Release of endothelial-derived kallikrein, kininogen and kinins

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The endothelial cell should be regarded as a highly active metabolic and endocrine organ interacting with the blood streak and the interstitium. Kinins are vasodepressor hormones that may participate in local blood flow regulation as part of an autocrine-paracrine system. We have previously reported that tissue kallikrein, its mRNA and kininogen are present in vascular tissue The present study was undertaken to examine the release of the components of this system from isolated perfused rat vessels. These vessels were perfused at 4 ml/min with physiological saline solution containing 3% Ficoll and 0.1% BSA. Kallikrein was released into the perfusate at a rate of 75 ± 5 ng Bk/100 g bw/30 min (n = 10). Kininogen was released at a rate of 55 ± 5 pg Bk/100 g bw/30 min. Pre-treatment with puromycin, a protein synthesis inhibitor, significantly reduces kallikrein and kininogen release. Vascular derived kinins were released at a constant rate of 38 ± 6 pg Bk/100 g bw/30 min for at least 120 min. This basal kinin release was increased 3-fold when perfused with the angiotensin converting enzyme inhibitor ramiprilat ($p < 0.05$). When purified kininogen was added to the physiological saline solution, immunoreactive kinins in the perfusate increased from 42 ± 7 to 3140 ± 210 pg Bk/100 g bw/30 min ($n = 6$; $p < 0.002$). Increase in flow rate (from 2 ml/min up to 4 ml/min and 8 ml/min) causes a parallel increase in the release of kinins (from 32 ± 4 up to 48 ± 6 and 62 ± 8 pg Bk/100 g bw/30 min, respectively; $p < 0.01$); the increase may be due to the effect of shear stress upon the endothelial cells. The present data confirm that vascular tissue synthesizes and releases continuously kallikrein, kininogen and kinins. Vascular kinins induce potent vasodilatation through the release of prostacyclin, nitric oxide and endothelium-derived hyperpolarization factor, and some of the converting enzyme inhibitors effect may be explained by potentiation of vascular-derived kinins.

Key terms: *angiotensin converting enzyme inhibitor, bradykinin, endothelium-derived, kallikrein, kinins, kininogen, perfused vessels, ramiprilat, rat*

INTRODUCTION

The vascular endothelium with its strategic location is not only a selective barrier between flowing blood and the underlying vessel wall, but also is able to sense humoral and hemodynamics changes and respond by secreting a variety of metabolic

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active substances. In order to understand the mechanisms involved in the endothelial-dependent vasomotor control, attention must be focussed not only in the level of blood pressure, but also in the blood flow and the effect of associated shear stress on endothelial cells. It seems now clear that in addition to the pressure component, this frictional mechanical stress makes an important hemodynamic regulation as blood goes through the vascular system. The endothelial cell response to these mechanical and chemical signals is probably mediated by the release of kinins as seen by Nolly *et al* (1993). Kinins acting as local hormones have been implicated to be among the factors that regulate blood pressure. Kinins are released from kininogen by the proteolytic action of kallikrein, and their potent vasodilator activity is mainly due to the release of prostacyclin, nitric oxide and endothelium-derived hyperpolarization factor (EDHF). However, kinins circulate at very low concentrations and are rapidly hydrolyzed by kininases (Margolius, 1996). Thus, it has been postulated by Nolly *et al* (1994) and others that if kinins participate in the regulation of blood pressure, they must be released as paracrine hormones at the arterial wall. Recently, a number of reports as one of Gavras (1992) have indicated that kinins mediate part of the effects of angiotensin-converting enzyme inhibitors (ACEI). However, the pathway by which endogenous kinins are generated is not still completely understood.

We have previously shown that kallikrein (KK) is present in and released from blood vessels (Nolly & Lama, 1982; Nolly *et al*, 1993). In addition, mRNA for tissue KK has been found in vascular and cardiac tissues, as previously described by Saed *et al* (1990) and Nolly *et al* (1994), suggesting local synthesis.

Previous data show that tissue KK is released from vascular and cardiac tissue. To further extend this observation, we examined: *i-* whether the vascular wall of isolated perfused vessels releases kallikrein, kininogen and kinins; *ii-* whether the release is inhibited by pretreatment with puromycin, a protein synthesis inhibitor, and enhanced after administration of ACEI; and *iii-* whether increases in shear stress augment the release of kinins.

MATERIALS AND METHODS

All procedures involving animals were in accordance with institutional guidelines for animal experimentation. Male Wistar rats weighing 250-300 g were employed. The animals were housed in a room under conditions of constant temperature (20°C) and a 12 h light/dark cycle.

Isolated perfused rat hind-quarters.

Rats were anesthetized with sodium pentobarbital (Abbott) and given 1,000 U heparin iv. After 3 min, each animal was decapitated to facilitate blood drainage. An abdominal incision was made and the aorta and vena cava carefully dissected from the renal vessels to the bifurcation. The rectum was cut between double ligatures and the distal sigmoid displaced to the upper abdomen. All major and minor tributaries of the descending aorta and abdominal vena cava were ligated except for the femoral and tail arteries. A 20 gauge catheter was placed in the aorta just below the renal arteries. The vena cava was also catheterized and warmed (37°C). Krebs-Henseleit solution, containing 3.5% Ficoll 70 and gassed with 95% O_2 + 5% CO_2, was perfused through the aorta. The buffer was passed through a 0.45 μm filter prior to use. In the perfusion system, the medium was oxygenated by passing it through the blood compartment of a C-DAK hollow-fiber artificial kidney and gassed with the O_2/CO_2 mixture. The isolated hind-quarters were perfused in a single-pass (non-recirculating) system using a peristaltic pump. Perfusion pressure was recorded constantly. Details of the procedure were similar to those reported previously (Nolly *et al*, 1997). During the initial perfusion period, the hind-quarters were thoroughly rinsed with buffer for 30 min until the effluent was consistently clear. The hind-quarter perfusates were collected during 30 min, concentrated 5 times and then dialyzed.

A group of rats (n = 10) was treated the day before surgery with puromycin (10 mg ip), a protein synthesis inhibitor.

Kallikrein release and kininogenase activity.

Kininogenase activity was measured by incubating 1 ml of the hind-quarter perfusate (previously concentrated 5 times and then dialyzed) for 5 h at 37°C with 200 µl of partially purified dog kininogen (2 µg kinin-releasing capability) in the presence of 1 ml fresh 0.1 mol/L Tris-HCl buffer (pH 8.5) containing EDTA (15 mg/ml), 1-10-phenantroline (1 mg/ml), 8-OH-quinoline (1 mg/ml) and Soybean trypsin inhibitor (SBTI) (100 µg/ml). Vascular kininogenase was inhibited by aprotinin and phenyl-methyl-sulfonyl fluoride (PMSF) but was resistant to SBTI, which is characteristic of glandular KK. SBTI was included in the incubation buffer to inhibit plasma KK and trypsin-like enzymes that could have contaminated the perfusates.

Kinins generated during incubation were measured by radioimmunoassay (RIA); the lower limit of sensitivity of the kinin RIA was 1 pg/sample. Activation of the kininogenase present in the sample as zymogen was allowed by incubating 500 µl of the perfusates with 20 µg trypsin for 30 min at 37°C. The reaction was stopped by adding 100 µg SBTI, after which the perfusates were incubated with kininogen as described above. Vascular kininogenase activity was expressed as the amount of kinins generated per ml per min of incubation with kininogen. Under these conditions, the kininogen itself released small amounts of kinins (kininogen blank). In every assay, duplicate tubes containing the reagents, but not homogenates, were run to generate the kininogen blank. Vascular kininogenase activity was calculated as kininogenase activity in the sample minus kininogen blank.

Kininogen release.

Kininogen released into the hind-quarters perfusate was measured by incubating 1 ml of the perfusate (previously concentrated 5 times and then dialyzed) for 5 h with an excess of purified urinary kallikrein in the presence of 1 ml fresh 0.1 mol/L Tris-HCl buffer (pH 8.5) containing EDTA (15 mg/ml), 1-10-phenantroline (1 mg/ml), 8-OH-quinoline (1 mg/ml) and SBTI (100 µg/ml). Kinins generated during the incubation were measured by RIA as mentioned above, and the kininogen content in the perfusates expressed as their kinin release capability (pg Bk/100 g bw/30 min).

Kinin release.

Kinins released from the isolated perfused rat hind-quarters were concentrated with on line Sep-Pak C18 cartridges (Waters Assoc, Milford, MA, USA) connected to the perfusion system. The cartridges were washed with 5 ml of 0.1% trifluoroacetic acid in distilled water and kinins were eluted with 2 ml of 60% acetonitrile and 0.1% trifluoroacetic acid in distilled water. The extracts were evaporated to dryness with nitrogen in a water bath at 70°C. The samples were stored at -20°C and immunoreactive kinins were measured by RIA, then the ACEI ramiprilat was added to the physiological saline solution (PSS) and immunoreactive kinins were dosed by RIA. The identity of immunoreactive kinins in the perfusate was examined by high performance liquid chromatography (HPLC) combined with RIA.

Drugs.

The compounds used were: trypsin TPCK (Worthington Biochem Co), 8-hydroxy-quinoline (Sigma); 1,10-phenantroline (Sigma), puromycin (Sigma), ramiprilat (HMR).

Partially purified dog kininogen was obtained as previously described (Nolly *et al*, 1985).

Statistics.

Values are expressed as means ± SEMs. Differences were tested for significance by one-way and two-way analyses of variance and multiple comparison Bonferroni's *t* tests. Significance was accepted at the 0.05 level of probability.

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RESULTS

Figure 1 shows total kallikrein released into the perfusate from the isolated perfused rat hind-quarters during a 30 min period after 30, 60, 90 and 120 min of perfusion. Pretreatment with puromycin significantly decreased the release of the enzyme.

Figure 2 shows the release of kininogen during the same period of time and the inhibitory effect of puromycin on the released amounts ($p < 0.001$).

Figure 3 shows immunoreactive kinins (IR-kinins) released into the perfusate of the isolated perfused rat vessels during a 30 min period after 30, 60, 90 and 120 min. In another group of animals (n = 6; data not shown), when purified kininogen was added to the PSS, immunoreactive kinins in the perfusate increased from 42 ± 7 to 3140 ± 210 pg Bk/100 g bw/30 min ($p < 0.002$).

Figure 4 shows that when the angiotensin converting enzyme inhibitor ramiprilat was added to the perfusing buffer, immunoreactive kinins in the perfusate increased almost three-fold ($p < 0.05$).

DISCUSSION

The present data and those published previously suggest that vascular tissue

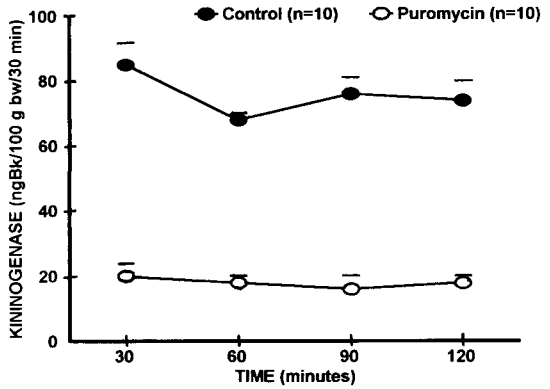

Fig 1. Release of kallikrein into the perfusate of isolated perfused hind-quarters from control rats (closed circles) and rats pre-treated with puromycin (open circles). Data expressed as means + SEMs.

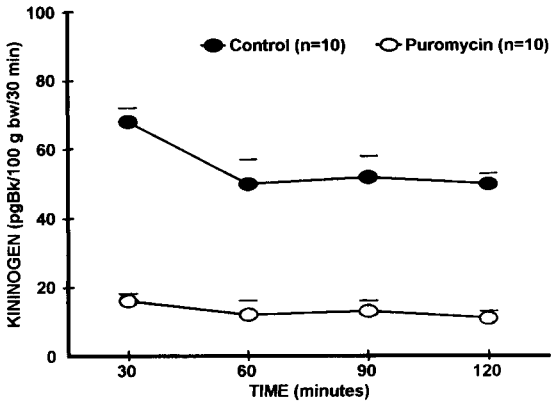

Fig 2. Release of kininogen into the perfusate of isolated perfused hind-quarters from control rats (closed circles) and rats pre-treated with puromycin (open circles). Data expressed as means + SEMs.

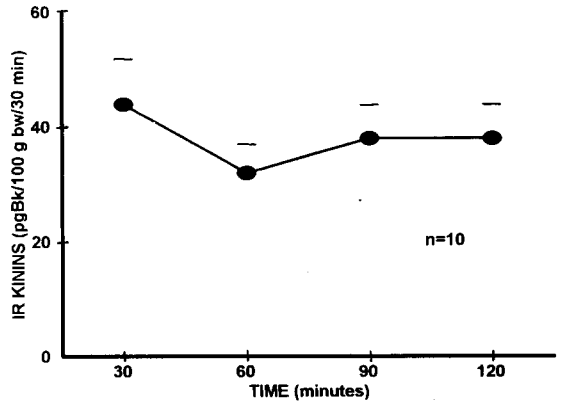

Fig 3. Release of immunoreactive kinins (IR Kinins) into the perfusate of isolated perfused rat hind-quarters. Values expressed in pg Bk/100 g bw/30 min. Data expressed as means + SEMs.

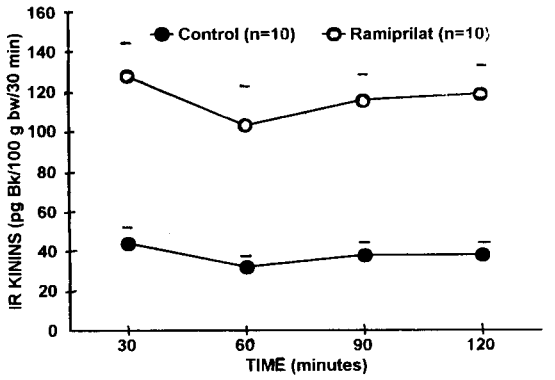

Fig 4. Release of immunoreactive kinins (IR kinins) into the perfusate of isolated perfused rat hind-quarters when ACEI ramiprilat was added to PSS. Values expressed in pg Bk/100 g bw/30 min. Data expressed as means + SEMs.

contains kallikrein capable of generating kinins from tissue or plasma kininogen.

Here, we found that KK appeared in the perfusate from isolated rat hind-quarters and remained more or less constant at least during 120 min. In addition, KK released into the perfusate was reduced almost 85% by pretreatment with the protein synthesis inhibitor puromycin suggesting that KK released originates from a pool that requires *de novo* synthesis. The present data show that vascular tissue synthesizes and releases KK into the perfusate and that *de novo* synthesis replenishes the pools of releasable KK.

Tissue KK is known to be present in plasma, albeit at low concentrations, as described by Vio *et al* (1982). It seems unlikely that vascular kininogenase activity could be due to trapped plasma, as extensive washing did not remove the kininogenase activity from the vessel wall as seen in the following data: kininogenase activity was 952 ± 31 pg Bk mg protein/min before perfusion, and 985 ± 28 pg/Bk/min after 2 h of perfusion (n = 7).

The hypothesis that KK released by vascular tissue is synthesized *de novo* and not adsorbed from plasma was strengthened because inhibition of protein synthesis suppresses release. We have found mRNA for tissue KK in rat vascular and cardiac tissues, suggesting that the kininogenase activity was due to locally synthesized KK. In addition, we used SBTI to inhibit the possible kinin-generating activity of plasma KK or trypsin-like enzymes. Up to now, attempts to locate KK in the vascular wall by immunohistochemistry have not been successful. This may be due to the fact that the concentration of KK is below the sensitivity of this technique, or because the epitopes recognized by the antibodies we used are not exposed. Kininogenase activity in vascular tissue from normotensive rats has been demonstrated in both the endothelium and the vascular smooth muscle layer (Nolly *et al*, 1994). Thus, vascular tissue may contain two separate kallikrein-kinin systems, one present in the endothelium and another of vascular smooth muscle origin. In fact, the KK found in the perfusate may be of endothelial origin, since it is difficult to see how smooth muscle KK would reach the lumen.

Kininogen, the kallikrein substrate, is also released in constant amounts to the perfusate in our experimental conditions. There was a bimodal pattern of release with an early phase of 30 min consisting in larger but quickly declining amounts of kininogen, followed by a second phase of a lower constant release (90 min). The first one may represent a washout of plasma derived kininogen plus the amount locally synthesized, and the second phase may indicate the locally synthesized KK-substrate. Here also, the kininogen releasable was significantly reduced by puromycin ($p < 0.001$).

Kinins are also released into the perfusate of the isolated perfused rat hind-quarters at more or less constant levels over 120 min. It has been postulated that kinins released in the vascular wall may act as local hormones through the regulation of vascular resistance and opposing hypertensive stimuli. Bradykinin (Bk) is a potent endothelium-dependent vasodilator. Endothelial cells produce and release Bk locally in response to flow. Bradykinin may then bind to Bk-B receptors on the endothelial cells and activate the L-Arginine-NO pathway, as reported among others by Regoli and Barave (1980) and by Bhoola *et al* (1992). Bradykinin has direct vasoconstrictor effects upon the vascular smooth muscle (Fasciolo *et al*, 1990) and indirect vasodilator effects by promoting the release of NO, prostaglandin I (PGI) and EDHF from the endothelium.

Endogenous bradykinin has been shown to have a role in mediating normal vasomotor responses under basal and flow-stimulated conditions in human resistance and epicardial coronary vessels.

We have seen that a significant increase in the amount of immunoreactive kinins was found in the perfusate when rat hind-quarter vessels were infused with PSS plus kininogen. This indicates that kininogen was converted into kinins by local kinin-forming enzymes. The shear stress of blood on the arterial wall has been postulated as one of the main factors in the release of endothelium-derived relaxing factors

(EDRFs). In preliminary studies, we observed a substantially increased kinin release in response to an increased flow rate in the perfused vessels. At an initial flow of 2 ml/min, a release of 32 ± 4 pg Bk/100 g bw/30 min was observed. By increasing the flow rate to 4 ml/min and 8 ml/min, the kinin release augmented up to 48 ± 6 and 62 ± 8 pg Bk/100 g bw/30 min, respectively ($p < 0.07$, and $p < 0.01$; $n = 8$). Rubanyi *et al* (1986), using a bioassay ring preparation, have also found that an increase in flow rate, specially if pulsatile, causes a parallel increase in the release of EDRF, resulting in flow-induced vasodilation. In both cases, the increased release of kinins and EDRF are best explained by the effect of shear stress on the endothelial cells.

The role of kinins in vascular tissue is determined by the rate of production of the peptides by kininogenases and their degradation by kininases, in particular angiotensin converting enzyme. Drugs such as angiotensin converting enzyme inhibitors potentiate the actions of kinins and normalize endothelial functions. In our experiments kinins were continuously formed and this basal kinin release was increased 3-fold when perfused with the ACEI ramiprilat. Inhibition of local angiotensin converting enzyme (ACE) not only reduces angiotensin II formation, but also bradykinin catabolism. The increase in kinin release is explained by the inhibition of local ACE by ramiprilat. Since ACEI protects the destruction of kinins locally, ACEI enhances relaxation through the increase in endothelium-derived vasodilator substances (NO, PGI_2 and EDHF) under kinin regulation. Kinins –through this pathway– play an important role in the therapeutic actions of ACEI.

In summary, all the components of the kallikrein-kininogen-kinin system are released from the isolated perfused rat hind-quarters vessels. This system –through the release of potent vasodilator compounds from the endothelium– may contribute to the regulation of vascular function. In addition, part of the acute and chronic effects of treatment with ACEI can be explained by potentiation of the vascular kallikrein-kinin system.

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