Changes in Vasoactive Properties of Blood Products with Time and Attempted Identification of the Spasmogens

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SUMMARY The contractile activity of various fresh, or incubated blood fractions was studied in vitro using the isolated canine basilar artery. Significantly greater contraction was induced by fresh platelet rich plasma (PRP) and serum compared to red blood cells (RBC). Following incubation, the contractile activity of RBC increased, reaching a plateau at day-3 and it was maintained for at least 14 days, while both PRP and serum lost most of their activity after 24 h of incubation. The contractions induced by fresh blood fractions were only partially blocked by desensitization of 5-hydroxytryptamine (5-HT) receptors or by the 5-HT antagonist methysergide. D-600 effectively antagonized the response to all blood fractions. Biochemical analysis of the incubated RBC by means of Sephacyr S-200 column chromatography and SDS-urea polyacrylamide gel electrophoresis revealed that the contractile substance possessed a molecular weight of about 60,000 daltons. Vasoactivity was only present in one peak of the chromatographically eluted fractions which was shown to possess a similar absorption spectrum to that of hemoglobin. Hemoglobin concentration was highest in day 3, 7, and 14 fractions and may be correlated with the contractile activity of incubated samples.

Stoke, Vol 12, No 6, 1981

MANY POSSIBLE CAUSES for the vasospasm which follows subarachnoid hemorrhage (SAH) have been considered. The role of serotonin (5-HT) has been extensively investigated because of the high sensitivity of cerebral vessels to this agent and the high concentration of 5-HT in the platelets. However, because the vasoconstrictor action of 5-HT both in vivo and in vitro is short lasting, it is unlikely to be the sole cause of chronic vasospasm.

More recently, the role of the erythrocyte and the by-products of its degradation have come under scrutiny. These studies have implied that products of the hemolysis of the red blood cells (RBC), especially hemoglobin (Hb), play a major role in chronic vasospasm.

The present study was undertaken to determine the time course of contractions elicited by various blood fractions and the effect of 5-HT on such contractions, using canine cerebral arteries. Also, the aim was to evaluate the role of RBC in vasospasm by determining whether the time course of vasospasm, induced by incubated RBC, parallels the measured Hb concentration of the fraction. Another purpose was to determine the identity of the active material in the fractions with vasoconstrictor activity and, finally, to study the effect of potential antagonists on such contractions.

Methods and Materials

Preparation of Arterial Strip

Adult dogs of either sex weighing 16 to 23 kg were anesthetized with intravenous sodium pentobarbital, 32 mg/kg, and were killed by bleeding from the femoral artery. The brain with the basilar artery attached was removed and a segment of the basilar artery was removed and placed immediately in ice cold Krebs bicarbonate solution gassed with 95% O2: 5% CO2.

The arteries were slipped onto a thin stainless steel wire and cut into spiral strips 30 to 40 mm long at an angle of approximately 45°. The spiral strips were then mounted in standard organ baths of 10 ml working volume filled with Krebs bicarbonate solution of the following composition: NaCl 118.0 mM, KCl 4.75, KH2PO4 1.19 mM, CaCl2 2.54 mM, MgSO4 1.19 mM, NaHCO3 25.0 mM and dextrose 5.6 mM (pH 7.4) and constantly aerated with a gas mixture of 95% O2 and 5% CO2. The temperature of the bath was maintained at 37°C. Contractions were recorded isometrically using Grass FT.03 strain gauges connected to ink-writing Grass polygraphs, model 7D or 5D. All preparations were suspended with a resting tension of 2 g and allowed to equilibrate for at least 90 min. During that time, the fluid was replaced every 20 min and the passive tension was readjusted. Contractions were obtained for each arterial strip at the onset of each experiment using 2 × 10-6 M 5-HT (ED 100) and 30 mM KCl. Values of contractions induced by test materials are expressed as a percentage of maximum 5-HT or KCl responses.

Preparation of Blood Fractions

Anesthetized dogs were bled at the time of sacrifice via a short polyethylene catheter placed in the femoral artery. Blood was collected into tubes cooled in ice and containing heparin (100 units per 10 cc whole blood). The various blood fractions were prepared as follows:

1. Platelet-rich plasma (PRP): whole blood was centrifuged at 105 × g for 20–30 min in a refrigerated centrifuge and the supernatant decanted.

2. Red blood cells were obtained from the packed red cells remaining after PRP was separated. The RBC were then washed 3 times with an equal volume
of cooled normal oxygenated Krebs solution. After each wash, the RBC were centrifuged at 105 × g and the supernatant discarded. The erythrocytes were suspended with 5 parts of sterilized oxygenated Krebs solution. This mixture is referred to as RBC.

3. Serum was obtained from whole blood collected without heparin and allowed to clot at room temperature; the supernatant was then separated.

Aging of the samples was achieved by incubating them at 37°C for 1, 3, 7 and 14 days under aseptic conditions.

Chromatography of RBC

The supernatant obtained from centrifugation of incubated RBC was eluted through a Sephacryl S-200 super fine column with either Tris HCl buffer (pH 7.4) or normal Krebs solution (pH 7.4). The protein concentration in the eluent was determined by measuring the absorbance of 280 nm. The peaks collected from the column were analyzed for spasmodenic activity by concentrating the samples by evaporation under reduced pressure and applying them directly to the arterial strip.

Molecular Weight Determination

The column (Sephacryl S-200) was calibrated using standard proteins. The elution volumes were used for molecular weight determination. Standard proteins used were ribonuclease A (M.Wt. 13,700) chymotrypsinogen A (M.Wt. 25,000), Ovalbumin (M.Wt. 43,000) and albumin (M.Wt. 67,000). Dextran and DNPS were used to determine the void and total bed volume respectively. Sodium dodecyl sulphate (SDS)-urea polyacrylamide slab electrophoresis was also used to determine the molecular weight of the fraction collected from the column. The molecular weight markers used were those used for column chromatography calibration described above.

Spectrophotometric Analysis

Both the incubated fractions of RBC eluted from the column and the incubated crude fraction were analyzed using a Bausch and Lomb scanning spectrophotometer. The samples were scanned through a wavelength range of 350 nM to 600 nM and the absorbance curves obtained were compared to that of standard hemoglobin.

Hemoglobin Determination

Hemoglobin concentration in supernatant from both the fresh and incubated samples were measured by the cyanmethemoglobin method. The hemoglobin determination kits were obtained from Sigma and the absorbance at 420 and 540 nM was measured using a micro-sample spectrophotometer 300-N.

Pharmacological Studies

In experiments designed to study desensitization, a concentration-response curve to 5-HT was first obtained and then the arterial strips were continuously exposed to 5-HT at a concentration that produced a maximum contraction (2 × 10⁻⁴M). A second concentration-response curve for 5-HT was then obtained in the presence of the desensitizing dose of 5-HT. To test the specificity of this desensitization, similar experiments were carried out with a number of other agonists, dose-response curves being obtained in the absence and then in the presence of a desensitizing concentration of 5-HT. The same experimental protocol was used in desensitization experiments involving various blood fractions.

Determination of the duration of contraction induced by incubated blood fractions was expressed as "residual force" calculated as follows:

\[
\text{Residual force (RF)%} = \frac{\text{Tension 10 min after maximum}}{\text{Maximum Tension}} \times 100
\]

In experiments in which the effects of antagonists on the contractile response to blood fractions were studied, the antagonists were allowed to remain in contact with the preparation for one hour prior to retesting with the sample. The antagonist was not washed out during the test period. Results are expressed as mean values ± standard errors of the means from at least 3 experiments. Significance was determined by the Student's t-test or analysis of variance.

Drugs and chemicals were 5-hydroxytryptamine creatinine sulfate (Sigma), methysergide (Sandoz), D-600 (Knoll), heparin (Sigma), pentobarbitone sodium (BDH), prostaglandins E, and F,a (Sigma), Sephacryl S-200, chymotrypsinogen A, ribonuclease A, ovalbumin and albumin (Pharmacia Fine Chemicals), Drabkin's reagent No. 525-2, Brij-35 solution No. 430AG-6, hemoglobin standard No. 525-18 (Sigma).

Results

Response of the Basilar Strip to Various Blood Fractions

Fresh samples (Day-0) of all blood fractions tested contracted the basilar artery. The contractions obtained with serum or plasma were significantly greater than those induced by RBC. Following incubation for 3, 7 and 14 days, both plasma and serum lost their activity producing only 10 to 20% of the maximum contraction to 5-HT. In contrast, the activity of incubated RBC increased gradually, reaching a plateau at about the third day and remained essentially unchanged over the 14 days of incubation. At 14 days a wider variability in the response of RBC was noted. These results are shown in figure 1.

The duration of the contractile response to PRP, serum and RBC is shown in figure 2. All fresh fractions produced contraction which reached a maximum within 1 to 2 min. Contractions produced by incubated fractions faded very rapidly with the rate of fade being dependent upon the duration of incubation. Mean residual force (RF) for fresh RBC was 51.0 ± 3.8% after 10 min, which was significantly different from
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**Figure 1.** Effects of storage on activity of RBC (• — •), plasma (● — ●) and serum (▲ — ▲). Percentage of contraction to 2 x 10^-6M, 5-HT as ordinate, incubation period (days) as abscissa. Bars represent standard errors.

Day 7 and 14 samples (R_s equalled 24.0 ± 5.0 and 4.1 ± 2.7% respectively, p > 0.05).

**Effect of Desensitization with 5-HT on Contractile Response to Drugs and Various Blood Fractions**

Serotonin caused dose-dependent contractions of canine basilar arteries that faded very rapidly, falling to initial tension within 15 to 25 mins. The rate of fade of 5-HT contractions was dependent on the concentration of the agonist, increasing with increased 5-HT concentration. In the continued presence of 5-HT (2 x 10^-6M), responses to subsequent application of 5-HT were markedly depressed. A gradual recovery of responses to test concentrations of 5-HT occurred after 30 to 40 min when the desensitizing concentration of the agonist had been thoroughly washed out from the bath.

To investigate whether the desensitization by 5-HT of its own responses was specific, the effect of a desensitizing concentration of 5-HT (2 x 10^-6M) on the response to PGF_2α (1 x 10^-6M), PGE_2 (5 x 10^-6M) and KCl (30 mM) was studied. In the presence of 5-HT the contractile response to each of these agonists was not significantly different from the control (fig. 3).

The contractile response of the basilar artery to fresh PRP, serum and RBC was attenuated in the presence of a desensitizing dose (2 x 10^-6M) of 5-HT; mean reduction of the responses were 23 ± 4.46 and 21.9 ± 6.67% respectively of the maximum contraction to 5-HT. When incubated samples were tested on tissues which had been desensitized to 5-HT, it was

**Figure 2.** Effects of storage on duration of response to RBC (● — ●), plasma (● — ●) and serum (▲ — ▲). Residual force (percentage decline in contraction after 10 min exposure — see text) as ordinate, incubation period (days) as abscissa. Bars represent standard errors.

**Figure 3.** Dose-response curves to 5-HT before (● — ●) and after (▲ — ▲) desensitization by prolonged exposure to 2 x 10^-6M 5-HT. % maximum response as ordinate, dose (M) as abscissa. The insert shows the response to other agents in tissues desensitized to 5-HT as a percentage of the control response in normal tissues. Bars represent standard errors.
found that the very small responses obtained with PRP or serum were enhanced, while the larger responses to RBC were significantly reduced but not abolished (fig. 4). The duration of the response to any fraction was not significantly affected by desensitization to 5-HT.

Effect of Desensitization by Blood Fractions on Contractile Response to 5-HT

The continued presence of all samples at Day 0 attenuated the contraction induced with 5-HT (2 × 10^{-4} M) without affecting the response to KCl (30 mM). PRP and serum reduced the 5-HT response to about 10% of control, while RBC was much less effective. After incubation PRP and serum largely lost their ability to alter the response to 5-HT, while prolonged exposure to RBC from incubated blood produced baseline changes which made the response to 5-HT difficult to measure. These results are shown in fig. 5.

Effect of Methysergide and D-600 on Blood Fraction-Induced Contraction

Methysergide (2 × 10^{-4} M) essentially eliminated the response to 5-HT (2 × 10^{-4} M) but was without effect on the contraction induced by KCl (30 mM). The contractions produced by fresh blood fractions were only partially blocked (fig. 6). Similarly, incubated RBC mixture was partially blocked by methysergide. In contrast, the contraction induced by incubated (Day 3 to Day 14) PRP or serum was not affected and there was a tendency for the responses to increase in the presence of methysergide. These data are also shown in figure 6.

D-600 (1 × 10^{-5} M) eliminated the contraction produced by 5-HT (2 × 10^{-4} M) on dog basilar artery and also produced a complete blockade of the contraction induced by fresh and incubated PRP, serum and RBC.
Biochemical Analysis

A. Column Chromatography

For isolation of the vasoactive substance, the supernatant from centrifugation of RBC was passed through a column of Sephacryl S-200. All samples yielded similar elution patterns consisting of 3 peaks. Significant contractile activity on the basilar artery was confined only to the second peak corresponding to a molecular weight of about 5.5 x 10^4 daltons. Polyacrylamide gel electrophoresis provided a similar picture to that of hemoglobin.

Spectrophotometric Analysis of Absorbance

The absorbance curve of this fraction was analyzed with a scanning spectrophotometer between the wavelengths of 350 to 650 nm. Prominent absorption bands occurred at 420, 540, and 580 nm and similar pattern was obtained for all the incubated samples tested (Day 0, 1, 3, 7 and 14) for RBC. The absorption spectrum obtained was similar to that of a hemoglobin standard.

Hemoglobin Concentration in the Fractions

The table summarizes the results obtained for RBC. Almost all the erythrocytes were already hemolysed by day 3 and, when compared to the contractile activity of the samples on the basilar artery, it can be seen that the response reached a plateau on day 3 and remained high thereafter.

Table: Comparison of Hemoglobin Concentration in RBC with Contractile Response Induced on Dog Basilar Artery

<table>
<thead>
<tr>
<th>Incubation Period (Days)</th>
<th>Hemoglobin Concentration (% Total Hb) in RBC</th>
<th>Response (% Max) S-HT 2 x 10^{-9} M to RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.6 ± 2.3</td>
<td>71.4 ± 4.4</td>
</tr>
<tr>
<td>1</td>
<td>20.2 ± 1.2</td>
<td>90.0 ± 5.7</td>
</tr>
<tr>
<td>3</td>
<td>79.6 ± 4.0</td>
<td>99.5 ± 4.6</td>
</tr>
<tr>
<td>7</td>
<td>86.0 ± 3.3</td>
<td>97.7 ± 8.0</td>
</tr>
<tr>
<td>14</td>
<td>86.1 ± 5.5</td>
<td>114.7 ± 9.7</td>
</tr>
</tbody>
</table>

Contraction induced by 2 x 10^{-9} M S-HT were taken as 100%.

Discussion

The major results of these experiments may be summarized as follows:
1. Platelet-rich plasma and serum can contract the isolated basilar artery, but on incubation the activity rapidly disappears. Desensitization to 5-HT or blockade with methysergide effectively inhibits the contractile activity of fresh samples, but may enhance the response to incubated samples.
2. Activity of the incubated erythrocyte suspension was initially low but after 3 days incubation had increased and remained elevated for at least 14 days. This response was always somewhat attenuated but not greatly reduced by procedures which inhibit 5-HT.
3. D-600 is an effective antagonist of all samples.

4. No sample produced a sustained maximum contraction.
5. Fractionation of the erythrocyte sample revealed that the vasoactive material had a molecular weight of about 60,000 and this was supported by studies using polyacrylamide gel electrophoresis.
6. The activity of the sample closely paralleled release of hemoglobin by incubated erythrocytes.

The implications of this study are clear and are in general agreement with other reported work. The activity of fresh samples of whole blood appears to be due largely to non-erythrocyte factors. 5-HT seems to be important in that procedures which interfere with 5-HT receptors also antagonize the action of the plasma fraction. Incubation of plasma or serum results in marked reduction of activity over the fourteen day period and finally the activity of these fractions is negligible. Whether this results from alterations of 5-HT itself by metabolism or chemical degradation, or from binding of 5-HT to other components in the plasma is not clear. One of the most curious findings is that in tissues desensitized to 5-HT, but not in tissues treated with methysergide, it appears that the small response to incubated samples of PRP and serum is enhanced. Other workers have examined hemoglobin-5-HT interactions, but this result implies that there is some slowly-developing spasmogen in plasma which is most effective in tissues which have been previously exposed to 5-HT. Further attempts to confirm this observation are in progress. This finding is of potential significance in late vasospasm where the blood vessels may well have lost their sensitivity to 5-HT.

The erythrocyte fraction is clearly of greater importance in that the time course of incubated erythrocytes is similar to that of clinical vasospasm. While some component of the activity seems to reside in 5-HT, or at least some compound which interacts with 5-HT receptors, neither methysergide nor desensitization were very effective in reducing the response to this fraction. Hemoglobin has frequently been implicated in vasospasm but there is some debate as to whether erythrocytes release another vasoactive protein. The study reported here implies strongly that hemoglobin is responsible; the only fraction with biological activity obtained from incubated erythrocytes has a molecular weight similar to that of hemoglobin, and an almost identical ultraviolet spectrum. Furthermore, release of hemoglobin from incubated erythrocytes follows a similar time course to the development of vasospastic activity in the samples.

Whether, in fact, incubated erythrocytes contract isolated blood vessels in a fashion closely analogous to the development of clinical vasospasm remains to be seen. D-600, the calcium antagonist, is an effective antagonist in the in vitro system while another calcium antagonist, nifedipine, appears to be effective in vasospasm. In fact, cerebral blood vessels in vivo and in vitro seem to have an important requirement for transmembrane calcium flux for all agonists. Thus, while these results would be expected if the model and the clinical syndrome are similar, it hardly provides...
strong support for hemoglobin as the mediator of vasospasm.

We have previously reported that tachyphylaxis to 5-HT takes place very rapidly in isolated arterial strips, while hemoglobin itself produces a more sustained contraction. The studies reported here imply, however, that the duration of the contraction produced by erythrocytes is also relatively short-lived, particularly after prolonged incubation. This observation has various possible explanations. First, the basilar artery strip, although very widely used in studies of cerebral vasospasm may have some limitations. In particular, desensitization may develop rapidly in this preparation but not in intact or in situ vessels. A second possibility is that changes in the spasmogen occur on incubation so that at 14 days (but not earlier) the potency is not significantly diminished (fig. 1) but the response desensitizes so rapidly that after 10 min exposure little response remains (fig. 2). This implies that a factor in the resolution of vasospasm is a change in the ability of the spasmogen to produce a sustained contraction. Finally, there is the strong likelihood that in vivo the spasmogen, which seems very likely to be hemoglobin, serves merely as the initiating factor and that further events sustain the vasospasm and ultimately, in some cases, resolve it.

Acknowledgment

We thank Dr. T. M. Allen and Ms. C. A. Krueger for advice and assistance. This work was supported by the Canadian Heart Foundation.

References

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F Okwuasaba, D Cook and B Weir

*Stroke*. 1981;12:775-780
doi: 10.1161/01.STR.12.6.775

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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