the absence of extracellular Ca\textsuperscript{2+} (74.4±1.3 \mu M vs 77.5±1.2 \mu M for bilirubin-free control) was observed. Alterations in Ca\textsuperscript{2+} uptake of dissociated arterial smooth muscle cells induced by 50 \mu M/L bilirubin are shown in Fig 4.

To examine further the cause of these changes, we asked the following questions. (1) Was the increased Ca\textsuperscript{2+} uptake induced by bilirubin due to greater Ca\textsuperscript{2+} binding, perhaps to membrane-bound bilirubin, or was it due to an increased permeability? (2) Did other solutes show a similar increase in uptake? To address these questions it was necessary to use cells that were completely depleted of ATP to eliminate energy-dependent ion movements but that still had an intact plasma membrane. We used this system because this state was well defined in previous studies in one of our laboratories using isolated adult rat heart cells.\textsuperscript{14} Fig 5 shows that bilirubin increased the uptake of Ca\textsuperscript{2+}, but no
increased permeation of sucrose was detected. The Ca\textsuperscript{2+} ionophore A23187 further increased the extent of Ca\textsuperscript{2+} uptake. Bilirubin, however, did not affect the final level of Ca\textsuperscript{2+} uptake after A23187.

**Discussion**

The primary findings of this study are that bilirubin accrues in subarachnoid hematoma and that it produces a direct contractile effect on arterial smooth muscle cells.

Although it has long been recognized that the xanthochromia of cerebrospinal fluid after subarachnoid hemorrhage is primarily due to the presence of bilirubin, the development of significant levels of this substance in hematoma has recently been questioned\textsuperscript{16,17}. One group of investigators has reported that no or minimal bilirubin was recovered from agar that had been soaked with hemoglobin and deposited in the subarachnoid compartment of monkeys\textsuperscript{16}. However, the adequacy of the technique used to extract bilirubin from agar, to which it avidly binds, was not demonstrated. In addition, the reagents and pH conditions used to convert bilirubin to its methyl ester were not described. Moreover, suggestions that bilirubin does not accrue in subarachnoid clot are difficult to reconcile with the documentation that significant levels of bilirubin develop in other forms of intracranial hematoma\textsuperscript{18}. The accrual is not surprising in view of the presence of heme oxygenase in arachnoid cells\textsuperscript{19}, macrophages\textsuperscript{20} that penetrate hematoma, and brain cells\textsuperscript{21}. The findings of the present study are, therefore, consistent with the weight of evidence.

Studies in the field of kernicterus have documented the toxic properties of bilirubin in a variety of biological tissues, including neurons, fibroblasts, and erythrocytes. The deleterious effects of bilirubin have been shown to depend on its concentration, duration of exposure, pH, and the presence or absence of albumin, to which bilirubin binds. Silberberg and Schutta\textsuperscript{22}, for example, reported that 8.5 \textmu mol/L bilirubin produced observable morphological changes in cultures of rat cerebellar cells within 3 hours and that by 18 hours virtually all cells were swollen or disrupted. Although certain alterations have been postulated to be the result of enzymatic interference, others may lie within bilirubin's ability to interact with phospholipid membranes, both mitochondrial and plasma\textsuperscript{23-25}. Given the documentation of the effect of bilirubin on Na\textsuperscript{+}-K\textsuperscript{+} ATPase\textsuperscript{26}, it is likely that a recent study reporting that bilirubin produced no alteration in K\textsuperscript{+} conductance in dissociated vascular smooth muscle cells as measured by patch clamp\textsuperscript{27} was the result of using solutions that were prepared by
adding the crystalline powder to solution at physiological pH, a method that yields an aqueous concentration of only 7 to 10 mmol/L.20 The absence of angiographic changes in monkeys subjected to subarachnoid injection of a similarly prepared solution29 is likewise not unexpected. The method we used for preparing bilirubin solution conforms to those conventionally used in bilirubin studies.22,24-26

With regard to the vasoconstrictive effect of bilirubin, the results indicate three important features. First, in contrast to the action of most substances known to control vascular tone, the contraction and Ca2+ uptake induced by bilirubin occurred gradually. These findings suggest that, at least under the conditions of these experiments, the dynamics of the action of bilirubin on the plasma membrane differ from those of agents having specific receptors. The lack of contraction in Ca2+-free media indicates that the bilirubin effect is dependent on influx of Ca2+, but the inability of verapamil to block the contraction shows that the influx is not primarily mediated by calcium channels. There are a number of possible reasons for the slower rate of contraction of cultured basilar artery smooth muscle cells, but two factors may be their adherence to the surface of the glass dishes and the presence of albumin, in which bilirubin binds.

Second, because bilirubin is known to bind Ca2+, 30 it was important to determine whether the increased uptake was due to binding of the ion to membrane-bound bilirubin or to a change in permeability. The results with heart cells suggest that the effects of bilirubin on Ca2+ uptake are not caused by an increase in Ca2+ binding, since such an increase would be expected to remain after A23187 treatment. It appears, therefore, that the effect of bilirubin is to increase membrane permeability. The evidence that bilirubin increases the permeability of the membrane to Ca2+ explains not only the increased uptake but also the morphological changes induced by bilirubin, since these cells are known to contract in response to increased levels of intracellular Ca2+.31 Further, this increased permeability does not appear to be nonspecific, inasmuch as the permeability to sucrose did not increase. Although these findings could be limited to cardiac myocytes, it is also possible that they apply to all membrane systems, including those of vascular smooth muscle cells.

Third, the results indicate that bilirubin does not act in precisely the same manner as the calcium ionophore A23187 because that agent further increased the uptake of Ca2+ by these cells. If bilirubin possesses ionophore-like activity, it may act only at the sarcolemma, whereas the more mobile A23187 will also conduct Ca2+ to intramitochondrial binding sites. The solubility characteristics and carboxyl groups of bilirubin render it a negatively charged amphiphile, and part of its action could be similar to that of other negatively charged amphiphiles that have been found to stimulate the Na+·Ca2+ exchanger.32

In summary, the principal findings of this study are (1) that bilirubin accretes in subarachnoid hematomas, (2) that bilirubin exerts a direct contractile effect on arterial smooth muscle cells, (3) that in these cells it causes an increased uptake of Ca2+, and (4) that this increase could be caused by greater permeability of the membrane to Ca2+, but not to certain other solutes, such as sucrose. The results of this investigation do not eliminate the possibility that the action of bilirubin on whole arteries may also be mediated through the release of vasoactive substances from nerve varicosities or from the endothelium. It is also possible that bilirubin may lead to the release of intracellular Ca2+ stores. These issues, as well as the precise role that bilirubin may play in the genesis of cerebral vasospasm, require further study.

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References
The results reported by Trost and colleagues are consistent with previous findings that bilirubin causes contraction of cerebral arteries. In the present study, the authors measured the concentration of bilirubin that was accumulated in clots 7 days after deposition of autologous arterial blood over canine internal and middle cerebral arteries. They detected a mean concentration of 263 μmol/L. A similar concentration of bilirubin was also detected in subarachnoid clots removed from two patients 8 and 10 days after hemorrhage. In vitro, bilirubin (10 to 100 μmol/L) produced contraction of smooth muscle cells from cerebral arteries. The authors also propose that contractions to bilirubin are due to selective increase in membrane permeability for calcium. The precise mechanism of the effect of bilirubin on cell membrane remains unclear.

What are the implications of these findings for understanding the mechanism of cerebral vasospasm after subarachnoid hemorrhage? First, it should be mentioned that the contribution of bilirubin as vasoconstrictor to chronic narrowing of cerebral arteries exposed to autologous blood has been questioned. In contrast to oxyhemoglobin, bilirubin did not produce significant vasospasm after intrathecal injections in the cynomolgus monkey. In addition, methemoglobin does not produce cerebral vasospasm despite the fact that it is converted to bilirubin in the subarachnoid space. However, the results of the present study suggest that bilirubin may contribute to an increase in cerebral arterial tone after subarachnoid hemorrhage. It appears that the role of bilirubin needs to be reassessed, particularly in view of the findings concerning the effect on arterial smooth muscle cell membrane permeability for calcium. Further comparative studies of the effects of oxyhemoglobin and bilirubin on cell membrane will certainly improve understanding of the complex mechanism underlying the chronic narrowing of cerebral arteries.

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