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Blood-Borne Vasoconstrictor Stimulates Release of [^{14}C]Arachidonate from Prelabeled Isolated Perfused Rabbit Gastrocnemius Muscle[1] (41493)

YUGI HAZEYAMA[2] AND RICHARD L. MORETTI[3]

Bruce Lyon Memorial Research Laboratory, Children's Hospital Medical Center, 51st and Grove Streets, Oakland, California 94609

Abstract. The effect of a blood-borne vasoconstrictor on vascular resistance and arachidonate release was examined using isolated, perfused rabbit gastrocnemius muscle. Replacement of blood by perfusion with Tyrode's solution caused a marked drop in vascular resistance. Addition of rabbit plasma or the plasma factor partially purified from human blood restored vascular resistance in a concentration dependent manner. The pressor effect of the plasma factor was unaffected by concentrations of phentolamine (2.5 $\mu g \cdot ml^{-1}$) and sar^1-ala^8-angiotensin II (75 $\mu g \cdot ml^{-1}$) sufficient to completely block the effects of norepinephrine and angiotensin II, respectively. However, the pressor effects of the factor were inhibited by indomethacin in a concentration-dependent manner. The factor produced a concentration dependent ($r = 0.95$, $n = 12$) increase in release of [^{14}C]arachidonate from prelabeled preparations. This release was blocked by indomethacin. These results are consistent with the hypothesis that the pressor effects of the factor result from its effects upon arachidonate metabolism.

Evidence indicates the existence of an unidentified humoral factor which is important in the maintenance of basal vascular resistance (1–4). Perfusion of the isolated dog gracilis muscle with a blood-free physiological salt solution causes vasodilation and a concomitant loss of the autoregulatory response to changes in perfusion pressure. Addition of blood plasma to the perfusate restores both the autoregulatory response and vascular resistance. Norepinephrine causes vasoconstriction but does not restore the autoregulatory response (1). Bohr and Sobieski obtained a substance from plasma which produces contraction in vascular smooth muscle strips isolated from a variety of sites (2). We have partially purified a substance from blood plasma which restores vascular resistance and the autoregulatory response in isolated rabbit hearts perfused with Tyrode's solution (3, 4).

The mechanism of action of this plasma pressor has not been completely delineated. Bohr and Johansson found the action of their plasma pressor to be unlike that of a variety of vasoactive agents (norepinephrine, angiotensin II, histamine, serotonin, and vasopressin) (5). We have found that the pressor effects of the plasma factor can be blocked with inhibitors of prostaglandin synthesis such as indomethacin (4). Additionally, the partially purified factor stimulates the conversion of arachidonate to prostaglandins and thromboxanes in isolated perfused rabbit hearts (3, 4), human platelet suspensions (6), and cell-free enzyme preparations (6–8). The factor also stimulates the conversion of arachidonate to 12-hydroperoxyeicosatetraenoic acid [12-HPETE] via the reaction catalyzed by lipoxidase (6). The factor alters the relative amounts of products formed from arachidonate, increasing formation of vasoconstrictors, such as prostaglandin $F_{2\alpha}$, with respect to vasodilators, such as prostaglandin I_2. This last effect could result from factor-stimulated synthesis of 12-HPETE

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[2] Present address: Center for Health Sciences, Chandler Laboratories, Bldg. 17, Lehigh University, Bethlehem, Pa. 18105.

[3] To whom reprint requests and correspondence should be addressed.

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which inhibits prostaglandin I_2 synthase (9). The increase in the ratio of vasoconstrictors to vasodilators might account for the pressor activity of the factor.

The factor may also increase the release of arachidonate from endogenous stores. In isolated rabbit hearts the factor increases prostaglandin E_2-like activity as assayed biologically with rat stomach fundus strips (3). Since exogenous arachidonate was not used in these experiments, prostaglandins must have been formed from endogenous arachidonate. The rate determining step in prostaglandin synthesis is the release of arachidonate from endogenous stores. Hence, the factor must have stimulated release of arachidonate.

To confirm this, we examined the effect of the factor on release of arachidonate in rabbit gastrocnemius muscle prelabeled with [^{14}C]arachidonate. Additionally, we compared the pressor effect of the factor with other vasoactive agents.

Methods. *Chemicals.* Stock solutions of all drugs and other vasoactive agents were prepared on the day of use. Norepinephrine (Levarterenol bitartrate, 0.2%, injection) was obtained from Winthrop; indigo carmine (0.8% injection) from Hynscott Wescott and Dunning; angiotensin II, histamine, serotonin, vasopressin, arachidonic acid and indomethacin from Sigma; phentolamine mesylate (injection) from Ciba; sar^1-ala^8-angiotensin II (saralasin) from Vega Biochemicals and rabbit serum albumin (Cohn fraction V) from ICN Pharmaceuticals. [^{14}C]Arachidonic acid (321 $Ci \cdot g^{-1}$) was purchased from New England Nuclear.

Indomethacin (100 mg) was dissolved in 2.0 ml 95% ethanol (37°) and diluted to 10 ml with modified Tyrode's solution (137 m*M* NaCl, 4 m*M* KCl, 1.8 m*M* $CaCl_2$, 0.5 m*M* $MgSO_4$, 0.35 m*M* Na_2HPO_4, 18 m*M* $NaHCO_3$, 5.6 m*M* glucose) containing 35 mg Na_2CO_3. Radiolabeled arachidonate was dissolved in the modified Tyrode's solution containing factor-free (4) albumin ($2.5\ mg \cdot ml^{-1}$).

Plasma factor. The plasma factor was prepared as described previously (4) except that it was obtained from human blood instead of rabbit blood. The factor is normally bound to albumin in blood plasma but can be extracted from albumin or plasma with chloroform methanol (2:1) and purified with thin-layer chromatographic techniques. The extraction procedure yields 60 to 70% of the original pressor activity in plasma. The partially purified factor was dissolved in methanol and stored at −20°. Before use, the methanol was evaporated with nitrogen and the residue dissolved in modified Tyrode's solution containing factor-free albumin ($2.5\ mg \cdot ml^{-1}$). For convenience, factor was reconstituted to the original volume from which it was extracted. Final factor concentrations are expressed as a percentage of the reconstituted stock solution.

Skeletal muscle and heart preparations. New Zealand male rabbits (2.2 to 3.2 kg) were anesthetized with sodium pentobarbital ($40\ mg \cdot kg^{-1}$). The anesthetic was supplemented as required. A tracheotomy was performed and the lungs were mechanically ventilated during surgery. The skin of the right leg was removed and the gastrocnemius muscle exposed. All blood vessels except the major artery and vein which supply and drain the muscle were ligated. Heparin ($1000\ unit \cdot kg^{-1}$) was administered before cannulation of the artery and vein. The excised muscle was placed in a humidified chamber and perfused with the modified Tyrode's solution (pH 7.4, 37°, saturated with 95% O_2, 5% CO_2).

Flow of the perfusate was maintained with the aid of a constant speed peristalic pump (Cole-Palmer). Pressure was measured with a pressure transducer (Statham P23Db) and recorded with a pen chart recorder (Cole-Palmer). Muscles were perfused for one hour to remove all residual blood before testing. In experiments using indomethacin, substances were tested in the following sequence: plasma, purified factor, indomethacin. Following the experiments the integrity of the vascular bed was examined by injecting indigo carmine solution (0.8%). Data from preparations in which partial blockage of the vascular bed was detected were discarded. Rabbit hearts were isolated and perfused as described

previously (3, 4). Briefly, the hearts were perfused through an aortic cannula with modified Tyrode's solution at 37°, pH 7.4, saturated with 95% O_2, 5% CO_2. Flow was adjusted to attain a basal pressure of 60 mm Hg. Aortic pressure was measured and recorded as in the experiments with the gastrocnemius muscle preparations.

In the experiments comparing the effect of the plasma factor to that of known vasoactive agents, the agonists were tested by bolus injection whereas the antagonists were infused at constant rate with the aid of a syringe pump (Harvard Instruments).

Radiolabeled prostaglandins and other products synthesized from [^{14}C]arachidonate were extracted from the venous effluent with ethyl acetate and identified by thin-layer chromatography as previously described (7). Identification was substantiated by comparing the migration rates of the free acids and their methylated derivatives to those of authentic standards using several solvent systems (7).

Results. *Pressor effects.* As perfusion with Tyrode's solution was started vascular resistance, which had dropped to a low value during cannulation of the artery, increased toward normal *in vivo* values. However, as the vascular system in the gastrocnemius muscle was purged of blood, vascular resistance decreased markedly, demonstrating that the net effect of switching from blood perfusion to perfusion with Tyrode's solution in this preparation is a decrease in vascular resistance (Fig. 1). When vascular resistance reached basal levels the perfusion pump speed was adjusted to produce a pressure of 30 mm Hg.

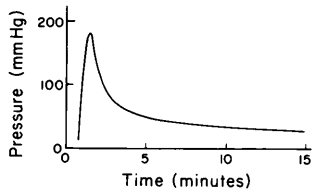

FIG. 1. Changes in perfusion pressure associated with the beginning of perfusion with Tyrode's solution in rabbit gastrocnemius muscle. Recorder tracing from one muscle preparation typical of all (15) preparations tested. Flow rate, 25 ml·min^{-1}·100 g^{-1}.

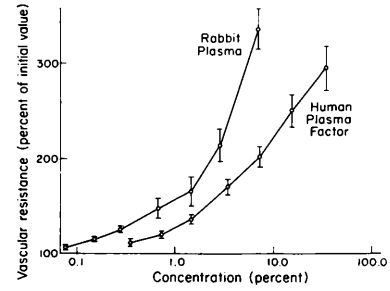

FIG. 2. Increases in vascular resistance produced by infusion of plasma and purified factor in isolated rabbit gastrocnemius muscle. Flow rate, 25 ± 4 ml·min^{-1}·100 g^{-1}, initial pressure = 30 mm Hg, resistance = pressure (mm Hg)·ml^{-1}·min·100 g, mean ± standard error. Data from six gastrocnemius muscle preparations.

The basal flow rate was 24.6 ± 3.9 ml·100 g^{-1}·min^{-1} (mean ± standard error, 15 gastrocnemius preparations). Infusion of rabbit plasma or partially purified human plasma factor caused a concentration dependent increase in vascular resistance (Fig. 2).

Comparison of factor with other vasoactive agents. Among the vasoactive agents tested (norepinephrine, angiotensin II histamine, serotonin, and vasopressin) only norepinephrine, angiotensin II, and histamine produced vasoconstriction in gastrocnemius muscle preparations (Fig. 3). Histamine produced vasoconstriction in high doses in gastrocnemius muscle but

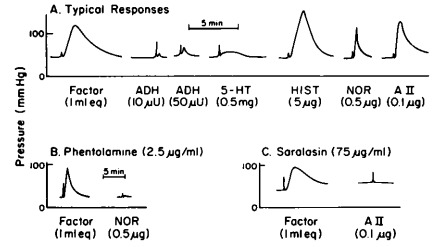

FIG. 3. Vascular effects of agonists and antagonists in isolated rabbit gastrocnemius muscle. Agonists were administered by bolus injection. Antagonists were infused at constant rates. Abbreviations: ADH, vasopressin; 5-HT, serotonin; HIST, histamine; NOR, norepinephrine; AII, angiotensin II. Recorder tracings from a single preparation are illustrative of the responses produced in all four muscle preparations tested. One milliliter equivalent (ml eq) is the amount extracted from one milliliter of blood plasma.

produced vasodilation in isolated rabbit hearts (Fig. 4). Serotonin also produced coronary vasodilation. The effects of norepinephrine and angiotensin II were completed blocked by phentolamine (2.5 $\mu g \cdot ml^{-1}$) and saralasin (75 $\mu g \cdot ml^{-1}$), respectively. Neither of these inhibitors had any effect upon factor-induced vasoconstriction (Fig. 3).

Effects on arachidonate metabolism. To study the release and metabolism of arachidonate the gastrocnemius muscle preparations were prelabeled with [^{14}C]arachidonate. Radiolabeled arachidonate (5 μCi in 1.0 ml) was injected into the perfusate in five aliquots at 2-min intervals. More than 90% of the label was retained by the preparation. Collection of samples was started 5 min after the last injection. Effluent (14 ml) was collected in a 4-min period. The radiolabeled substances identified in the venous effluent were arachidonate, prostaglandin E_2, and the stable derivative of prostaglandin I_2, 6-keto-prostaglandin $F_{1\alpha}$. Because of the low levels of prostaglandin E_2 and 6-keto-prostaglandin $F_{1\alpha}$ recovered from the venous effluents using this prelabeling technique, effects of the factor on venous levels of these products could not be determined. Although both these products were detected in the venous effluent of all four of the muscle preparations tested, factor-induced statistically significant effects were not obtained. However, a correlation ($r = 0.95$, $n = 12$) between factor-induced vasoconstriction and release of [^{14}C]arachidonate was seen (Fig. 5, Table I). The release of arachidonate was not due to vasoconstriction per se because there was no correlation ($r = 0.44$) between the vasoconstriction produced by

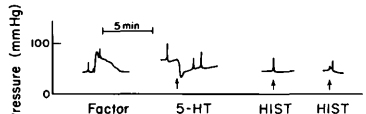

FIG. 4. Effects of factor, serotonin (5-HT), and histamine (HIST) on vascular resistance in isolated perfused rabbit hearts. Recorder tracings from one heart are typical of those seen in the five isolated hearts tested. Flow rate, 20 $ml \cdot min^{-1}$.

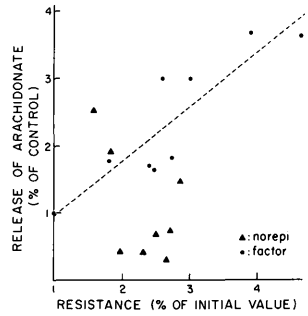

FIG. 5. Correlation between release of [^{14}C]arachidonate and factor-induced vasoconstriction. For factor: $y = 1.1X + 13$, $r = 0.95$ for norepinephrine: $y = 0.6X + 108$, $r = 0.44$. Data from four isolated gastrocnemius muscle preparations.

norepinephrine and the release of [^{14}C]-arachidonate (Fig. 5).

Indomethacin counteracts factor-induced vasoconstriction and prostaglandin synthesis in isolated rabbit hearts (3, 4). In gastrocnemius muscle preparations indomethacin had little effect on vascular resistance in the absence of factor, but did suppress factor-induced vasoconstriction in a concentration-dependent manner (Table II). Inhibition was proportional to the logarithm of indomethacin concentration ($y = 36.3x + 6.9$, $r = 0.97$, $n = 60$). The indomethacin concentration for 50% inhibition was 15 μM. A period of about 20 min was required for stabilization of vascular resistance after indomethacin infusion was started. After stabilization [^{14}C]arachidonate was administered by bolus injection 1 min before sample collection was started. Indomethacin reduced radiolabeled venous ^{14}C-prostaglandins both in the presence and absence of factor. It is of particular interest that indomethacin blocked the factor-induced increase in release of [^{14}C]arachidonate (Table I).

Discussion. Our results show that the factor which restores coronary vasoconstriction in isolated rabbit hearts (3, 4) also restores vasoconstriction in skeletal muscle. Our results confirm those of Bohr and Johansson (5) which show that the action of the plasma factor is unlike that of a variety of vasoactive agents.

The results reported herein substantiate

TABLE I. EFFECT OF VASOACTIVE FACTOR AND INDOMETHACIN ON RELEASE OF [^{14}C]ARACHIDONATE FROM LABELED RABBIT GASTROCNEMIUS MUSCLE

Factor concentration (%)	Indomethacin (mM)	Vasoconstriction (% of control)	Arachidonate (% of control)
0	0	100	100
15	0	212 ± 28	174 ± 9
35	0	285 ± 18	326 ± 23
35	0.2	132 ± 23	30 ± 5

Note. Repeated measurements were made on four muscle preparations, means ± standard error.

results we obtained using isolated rabbit hearts (3) which suggest that the factor stimulates release of arachidonate from endogenous stores. In this regard the relationship between indomethacin and factor is important. Indomethacin counteracts the effects of the factor on the cyclooxygenase reaction in a competitive manner (7). Indomethacin has been shown to block the release of arachidonate by phospholipases (10). Hence, indomethacin and the plasma factor have opposing effects on both the release of arachidonate and its conversion to endoperoxides. This finding is compatible with recent evidence for enzymatic coupling of phospholipase A_2 and cyclooxygenase activities (11).

The correlation between factor-induced arachidonate release and factor-induced vasoconstriction is consistent with the hypothesis that vasoaction of the factor results from its effects upon arachidonate metabolism. In addition to stimulating the release of arachidonate and its conversion to endoperoxide prostaglandins via the cyclooxygenase reaction (3, 4) the factor stimulates conversion of arachidonate to 12-HPETE by lipoxidase (6). This hydroperoxy fatty acid inhibits the formation of the vasodilator prostaglandin I_2 from endoperoxide prostaglandins (9). Lipoxidase activity has been found in vascular tissue (12). Hence the factor stimulates release of arachidonate but can simultaneously divert it from the formation of prostacyclin, a vasodilator, to vasoconstrictor prostaglandins (e.g., $PGF_{2\alpha}$).

Administration of exogenous arachidonate can produce vasodilation, presumably through formation of prostaglandin I_2 (13). However, we have shown that the plasma factor attenuates arachidonate-induced vasodilation while concurrently increasing the ratio of prostaglandin $F_{2\alpha}$ to prostaglandin I_2 which is formed (8). Arachidonate is transformed to prostaglandins, thromboxanes, hydroperoxy fatty acids, and leukotrienes (14). Both vasodilators and vasoconstrictors can be formed simultaneously, depending upon the activity of the enzymes present. The plasma factor has a marked effect on the activity of some of these enzymes. Thus, it could act by maintaining a balance between the vasocon-

TABLE II. INDOMETHACIN INHIBITION OF FACTOR-INDUCED VASOCONSTRICTION

Indomethacin (μM)	Factor concentration (%)			
	3.5	7.0	15	35
	Percentage inhibition			
0	0	0	0	0
6	32 ± 2	42 ± 2	28 ± 3	37 ± 4
50	84 ± 14	75 ± 12	68 ± 11	65 ± 24
200	94 ± 13	98 ± 14	89 ± 11	83 ± 6

Note. Percentage inhibition was calculated by dividing the factor-induced increase in vascular resistance in the presence of indomethacin by the increase in the absence of indomethacin, subtracting the quotient from 1.0, and multiplying that difference by 100. Data from four gastrocnemius preparations (mean ± standard error).

strictors and vasodilators formed from arachidonate.

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