Role of Intrinsic Arachidonate Metabolites in the Vascular Action of Erythrocyte Breakdown Products

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SUMMARY In helically-cut strips of dog basilar and mesenteric arteries, the isometric tension developed by application of ghost-free hemolysate from dog erythrocytes was recorded. The hemolysate contracted basilar arteries in a concentration-dependent fashion, the response being attenuated by treatment with either aspirin or polyphloretin phosphate, a prostaglandin antagonist. Mesenteric arteries were contracted only slightly by high concentrations of hemolysate. When the mesenteric arteries had partially been contracted with prostaglandin F2α or norepinephrine, the hemolysate induced relaxations, which were abolished by aspirin in approximately half the preparations used. Studies on rat stomach strips exposed to superfusate of dog cerebral arteries showed a release of prostaglandin-like substance by the hemolysate application.

It may be concluded that the hemolysate contracts basilar arteries and relaxes mesenteric arteries, mainly through prostaglandins synthesized in and released from the vascular wall. Such a mechanism may be involved in the pathogenesis of cerebral vasospasm following a subarachnoid hemorrhage.

Stroke Vol 15, No 1, 1984

ONE OF THE MOST important features of cerebral vasospasm following a subarachnoid hemorrhage (SAH) is its delayed onset after the aneurysmal rupture. No vasospasm occurs within a few days after the initial attack of SAH. Timing of the blood clot lysis matches the occurrence of delayed cerebral vasospasm after SAH. Furthermore, there is a close relationship between the incidence of vasospasms and the intracerebral high density area observed by the CT scan.

These facts suggest that subarachnoid blood clots are involved in the pathogenesis of vasospasm.

Since Osaka demonstrated that hemolysed erythrocytes have a remarkable vasoconstrictor activity, quite a few investigators provided evidences that supported an important role of constituents of erythrocytes or oxymeglobinins in the pathogenesis of vasospasm. Recently, the vascular action of blood constituents was pharmacologically analysed. However, the data were not confined to those with erythrocyte hemolysate. Hemoglobin, the main constituent of erythrocyte hemolysate, is one of the cofactors required for the action of cyclo-oxygenase which converts arachidonic acid to prostaglandin (PG) endoperoxides in vitro. The present study was thus undertaken to determine the mechanism of action of breakdown products of erythrocytes in isolated dog cerebral and mesenteric arteries, the special interest being focused on the involvement of PG synthesis.

Materials and Methods

Mongrel dogs of either sex, weighing 8 to 12 kg, were anesthetized with intraperitoneal injections of thiopental sodium (50 mg/kg) and sacrificed by rapid exsanguination from the common carotid arteries. The brain and the superior mesenteric artery were rapidly removed. The basilar artery and distal portions of the superior mesenteric artery of about 15 mm long were dissected free and cut helically into strips. Each arterial strip was vertically mounted between hooks in a muscle chamber; the lower end was fixed on the rigid arm and the upper end was connected to the lever of a strain gauge transducer (Model T7-8-240, Toyo-Baldwin Inc., Tokyo, Japan). The chamber was filled with the following composition (mM): Na+ 139.7, K+ 5.4, Ca2+ 2.2, Mg2+ 1.0, Cl- 131.5, HCO3- 20.0, and glucose 5.6. A gas mixture of 95% O2 and 5% CO2 was bubbled continuously, and the temperature was maintained at 37 ± 0.5 °C. The pH of the solution was approximately 7.3. The resting tension was adjusted to 1.5 g, then the specimen was allowed to equilibrate for 90 to 120 minutes.

About 200 ml of homogenous arterial blood was collected from the common carotid artery in a plastic bag for blood transfusion (JMS Blood Bag, Japan Medical Supply Co., Ltd., Hiroshima, Japan) which contained 0.66 g of sodium citrate, and stored in a refrigerator at 4 °C. The blood was centrifuged for 20 minutes at 500 rpm. Supernatant platelet-rich plasma as well as upper one third of blood cells were discarded. After the addition of the same volume of saline, suspended blood cells were centrifuged again for 10 minutes at 3000 rpm. and packed erythrocytes were isolated. The erythrocytes were washed with 5 volumes of saline three times. Finally the equal volume of distilled water was added and stored overnight at 4 °C to promote hemolysis. The hemolysate solution was then centrifuged for 20 minutes at 10,000 rpm., and the ghost-free supernatant was used as "hemolysate." The concentration of the hemolysate was expressed as the hemoglobin content which was assayed by a hemoglobin cyanide method.

Isometric tension developments were recorded on an ink-writing oscillograph (San-Ei Instrument Co., Tokyo, Japan). The contractile response to 30 mM K+ was first obtained in each specimen. Hemolysate was added directly to the bathing media in a cumulative manner. Contractile responses were presented as rela-
tive values to those induced by 30 mM K⁺. To evaluate relaxant responses, the arteries were partially contracted with low doses of norepinephrine, PGF₂α or K⁺. After the dose-response relationship for the hemolysate was completed, 10⁻⁴ M papaverine was added to attain the maximum relaxation.¹⁴ Each relaxant response was presented as a percentage of the papaverine-induced relaxation. To remove drugs or hemolysate, the specimen was washed three times with bathing solutions and allowed to equilibrate for 30 to 60 minutes, during which time the solution was replaced every 10 to 15 minutes. Preparations were treated for 60 minutes with polyphloretin phosphate (PPP) or 20 minutes with the other antagonists, prior to the addition of hemolysate.

The release of PG-like substance from cerebral arteries by hemolysate was evaluated with a superfusion technique using rat stomach strip (RSS). The principle of the experimental method was described by Vane.¹⁵ Pial arteries were obtained from two dogs for each experiment. The procedures were the same as those described in an earlier report.¹⁶ The RSS was treated with a mixture of antagonists, including atropine (10⁻⁷ M, final concentration in RSS superfusate, an anticholinergic agent), chlorpheniramine (10⁻⁶ M, a histamine H₁ blocker), cinanserin (10⁻⁷ M, a serotonin blocker), indomethacin (3 x 10⁻⁵ M, PG synthesis inhibitor) and propranolol (10⁻⁶ M, a β-adrenergic blocker). The contraction of RSS was recorded via an isotonic transducer (Nihon Koden Kogyo Co., Tokyo, Japan). The direct bolus application of hemolysate to RSS (40 μl of 10 g/dl hemoglobin-containing hemolysate) induced a transient contraction. When the strip was continuously superfused with hemolysate-containing solutions (0.1 g/dl hemoglobin in final concentration), the bolus application of hemolysate to RSS did not alter the tension. Then, hemolysate was applied to cerebral arteries, and contractions of RSS were recorded. Responses of RSS to different concentrations of authentic PGE₂ were obtained after each trial of hemolysate, and the concentration of PG-like substance was expressed as a relative value to the concentration of PGE₂. Results were expressed as mean values ± standard error of the mean, the Student's t-test being used for statistical analysis.

Drugs used were aspirin, sodium polyphloretin phosphate, cinanserin, catalase (Sigma, Saint Louis), superoxide dismutase (Sigma), dl-α-tocopherol (Nakarai, Kyoto, Japan), dl-norepinephrine hydrochloride, prostaglandins E₂ and F₂α (Ono Pharmaceutical Co., Osaka, Japan), atropine sulfate, d-chlorpheniramine maleate, indomethacin, dl-propranolol hydrochloride and papaverine hydrochloride.

Results

Vascular Actions of Hemolysate

Intact erythrocytes which were washed with saline did not significantly alter the tension of basilar arteries, unless they were hemolyzed (fig. 1). Hemolysate contracted basilar arteries in a concentration-dependent fashion. Contractions of mesenteric arteries were induced only with high concentrations. The maximal contraction of basilar arteries was markedly greater than that of mesenteric arteries (fig. 2, left). The concentration-response relationship could not be completed because of a limitation in preparing the condensed hemolysate. Mean values of the apparent median effective concentration (ED₅₀) in basilar and mesenteric arteries were 0.172 ± 0.057 g/dl and 0.403 ± 0.037 g/dl, respectively.

When the mesenteric arteries had partially been contracted with norepinephrine (5 x 10⁻⁸ to 2 x 10⁻⁷ M) or PGF₂α (10⁻⁷ to 5 x 10⁻⁷ M), relaxant responses to the hemolysate were obtained (fig. 2, right). The ED₅₀ value was 0.0447 ± 0.0061 g/dl. Basilar arteries were not relaxed by hemolysate even if they had gained active tension by PGF₂α or 15 mM K⁺.

Contractile responses of the basilar arteries to the hemolysate were significantly attenuated by treatment with aspirin (5 x 10⁻³ M), an inhibitor of PG biosynthesis, and PPP (3 x 10⁻⁵ g/ml), a PG antagonist (fig. 3). The vasoconstrictor activity of the hemolysate was not antagonized by 10⁻⁶ M cinanserin (N = 6), a serotonin antagonist, nor by free radical scavengers such as superoxide dismutase (1.1 x 10³ U/dl, N = 5), catalase (1.9 x 10⁴ U/dl, N = 5) and dl-α-tocopherol (10⁻⁴ M, N = 6) (fig. 4).

In order to eliminate the participation of PG's derived from erythrocytes, the erythrocytes were washed by guest on November 10, 2017 http://stroke.ahajournals.org/ Downloaded from
Concentration of hemolysate (g/dl Hb)

**FIGURE 2.** (a) The contractile response of basilar and mesenteric arteries induced by hemolysate. The contraction induced by 30 mM K⁺ was taken as 100%: mean absolute values in basilar and mesenteric arteries in response to 1.0 g/dl hemoglobin-containing hemolysate were 2.64 ± 0.28 g and 0.224 ± 0.040 g, respectively. Mean ± SEM. Note that the hemolysate (10⁻⁴ to 1.0 g/dl hemoglobin) contracted basilar arteries in a concentration-dependent fashion, whereas contractions of mesenteric arteries in high concentration (3 × 10⁻¹ and 1.0 g/dl hemoglobin) were only slight. (b) The hemolysate-induced relaxation of mesenteric arteries which had been partially contracted with prostaglandin F₂α or norepinephrine. The maximal relaxation induced by 10⁻⁴ M papaverine was taken as 100%: mean absolute values in response to 10⁻¹ g/dl hemoglobin-containing hemolysate was 0.336 ± 0.036 g. Mean ± SEM.

In 11 out of 23 mesenteric arterial strips, the relaxation induced by hemolysate was converted to a contraction by treatment with 2 × 10⁻⁴ M aspirin (fig. 5), whereas in the remaining 12, aspirin slowed the development of the vasoconstrictor activity of the hemolysate prepared in such a way did not differ from the activity of control hemolysate (N = 5).

**FIGURE 3.** Effects of aspirin and polyphloretin phosphate (PPP) on the contractile response of basilar arteries induced by hemolysate. The maximal contraction induced by hemolysate in control media was taken as 100%. Mean ± SEM. Note that the contractile responses of basilar arteries were significantly attenuated by aspirin and markedly suppressed by PPP. Significantly different from controls: * p < 0.05, ** p < 0.01.

**FIGURE 4.** Effects of superoxide dismutase (SOD), catalase, and dl-α-tocopherol on the contractile response of basilar arteries induced by hemolysate. The maximal contraction induced by hemolysate in control media was taken as 100%. Mean ± SEM.

In 11 out of 23 mesenteric arterial strips, the relaxation induced by hemolysate was converted to a contraction by treatment with 2 × 10⁻⁴ M aspirin (fig. 5), whereas in the remaining 12, aspirin slowed the development of the vasoconstrictor activity of the hemolysate prepared in such a way did not differ from the activity of control hemolysate (N = 5).
RBC HEMOLYSATE AND CEREBROARTERIAL CONTRACTION/Okamoto et al

FIGURE 6. Relaxant responses of a mesenteric artery induced by hemolysate containing 0.1 g/dl hemoglobin (H) before (upper trace) and after (lower trace) treatment with 2 x 10^-4 M aspirin. The mesenteric artery was partially contracted by prostaglandin F2α prior to the hemolysate application. Horizontal bars indicate the level of resting tension. P = papaverine 10^-5 M. Note that the time required to induce half the maximum relaxation is markedly prolonged by aspirin treatment.

opment of relaxations but did not reduce the magnitude of relaxations (fig. 6). The time required to attain the half maximum relaxation was significantly prolonged (table 1).

Release of PG-like Substances from Cerebral Arteries

The release of PG-like substances from cerebral pial arteries was determined by superfusion experiments. A bolus injection of 40 μl hemolysate (10 g/dl hemoglobin-containing) to the arterial specimen induced a marked contraction of RSS (fig. 7, a), whereas the hemolysate injected to RSS did not significantly alter the tension (fig. 7, b). The contractile response of RSS was markedly attenuated by treatment of the arterial specimen with indomethacin (3 x 10^-7 M) (fig. 7, d). The response was reversed after 30 minutes exposure to control superfusate (fig. 7, f). The RSS was contracted with authentic PGE2 (5 and 20 p mol) in a concentration-dependent manner, and the contraction was not affected by indomethacin (fig. 7, c and e) (table 2).

Discussion

The present study confirmed an intense vasoconstrictor action of hemolysate on isolated basilar arteries which has been reported in vivo^6-9 or in vitro. The contractions of basilar arteries induced by hemolysate are markedly attenuated by treatment with aspirin and PPP. Aspirin blocks the synthesis of PG’s from arachidonic acid by an inhibition of cyclo-oxygenase. PPP antagonizes contractile actions of PG’s but not K+ on arterial smooth muscle. Incubation of hemolysate with aspirin did not reduce the contractile response to hemolysate. These findings clearly indicate that the hemolysate-induced contractions of basilar arteries are mainly associated with the synthesis of vasoconstrictor PG’s in the arterial wall. In the superfusion experiment, the RSS made insensitive to catecholamines, histamine, serotonin and acetylcholine by treatment with antagonists was contracted by hemolysate applied to pial arteries. The hemolysate injected directly to RSS did not alter the tension. The response was abolished by treatment of the arteries with indomethacin. The release of PG-like substance from dog pial arteries in response to hemolysate was thus determined.

When oxyhemoglobins undergo autoxidation and are converted to methemoglobins, superoxide anions (O2^-) emerge simultaneously, which trigger a chain of free radical reactions. The present study confirmed an intense vasoconstrictor action of hemolysate on isolated basilar arteries by guest on November 10, 2017 http://stroke.ahajournals.org/ Downloaded from
the products obtained from subsequent free radical re-
actions constrict cerebral arteries.20,21 However, eryth-
rocytes contain free radical scavengers such as super-
oxide dismutase, catalase and glutathione peroxidase in the sufficient amount, the activities of which are not decreased by hemolysis.20,21 In fact, superoxide dismutase, catalase and dl-α-tocopherol which scavenge superoxide anions, hydroperoxides and hydroxyl radicals, respectively, did not inhibit the arterial contraction induced by the hemolysate in the present study.

The present study revealed that the mesenteric arteries responded to hemolysate with a relaxation in lower concentrations (10^{-2} to 10^{-1} g/dl hemoglobin) and with a slight contraction in higher concentrations (3 × 10^{-1} to 1.0 g/dl hemoglobin). In 11 out of 23 strips, the relaxant responses were abolished by treatment with aspirin, while in the remaining 12, the development of relaxant responses were abolished by treatment with indomethacin (-), p < 0.001.

The mechanism of action of hemolysate in both basilar and mesenteric arteries is principally the same; that is, through a release of intrinsic PG's. The opposite responses of these arteries may be due to a different ratio of vasoconstrictor PG's to vasodilator PG's synthesized or to different sensitivity of the arteries to PG's released. According to Toda,22 the conversion of PGH_3 to I_2 and the sensitivity to PGI_2, are less in dog cerebral arteries than in mesenteric arteries.

It may be implied that in the subarachnoid hemorrhage the erythrocyte breakdown products released by the lysis of subarachnoid blood clot facilitate the synthesis and release of intrinsic vasoconstrictor PG's in the affected arteries, and that the latter may be involved in the development of cerebral vasospasm.

Acknowledgments
We thank Dr. S. Hayashi for his excellent technical assistance. This work was supported in part by Scientific Research Fund 56480102 from the Ministry of Education, Science and Culture of Japan.

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Role of intrinsic arachidonate metabolites in the vascular action of erythrocyte breakdown products.
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Stroke. 1984;15:60-64
doi: 10.1161/01.STR.15.1.60
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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