PLASMA AND TISSUE KALLIKREINS CLEAVE KININOGENS RELEASING BRADYKININ AND RELATED PEPTIDES THAT ARE DEGRADED BY VARIOUS KININASES INCLUDING ANGIOTENSIN-CONVERTING ENZYME (KININASE II; 6, 7, 14). KININS SERVE VASORELAXANT FUNCTIONS AND CONTRIBUTE TO THE ANTIHYPERTENSIVE EFFECT OF CONVERTING ENZYME INHIBITORS (2, 4, 13). A RECENT STUDY REVEALED THAT THE CONCENTRATION OF FREE KININS IN THE HEART, AORTA, ADRENAL GLANDS, LUNG, AND BRAIN OF RATS EXCEEDS THAT IN CIRCULATING BLOOD (5), SUGGESTING THAT THE ACTIVITY OF THE KALLIKREIN-KININ SYSTEM OF TISSUES IS MORE PROMINENT THAN THAT OF THE CIRCULATING BLOOD. ALONG THIS LINE, REPORTS THAT KININS GENERATED WITHIN VASCULAR STRUCTURES STIMULATE NITRIC OXIDE SYNTHESIS (23, 29) AND MEDIATE THE VASORELAXING EFFECT OF CONVERTING ENZYME INHIBITORS (9) EMPHASIZE THE FUNCTIONAL RELEVANCE OF THE TISSUE KALLIKREIN-KININ SYSTEM.

FREE KININ PEPTIDES CAN BE RECOVERED FROM THE VENOUS EFFLUENT OF ISOLATED HEARTS AND RAT HINDQUARTERS PERFUSED WITH MEDIA LACKING IN KALLIKREIN AND KININOGEN (3, 28). THE RATE OF KININ RELEASE FROM ISOLATED ORGANS PERFUSED IN SUCH A MANNER MAY REFLECT THE NET ACTIVITY OF THE TISSUE KALLIKREIN-KININ SYSTEM. THIS STUDY WAS UNDERTAKEN TO GAIN KNOWLEDGE OF THE FACTORS INFLUENCING THE RATE OF RELEASE OF FREE KININS FROM ISOLATED RAT HINDQUARTERS PERFUSED WITH AN ARTIFICIAL MEDIA.

METHODS

Animals

EXPERIMENTS WERE CONDUCTED ON MALE SPRAGUE-DAWLEY RATS (280 ± 5 g) PURCHASED FROM CHARLES RIVER (WILMINGTON, MA). THE EXPERIMENTAL PROTOCOLS WERE APPROVED BY THE INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE. THE ANIMALS WERE FEED A REGULAR CHOW AND DRANK TAP WATER.

Hindquarter Perfusion

THE ABDOMINAL CAVITY OF RATS ANESTHETIZED WITH PENTOBARBITAL SODIUM (50 mg/kg ip) WAS EXPOSED THOUGH A MIDLINE INCISION, AND THE TESTES AND INTESTINES WERE LIGATED AND EXCISION. THE AORTA AND VENA CAVA DISTAL TO THE RENAL VESSELS WERE DISSECTED FROM THE SURROUNDING TISSUE, AND ALL VISIBLE BRANCHES WERE LIGATED. SUBSEQUENTLY, HEPARIN (1,000 U) WAS INJECTED INTRA-AORTICALLY, THE ABDOMINAL AORTA AND THE INFERIOR VENA CAVA WERE LIGATED JUST BELOW THE LEFT RENAL PEDICLE, AND POLYETHYLENE CANULAS WERE INTRODUCED INTO THE AORTA (PE-60) AND VENA CAVA (PE-100) AND ADVANCED TO A POSITION 2 CM ABOVE THE ILIAC BIFURCATION. THEREAFTER, THE ANIMAL WAS TRANSPLANTED CRANIAL TO THE SITES OF VASCULAR CANNULATION, AND THE ISOLATED HINDQUARTER WAS PERFUSED THROUGH THE AORTIC CANULA AT 10 ml/min WITH ARTIFICIAL MEDIA DELIVERED BY MEANS OF A PERISTALTIC PUMP (HARVARD INSTRUMENTS, SOUTH NATICK, MA). THE PERFUSION MEDIA CONSISTED OF KREBS BICARBONATE BUFFER CONTAINING 4.0% FICOLL 70, WITH OR WITHOUT ADDED TEST AGENTS. THE COMPOSITION OF THE KREBS BICARBONATE BUFFER WAS (IN MMOL/L) 118.5 NaCl, 4.7 KCl, 2.8 CaCl₂, 1.2 KH₂PO₄, 1.1 MgSO₄, 25.0 NaHCO₃, AND 11.1 DEXTROSE. THE PERFUSION MEDIA, MAINTAINED AT 37°C, WAS GASED WITH 95% O₂-5% CO₂ AND WAS NOT RECIRCULATED.

PERFUSION PRESSURE WAS MONITORED VIA A SIDE BRANCH OF THE AORTIC CANULA BY A TRANSDUCER (MODEL P231D, STATHAM DIVISION, GOULD, OXNARD, CA) CONNECTED TO A POLYGRAPH (GRASS, MODEL 7D, GRASS INSTRUMENTS, QUINCY, MA). PERFUSION PRESSURE AVERAGED 81 ± 2 mmHg. EXPERIMENTAL PROTOCOLS WERE STARTED 90 min AFTER THE ONSET OF PERFUSION, ONCE THE VENOUS EFFLUENT HAD BEEN SHOWN TO BE FREE OF CONTAMINATING BLOOD (HEMOSTIX, AMES, ELKHART, IN) FOR AT LEAST 45 min. IN THREE EXPERIMENTS USING HINDQUARTERS OF RATS INJECTED WITH [¹²⁵I]-LABELED HUMAN SERUM ALBUMIN (1.0 µCi/kg iv), 15–25 min BEFORE PREPARATION FOR PERFUSION, THE VENOUS EFFLUENT CONTAINED RADIOACTIVITY DURING THE FIRST 30 min OF PERFUSION ONLY. HENCE, 90 min AFTER THE ONSET OF PERFUSION, WHEN THE EXPERIMENTAL PROTOCOLS WERE STARTED, THE VENOUS EFFLUENT IS FREE OF CIRCULATING ALBUMIN AND PRESUMABLY OF OTHER BLOOD PROTEINS.

Measurement of Kinin Release

THE VENOUS EFFLUENT OF PERFUSED HINDQUARTERS WAS DIRECTED INTO THE BARREL OF A 30-ML SYRINGE CONNECTED TO A C₁₈-Sep-Pak CARTRIDGE (WATERS ASSOCIATES, MILFORD, MA) PREVIOUSLY PRIMED BY SEQUENTIAL WASHING WITH METHANOL (4 ml) AND WATER (12 ml). THE OTHER END OF THE CARTRIDGE WAS CONNECTED TO A FLASK LINKED TO A VACUUM SOURCE TO MAINTAIN FLOW THROUGH THE CARTRIDGE AND PREVENT BACK UP ACCUMULATION OF VENOUS EFFLUENT. IN THIS MANNER, KININS RELEASED FROM THE PERFUSED HINDQUARTERS WERE EXTRACTED FROM THE VENOUS EFFLUENT DURING PASSAGE THROUGH THE C₁₈-Sep-Pak CARTRIDGE, AN APPROACH
similar to that used to extract angiotensin peptides from the effluent of perfused organs (11). The cartridge was replaced at 15-min intervals. After replacement, the cartridge was washed sequentially with water (8 ml) and 0.1 M acetic acid (4 ml), followed by elution of kinin peptides with 6 ml of acetonitrile: 0.1 M acetic acid (80:20, vol/vol). The kinin-containing eluate was dried under nitrogen, and the resultant residue was dissolved in 1 ml of 0.01 M phosphate buffer (pH 7.0) containing 0.15 M NaCl, 0.03 M EDTA, 0.003 M 1,10-phenanthroline, and 1 mg/ml chicken egg albumin. The concentration of kinins in the reconstituted samples was measured by radioimmunoassay as previously described (1, 4), using bradykinin antibody supplied by Dr. Kazuaki Shimamoto (Sapporo Medical College, Sapporo, Japan) and 125I-[Tyr12]bradykinin (DuPont NEN, Boston, MA). Kinin release is expressed as picograms of bradykinin per 15-min perfusion period. Kinins were not detected in blank samples obtained by passing 150 ml of perfusion media not exposed to tissues through C18-Sep-Pak cartridges over a 15-min period. Samples obtained by passing through the cartridges 150 ml of perfusion media containing 2 pg/ml of authentic bradykinin yielded 258 ± 26 pg kinin (n = 4), indicating that 86 ± 9% of the bradykinin added to the perfusion media was extracted and subsequently eluted from the cartridge.

The identity of kinin peptides in the venous effluent of perfused hindquarters was examined using high-performance liquid chromatography of 18 samples extracted as described above and subsequently pooled. The sample or standards (bradykinin triacetate, [Lys]bradykinin and [MetLys]bradykinin), dissolved in 0.1 M acetic acid (50 µl), was applied to a Bond-Pak C18 column (30 × 39.9 cm; Waters Associates), and the column was eluted using a linear gradient of 18–25% acetonitrile in 0.1% trifluoroacetic acid over a 24-min period at a flow rate of 1.5 ml/min. Twenty-second fractions of eluate were collected, dried under nitrogen, and reconstituted in buffer before radioimmunoassay (1, 4).

Experimental Protocols

Protocol 1. The time course of kinin release from isolated rat hindquarters was examined in preparations perfused with media containing (n = 4) and not containing (n = 4) inhibitors of kininases to protect kinins from degradation. The agents used were captopril (10 µM), phosphoramidon (1 µM), and 2-mercaptomethyl-3-guanidinoethylthio propanoic acid (MGTA, 10 µM), which inhibit angiotensin-converting enzyme (kininase II; 4, 7), neutral endopeptidase 24.11 (26), and kininase I (19), respectively.

Protocol 2. The effect of kallikrein inhibitors aprotinin (25) and soybean trypsin inhibitor (17) on kinin release was studied in rat hindquarters perfused with media containing kininase inhibitors as described in protocol 1. Kinin release was measured for three consecutive 15-min periods before and after addition of aprotinin (500 KIU/ml; n = 6) or soybean trypsin inhibitor (100 µg/ml; n = 4) to the perfusion media.

Protocol 3. The effect of kinogen on kinin release was investigated in rat hindquarters (n = 4) perfused with media containing kininase inhibitors as in protocol 1. Kinin release was measured for two consecutive 15-min periods before, during, and after perfusion with media containing bovine kininogen (Seikagaku Kogyo, Tokyo; sp act 19.8 µg of bradykinin/mg protein; 3.3 µg/ml). The effect of kinogen on kinin release also was examined in preparations perfused from the outset with media containing kininase inhibitors and either aprotinin (500 KIU/ml; n = 4) or soybean trypsin inhibitor (100 µg/ml; n = 4).

Protocol 4. The effect of the protein synthesis inhibitor cycloheximide (12) on kinin release was examined in rat hindquarters perfused with media containing kininase inhibitors as in protocol 1. Kinin release was measured for six consecutive 15-min periods in preparations perfused from the outset with media containing (n = 6) and not containing (n = 6) cycloheximide (10 µg/ml). In separate experiments, after perfusion of the preparations with cycloheximide containing (10 µg/ml) and not containing media for 150 min, kinin release was measured for 15 min before and after addition of bovine kininogen (3.3 µg/ml; n = 4) or pancreatic kallikrein (5 U/ml; n = 4) to the perfusion media.

Statistics

All results are expressed as means ± SE. The data were analyzed as appropriate by one-way or two-way analysis of variance followed by the Newman-Keuls test or by Student’s t-test; P < 0.05 was considered significant.

RESULTS

Figure 1 depicts the time course of kinin release from isolated rat hindquarters perfused with media containing and not containing inhibitors of kinin-degrading enzymes. The rate of kinin release was stable over the 90-min observation period, ranging from 14.6 ± 2.5 to 20.7 ± 6.7 pg/15 min in preparations perfused with control media lacking kininase inhibitors and from 243 ± 53 to 276 ± 78 pg/15 min in preparations perfused with media containing kininase inhibitors. The rate of kinin release from hindquarters perfused with kininase inhibitors containing media exceeded (P < 0.05) that obtained in preparations perfused with control media. High-performance liquid chromatography of material extracted from a pool of eighteen 15-min samples obtained in three preparations revealed that the elution profile of kinin immunoreactive material was virtually identical to the elution profile of authentic bradykinin (Fig. 2).

![Fig. 1. Time course of kinin release into the venous effluent of rat hindquarters perfused from the outset with Krebs bicarbonate buffer not containing (A) and containing (B) kininase inhibitors. Results are mean ± SE; n = 4 experiments. *P < 0.05 relative to corresponding results in preparations perfused with media not containing kininase inhibitors.](http://ajpregu.physiology.org/)
Figures 3–6 illustrate the results of experiments examining the effect of kallikrein inhibitors, kininogen, cycloheximide, and pancreatic kallikrein on kinin release from rat hindquarters perfused with media containing inhibitors of kinin degradation. Kallikrein inhibitors aprotinin (500 KIU/ml) and soybean trypsin inhibitor (100 µg/ml) reduced (P < 0.05) the rate of kinin release to 42 and 71% of pretreatment values, respectively (Fig. 3). Kinin release increased (P < 0.05) during perfusion of the hindquarters with media containing kininogen (3.3 µg/ml) (Fig. 4). The kininogen-induced elevation of kinin release (1,792 ± 591 pg/15 min) was attenuated (P < 0.05) in preparations perfused with media containing aprotinin (334 ± 32 pg/15 min) or soybean trypsin inhibitor (631 ± 78 pg/15 min) (Fig. 4). The rate of kinin release in hindquarters perfused from the outset with the protein synthesis inhibitor cycloheximide (10 µg/ml) was decreased (P < 0.05) relative to the rate of kinin release in control preparations (Fig. 5). The kininogen-induced kinin release in preparations perfused with media containing cycloheximide (560 ± 136 pg/15 min) was diminished (P < 0.05) relative to that in preparations perfused with control media without cycloheximide (2,006 ± 533 pg/15 min) (Fig. 6). In contrast, the release of kinins induced by addition of pancreatic kallikrein to the perfusion media was comparable in hindquarters perfused with media containing and not containing cycloheximide (Fig. 6).

DISCUSSION

This study demonstrates occurrence of bradykinin in the venous effluent of isolated rat hindquarters perfused with media lacking in kininogen and kinin-forming enzyme(s). The study also shows that the rate of kinin release is greatly increased in preparations containing kininogen (3.3 µg/ml) (Fig. 4). The kininogen-induced elevation of kinin release (1,792 ± 591 pg/15 min) was attenuated (P < 0.05) in preparations perfused with media containing aprotinin (334 ± 32 pg/15 min) or soybean trypsin inhibitor (631 ± 78 pg/15 min) (Fig. 4). The rate of kinin release in hindquarters perfused from the outset with the protein synthesis inhibitor cycloheximide (10 µg/ml) was decreased (P < 0.05) relative to the rate of kinin release in control preparations (Fig. 5). The kininogen-induced kinin release in preparations perfused with media containing cycloheximide (560 ± 136 pg/15 min) was diminished (P < 0.05) relative to that in preparations perfused with control media without cycloheximide (2,006 ± 533 pg/15 min) (Fig. 6). In contrast, the release of kinins induced by addition of pancreatic kallikrein to the perfusion media was comparable in hindquarters perfused with media containing and not containing cycloheximide (Fig. 6).
perfused with media containing a combination of captopril, phosphoramidon, and MGTA to inhibit angiotensin-converting enzyme, neutral endopeptidase 24.11, and kininase I, respectively (7, 19, 26). Previous studies ascribed to these enzymes a major role in kinin degradation (7, 26). Hence, our findings suggest that the rate of kinin release from perfused rat hindquarters is significantly influenced by the activity of the kinin-degrading pathways.

According to our study, the rate of kinin release from isolated rat hindquarters falls during perfusion with media containing soybean trypsin inhibitor or aprotinin. Soybean trypsin inhibitor inhibits plasma kallikrein (17), whereas aprotinin inhibits both plasma and tissue kallikrein (17, 25). Hence, it may be concluded that the activity of the kinin-generating pathways contributes importantly to set the rate of kinin release from perfused rat hindquarters. Along this line, the increased kinin release prompted by perfusion with media containing exogenous kininogen or pancreatic kallikrein may be ascribed to an enhancement of kinin generation brought about by the availability of kallikrein-kinin system components in the perfusion media. That kinin release from rat hindquarters perfused with media lacking in kinin-forming enzymes is increased by addition of exogenous kininogen to the perfusate implies that the protein precursor of kinins has appropriate access to an endogenous kinin-forming enzyme. Likewise, the observation that kinin release from rat hindquarters perfused with media lacking in kininogen is increased by addition of pancreatic kallikrein to the perfusate suggests that the kinin-forming enzyme has appropriate access to an endogenous kininogen.

Kinin release into the venous effluent of rat hindquarters perfused with media lacking in kininogen and kinin-forming enzymes may depend on kinin generation by components of the plasma kallikrein-kinin system present in the perfused tissues, by components of the tissue kallikrein-kinin system, or by components of both systems. That the samples of venous effluent used for measurement of kinins were free of contaminating blood minimizes, but does not exclude, the possibility that the kinins released into the venous effluent depend for their formation on one or more components of the plasma kallikrein-kinin system. In this regard, it is well documented that endothelial cells express high-affinity binding sites for high- and low-molecular-weight kininogens (10, 15, 22, 27) and that kininogen bound to endothelial cells can be cleaved by kallikrein to liberate kinins (15). It is also possible that, in perfused hindquarters, one or more components of the plasma kallikrein-kinin system exits the intravascular space and contributes to kinin generation at perivascular sites. The observation that basal kinin release is diminished in preparations perfused with soybean trypsin inhibitor, an inhibitor of trypsin-like enzymes, including plasma kallikrein (17), may be taken as an indication that plasma kallikrein contributes to the generation of kinins released into the venous effluent of perfused rat hindquarters. However, this observation also may signify that soybean trypsin inhibitor interferes with the activation of tissue prekallikrein by trypsin-like enzymes, with attendant reduction in tissue kallikrein-catalyzed generation of kinins.

The present study demonstrates that basal kinin release is decreased in rat hindquarters perfused with media containing cycloheximide, an inhibitor of protein synthesis. Another inhibitor of protein synthesis, puromycin, also was reported to reduce basal kinin release...
in perfused rat hindquarters (28). These observations raise the possibility that kinin generation and basal release in rat hindquarters perfused with media lacking in kininogen and kinin forming enzymes is sustained by kallikrein-kinin system components, kininogens and/or kallikrein, manufactured de novo in the perfused preparation. In this regard, previous studies documented the presence of kininogen in aortic smooth muscle cells cultured in serum-free media (18) and of kininogen mRNA in cultured endothelial cells (22). However, a report that the kininogen present in endothelial cells is manufactured within the cells (22) conflicts with a report that it is not (27). Our finding that pancreatic kallikrein-induced kinin release is not diminished in rat hindquarters perfused with cycloheximide-containing media argues against the possibility that tissue kininogen(s) manufactured de novo contribute significantly to the generation of kinins released into the venous effluent. Rather, this observation lends credence to the view that pancreatic kallikrein-induced kinin generation and release in this preparation is sustained by preexisting kininogen(s), possibly by plasma kininogen bound to endothelial cells (8, 27).

Previous studies documented the occurrence of a kinin-forming enzyme resembling tissue kallikrein and mRNA coding for tissue kallikrein in blood vessels (17, 20). Tissue kallikrein also was identified in cultured rat aortic smooth muscle cells (18), cultured endothelial cells from human umbilical veins and pulmonary artery (8), and in the venous effluent of isolated perfused rat hindquarters (16). The venous effluent of perfused rat hindquarters contains both active tissue kallikrein and an inactive form of the enzyme (prekallikrein), which can be activated by trypsin (16). Treatment with puromycin decreases the release of tissue kallikrein and prekallikrein, suggesting that the release is sustained by de novo synthesis of the enzyme and its zymogen (16). Therefore, it is conceivable that a tissue kallikrein manufactured de novo in vascular and perhaps nonvascular elements of perfused rat hindquarters (16, 24) contributes to the generation of kinins released into the venous effluent. Such a notion is supported by our observations that cycloheximide attenuates both basal and kininogen-induced kinin release in perfused rat hindquarters, but is without effect on the release of kinins induced by exogenous pancreatic kallikrein. However, a role for tissue kallikrein in the generation of kinins released basally and in response to kininogen is questionable because of the finding that kinin release from perfused rat hindquarters is significantly attenuated by soybean trypsin inhibitor, an agent that at the concentration used in our study was reported not to inhibit vascular tissue kallikrein directly (17). Reconciliation between such a role and the aforementioned finding rests on the possibility that soybean trypsin inhibitor interferes with the activation of tissue prekallikrein by a trypsinlike enzyme(s), with attendant reduction in tissue kallikrein-catalyzed generation of kinins in the perfused hindquarter.

In summary, this study demonstrates occurrence of bradykinin in the venous effluent of isolated rat hindquarters perfused with media lacking in kininogen and kallikrein. The rate of kinin release from perfused hindquarters is influenced significantly by the activity of both the kinin degrading- and the kinin-generating pathways. That perfusion with cycloheximide-containing media attenuates the release of kinins elicited by exogenous kininogen suggests that a kallikrein manufactured de novo contributes to kinin generation and release in the isolated rat hindquarter. That perfusion with cycloheximide-containing media is without inhibitory effect on the release of kinins induced by pancreatic kallikrein suggests that kinin generation and release in this preparation is sustained by preexistent kininogen(s), possibly by plasma kininogen bound to endothelial cells.

Perspectives

The tissue kallikrein-kinin system has long been implicated in blood pressure homeostasis (6, 14). Published reports suggest that a kallikrein-kinin system intrinsic to vascular tissue promotes synthesis of nitric oxide (23, 29) and eicosanoids (21) and mediates vasodilation (9). Our study offers insights into the factors influencing tissue kinin generation and release. That pharmacological inhibition of kinin-degrading enzymes results in elevation of tissue kinin release agrees with suggestions that tissue kinins contribute to the antihypertensive effect of converting enzyme inhibitors (4, 13).

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