Arteriolar Constriction and Local Renin-Angiotensin System in Rat Microcirculation

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Intravital microscopy was used in a preparation of rat cremaster muscle that was isolated from its normal blood supply and externally perfused with a physiological solution, thus allowing exclusion of circulating converting enzyme, renin, and angiotensinogen. The arterioles studied were classified as second-, third-, and fourth-order arterioles with mean diameters of 60.5, 29.9, and 14.8 μm, respectively. Topical administration of 1 nmol/mL angiotensin I or 1 nmol/mL tetradecapeptide renin substrate induced marked vasoconstrictions (i.e., 38.5%, 61.5%, and 90.1% and 25%, 34%, and 88% for second-, third-, and fourth-order arterioles with angiotensin I and tetradecapeptide renin substrate, respectively). The angiotensin converting enzyme inhibitor quinapril significantly inhibited the vasoconstrictions caused by either angiotensin I or tetradecapeptide renin substrate. Almost no vasoconstriction was found when angiotensinogen-rich renin-free plasma containing either 2.45 nmol/mL of angiotensinogen or 1.2 μg/mL renin was administered. Conversely, these two compounds induced significant constrictions in cremaster muscle preparations in which normal blood perfusion (and thus circulating renin and angiotensinogen) was left in place. We concluded that, in skeletal muscle, 1) the microvascular network is a very effective site of local angiotensin converting enzyme activity and consequently an important target site of angiotensin converting enzyme inhibitors; 2) the effects of tetradecapeptide renin substrate are very different from those of angiotensinogen from plasma and suggest that a large part of the effect of tetradecapeptide renin substrate was due to its nonspecific hydrolysis; and 3) at the microvascular level, circulating renin and angiotensinogen are more effective in inducing arteriolar constriction, in the presence of their substrate or associated enzyme, than local renin and angiotensinogen. (Hypertension 1993;21:491–497)

KEY WORDS • microcirculation • renin-angiotensin system • renin • angiotensins • kininase II • angiotensin converting enzyme inhibitors

It is now well recognized that the renin-angiotensin system (RAS) is not only an endocrine system but that some of its components are generated or activated in several tissues. They exert autocrine and paracrine effects on local regulatory mechanisms, contribute to cardiovascular and volume homeostasis, and may affect vascular wall structure. Several authors have shown the presence of a local RAS in large vessels: thus, angiotensin converting enzyme (ACE) has been reported to be widespread in endothelial cells,1–3 and the presence of renin has been suggested both by immunohistochemical methods and by the existence of renin messenger RNA (mRNA) in vascular smooth muscle cells.4–6 The presence of this local renin was also suggested by the production of angiotensin I (Ang I) by vessels perfused with tetradecapeptide renin substrate (TDP)7,8 and by the increased resistance of isolated muscle preparations when perfused with TDP.9–12 As regards angiotensinogen, its local production was suggested by its presence in vascular tissue13 and by the presence of angiotensinogen mRNA in different tissues.11,14 More recently, local production of angiotensinogen has also been demonstrated in isolated perfused beating heart.15

Although a large body of data has been acquired for the different components of the local RAS in isolated large arteries and in cultured vascular cells, as well as by whole-organ perfusion, very little is known about the characteristics of this system in the microvascular network. This is mainly due to the difficulty of separating the local from the circulating components of the RAS when studying the microcirculation. However, exploration of the microvascular network RAS seemed important to us because a significant part of the pressure gradient exists in the arteriolar network, which is consequently an important site for pressure regulation and because the terminal part of the microvascular network has been demonstrated to be the most sensitive to several vasopressive substances, including angiotensin II (Ang II).16–18 In addition, the mechanism of action of several classes of antihypertensive drugs is based on the inhibition of the activity of the different components of the RAS, but the exact part of local versus systemic mechanisms in the actions of these drugs has not been completely determined. The purpose of the present study was, therefore, to evaluate directly the capacity of local ACE, local renin, and local angiotensinogen to induce effective vasoconstriction in the presence of their associated substrate or enzyme. Vasoconstriction was

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precisely quantified by direct visualization in an original preparation of cremaster muscle whose main characteristics were the following: 1) The muscle was prepared for intravital microscopy by a new technique, which allowed it to be kept intact and reduced the risk of hemorrhages to a minimum. This permitted in vivo study of the microvascular network in a situation as close as possible to physiological conditions. 2) The muscle was isolated from its normal blood supply and perfused at a constant pressure via a femoral artery with a physiological buffer, thus enabling the circulating RAS to be excluded and the vasomotor responses to be limited to the effects of the local RAS. The responses observed in these conditions can be compared with those obtained in other cremaster muscles for which the normal blood supply of the muscle was left in place, thus allowing the presence of the circulating components of the RAS.

We used this muscle preparation to test the effects of Ang I and Ang II, TDP, angiotensinogen-rich plasma (from binephrectomized rats), and renin.

Methods

Male Sprague-Dawley rats (from Iffa-Credo, L'Arbresles, France) weighing 205±4 g were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentothiobarbital (Nesdona). A patent airway was maintained with a tracheotomy tube. The carotid artery was cannulated for measurement of systemic mean arterial blood pressure with a Statham P23DB transducer. All animals whose mean pressure fell to less than 90 mm Hg were excluded.

Preparation of Rat Cremaster Muscle

After anesthesia, the right cremaster muscle was surgically prepared for in vivo visualization by a new technique described in detail elsewhere. Briefly, the muscle was detached from the scrotum, then a transverse buttonhole slit approximately 5 mm long was made at the proximal part of the cremaster pouch. The testicle and epididymis and the cremaster itself were then drawn out through the buttonhole. The small pedicle that attaches the cremaster to the testicle was ligated with two stitches and cut between them so as to separate the cremaster completely from the testicle, which was reincorporated into the abdominal cavity. To prepare the cremaster muscle for transillumination microscopy, a flexible extendable ovoid ring 0.1 mm in diameter was made of metal wire covered by Silastic rubber and was introduced longitudinally into the cremaster pouch. When the clamp was removed, the ring expanded gently, spreading out the cremaster, which acquired a racket shape. This procedure involves minimal incision of the cremaster and consequently reduces considerably the risk of hemorrhage and of lesions to the muscle and its microcirculation.

The muscle chamber was filled with Krebs solution at a flow of 2 mL/min and a temperature of 34.5°C in the cremaster chamber. By bubbling the solution with a 6% CO₂–94% N₂ gas mixture, the pH, P₀₂, and Pco₂ of this solution (within the muscle chamber) were fixed at 7.40±0.05, 15±1.4 mm Hg, and 40±0.5 mm Hg, respectively. The chamber was covered with a Plexiglas plate to isolate it from the atmosphere.

To allow external perfusion of the cremaster muscle, we introduced a catheter into the femoral artery homolateral to the cremaster studied. A ligature was placed on the common iliac artery just upstream of the bifurcation with the pudic epigastric trunk (from which runs the feeding artery of the cremaster muscle). At this time, the ligature was not secured tightly. To reduce the duration of surgery as much as possible, we did not ligate all the arteries issued from the common iliac artery and pudic epigastric trunk. Consequently, the external perfusion not only perfused the cremaster muscle but also the muscles depending on these arteries. To exclude collateral perfusion of the cremaster muscle, several ligatures were made in the lateral fascias of the muscle that connect it with the pelvic wall. Then, the ligature on the common iliac artery was tightened and the muscle perfused at a controlled constant perfusion pressure of 100 mm Hg using a controlled pump (Fluid Metering Inc., Oyster Bay, N.Y.) with a pulse shock absorber. Perfusion pressure was recorded with a Statham pressure transducer (model P231D, Spectramed, Bilthoven, The Netherlands). Perfusion medium was a Krebs solution containing 0.5% bovine serum albumin (wt/vol) gassed at 5% CO₂–95% O₂ and kept at 37°C. The muscle was gently washed externally three times, and a washout period of 45 minutes of perfusion was allowed before any drug was administered.

In some sections of the present study, the results obtained with the cremaster preparation externally perfused were compared with those obtained in cremaster muscle prepared similarly except that the normal blood supply was kept in place, thus allowing the presence of the circulating RAS.

Arteriolar Network Visualization

For visualization of the microcirculation, the chamber was placed on the movable stage of a modified Leitz microscope, and the cremaster muscle was transilluminated with a 100-W tungsten-halogen lamp. The image, magnified by a x20 water-immersion objective and x10 oculars, was projected into a CCD camera (Sony 101) connected to a videocassette recorder (Sony VP 3600) and a video monitor. Total magnification from tissue to video monitor was x1,210 or x650. The image was positioned by an optical system in such a way that the images of most of the arterioles were vertical. The field studied was usually centered on an arteriolar bifurcation after identification of the order of the arterioles by their relative locations in the network, according to the morphological criteria derived from Zweifach. The arterioles studied in the present experiments were second-, third-, and fourth-order arterioles (A2 to A4, respectively). Measurements were made at the site of maximal arteriolar constriction in the field considered and during the period of maximal response. To be sure that a constriction was observed even if its kinetics would be slow, observation time after any intervention was at least 60 minutes (however, all the constrictions reported in the present study were maximal within less than 15 minutes). Arteriolar internal diameters were measured by playback analysis of the video record using a video-dimension analyzer (model 303 or 908, IPM,
Experimental Protocols

In a first group of rats, we compared the vascular tone of the cremaster isolated from its normal blood supply and perfused with Krebs (n = 8; n refers to the number of rats used in each group) to that of a normal cremaster preparation (n = 8). Two doses of Ang II were studied (0.01 and 0.1 nmol/mL). These two doses were chosen because they induced vasoconstrictions in a range similar to those observed with the other compounds used in the following protocols. In this protocol and in the following ones, results were expressed as percent vasoconstriction calculated as (di – dj)/di, where di is the basal inner diameter and dj the inner diameter at the peak vasoconstriction.

The activity of local microvascular ACE was studied by topical administration of 1 nmol/mL Ang I in cremaster from which circulating RAS (and therefore circulating ACE) was excluded by the procedure described previously. Ten minutes before Ang I administration, rats were randomly divided into two groups for pretreatment with Krebs buffer (n = 8) or 10 nmol/mL quinapril (10 nmol/mL quinapril, n = 8).

In other rats, we studied the effect of topical administration of 1 nmol/mL TDP in cremasters from which circulating RAS (and therefore circulating renin) was excluded. Ten minutes before administration of TDP, rats were randomly allocated into two groups pretreated with Krebs buffer (n = 10) or 10 nmol/mL quinapril (n = 9).

The effect of topical administration of plasma from binephrectomized rats containing 2.45 ± 0.15 nmol/mL angiotensinogen and no renin (see References 22 and 23 for details on dose) was also studied. For this series, we compared the results obtained in a group of cremaster muscles isolated from normal blood supply and perfused with Krebs (n = 6) with those obtained with the same plasma samples in another group of rats for which the normal blood supply of the cremaster muscle was left in place (n = 6) (i.e., in the first group, circulating RAS and therefore circulating renin was excluded, and in the second group, circulating angiotensinogen was present). Because the preparation of cremaster muscle for external perfusion implied a washout period, we waited for an equivalent period of time before any drug administration in the groups of rats for which normal perfusion of the cremaster muscle was left in place.

Results

All results are expressed as mean ± SEM. In all protocols, results in treated and control groups were analyzed by two-way analysis of variance for two between-factors; i.e., one factor was the arteriolar order and the other was the treatment. To compare the effect of quinapril on the Ang I- and TDP-induced vasoconstriction, we analyzed the results of the two series of experiments by a three-way analysis of variance (i.e., two factors were the same as previously, and the third was the type of substrate inducing the constriction). All tests were done with BMDP software (University of California at Los Angeles) with a significance level fixed at 5%.

Vascular Tone in Normal and Krebs-Perfused Cremaster Muscle

As shown in Figure 1, no significant differences in vascular tone were found between the cremaster muscle perfused with Krebs and the cremaster muscle in which normal blood perfusion was left in place.

Effect of Angiotensin II in Normal and Krebs-Perfused Cremaster Muscle

As shown in Figure 2, in all arteriolar orders studied and for the two doses, no significant differences were found between the response to Ang II by the cremaster muscle perfused with Krebs and the cremaster muscle in which normal blood perfusion was left in place (basal diameter of the studied arterioles was not significantly different between the two preparations).

Effect of Angiotensin I in Krebs-Perfused Cremaster Muscle

As shown in Figure 3, administration of Ang I induced marked arteriolar constrictions whose average values for the three arteriolar orders are reported in Figure 4 (hatched columns). Vasoconstriction was greatest for the smallest arterioles, which were almost completely closed. The maximal degree of constriction was obtained in a period ranging from 25 seconds to 2 minutes.

No significant effect of quinapril on basal diameter was found, but as shown in Figure 4 (black columns) for
all arteriolar orders studied, vasoconstriction was greatly inhibited by administration of quinapril 10 minutes before Ang I (p<0.001).

Effect of Tetradecapeptide Renin Substrate in Krebs-Perfused Cremaster Muscle

Administration of TDP also induced significant arteriolar constriction (Figure 5, hatched columns), and a longitudinal gradient of constriction was also found with this substrate. The maximal constriction was obtained within a period of time very similar to that found for Ang I. No significant effect of quinapril on basal diameter was found, but as shown in Figure 5 (black columns), the vasoconstriction induced by TDP was also greatly inhibited (p<0.001) by pretreatment with quinapril in all arteriolar orders. Despite the significant inhibition of TDP-induced constriction by quinapril, higher degrees of constriction were obtained with TDP plus quinapril than with Ang I plus quinapril. This difference was significant because a significant interaction (p<0.02) was found in the three-way analysis of variance between the factor “type of substrate” and the factor “presence or absence of quinapril.”

Administration of Plasma From Binephrectomized Rats in Normal and Krebs-Perfused Cremaster Muscle

In the cremaster muscle isolated from its blood supply and perfused with Krebs, very limited vasoconstriction was seen in the arterioles after topical administration of plasma from binephrectomized rats (Figure 6, hatched columns). The long time of observation allowed us to exclude any constriction that could have exhibited very slow kinetics. Conversely, when the same experiment was made without excluding the normal blood perfusion of the muscle, significant vasoconstriction was found in the minutes after topical administration of binephrectomized rat plasma (Figure 6, black columns).

Administration of Renin in Normal and Krebs-Perfused Cremaster Muscle

In the preparation from which circulating RAS was excluded, no vasoconstriction was observed after administration of renin, despite the high dose applied. Conversely, when circulating RAS was present, the same dose of renin induced significant constrictions of all arterioles (Figure 7, black columns).

Discussion

The effects of Ang I and TDP in the arteriolar network already have been observed by Cornish and coworkers. However, these authors studied a cremaster muscle in which normal blood supply was left in place and consequently did not distinguish between local and circulating RAS. In contrast, the present results constitute, to our knowledge, the first evaluation of the capacity of the different components of the local...
RAS of skeletal muscle to induce acute vasomotor effects in the presence of their associated substrates or enzymes. As stated previously, this evaluation seemed important to us, because the arteriolar network is crucial for regulation of pressure and tissue perfusion and because pharmacological compounds that can antagonize circulating or local components of the RAS are proposed for clinical use.

In the present study, we used an original preparation of cremaster muscle isolated from its normal blood supply and perfused with Krebs solution. For measurements, we used the technique of intravital microscopy usually applied to blood-perfused organs. Although the presence of blood enhances the contrast between the vessels and their surrounding tissues, it may be stressed that it was easy when using a long-distance condenser to visualize clearly the limits of the arterioles, even when the muscle was perfused with a transparent solution (see photographs in Figure 3). We checked that the tone of this new preparation was not different from that of a normal cremaster muscle preparation (see Figure 1). Despite a certain degree of heterogeneity in the results found by different groups, it may be noted that for all arteriolar orders, the mean vascular tone in the two preparations used in the present study was in the range of tone reported by others in cremaster muscle.19,25-30 We also checked that the reactivity to Ang II of this new preparation was not different from that of a normal cremaster muscle preparation (Figure 2). As regards the sensitivity of this preparation for the detection of local peptide conversion versus that of other experimental models, a general difficulty exists in comparing different routes of administration and relating quantitatively the

**FIGURE 3.** Photographs show typical video images of arteriole in control state (top left panel) and a few seconds after administration of 1 nmol/mL angiotensin I (bottom left panel) in muscle from which circulating renin-angiotensin system was excluded. Right panels: Corresponding recordings of changes in diameter, perfusion, and blood pressure (bottom, middle, and top panels, respectively).
vasoconstriction observed in the arterioles to the increase in pressure measured by others in a whole-organ preparation. However, in preliminary studies with our preparation, we measured significant vasoconstriction after administration of Ang I at concentrations of 5–10 pmol/mL. It is worth noting that these concentrations were of the same order of magnitude as the 4 pmol/mL perfused for 5 minutes by Hilgers and coworkers, who obtained a significant increase of pressure in rat hindlimb with this concentration.

From our first series of experiments, it was clear that the system of Ang I conversion to Ang II is very effective at the microvascular level, because almost complete vasoconstriction of the most distal arterioles was found. The most distal arterioles were those most affected by this local Ang II production. However, the present experiments did not show whether the conversion of Ang I occurred throughout the entire arteriolar network or whether the vasoconstriction of the most distal arterioles was due to the transport of the Ang II that had been produced in the proximal ones. In addition, because substrates were administered in the suffusion bath, changes in diameter of the observed vessel may be influenced by autoregulatory mechanisms due to changes in pressure or flow induced by the response of the other microvessels in the network. These possible interactions complicate the interpretation of the observed segmental differences.

The vasoconstrictions induced by Ang I were greatly inhibited by pretreatment with quinapril. This inhibition was very effective, even in the most distal parts of the microcirculatory network, which were almost completely closed without quinapril pretreatment but exhibited less than 25% vasoconstriction with quinapril pretreatment (see Figure 4).

Significant vasoconstriction was also found when TDP was administered (Figure 5). These results are in accordance with microvascular constrictions after TDP administration reported by Cornish et al and with the pressor effect and Ang II formation reported by several authors after TDP infusion. As regards the possibility of suppressing TDP-evoked vasoconstriction by ACE inhibitors, some groups reported that captopril was effective in this respect and others that it was not. We did not test captopril on the present preparation but found a significant inhibition of the TDP-induced vasoconstriction with quinapril. It should be noted that quinapril is a more lipophilic ACE inhibitor than captopril and consequently may more effectively suppress Ang II formation by the vasculature than the latter. Indeed, these findings indicated that, in the present experimental model, a large part of the Ang II formed from TDP was generated by an enzymatic cascade whose one step involved converting enzyme. However, it should also be noted that the inhibition of the Ang I–induced constriction was more important than that of the TDP-induced constriction. This suggests that a part of the Ang II was also formed from TDP independently of ACE activity, as reported by several authors in other experimental models. In addition, because different enzymes are capable of generating Ang I and II from TDP, the vasoconstriction induced by TDP cannot be unequivocally related to the presence of active renin. Consequently, to provide to the system a substrate that could require an enzymatic activity more specific of the renin, it seemed worthwhile to us to study the effects of plasma from binephrectomized rats containing 2.45 nmol/mL angiotensinogen. Hatched columns correspond to results found in muscle from which circulating renin-angiotensin system was excluded (n=5, 9, 8 for A2, A3, and A4, respectively) and black columns to those found in muscle from which circulating renin-angiotensin system was not excluded (n=5, 9, 8 for A2, A3, and A4, respectively). Results are expressed as percent vasoconstriction (mean±SEM). n, Number of arterioles studied.

**Figure 6.** Bar graph shows vasoconstrictor responses of second- to fourth-order arterioles (A2 to A4) to topical administration of plasma from binephrectomized rats containing 2.45 nmol/mL angiotensinogen. Hatched columns correspond to results found in muscle from which circulating renin-angiotensin system was excluded (n=5, 9, 8 for A2, A3, and A4, respectively) and black columns to those found in muscle from which circulating renin-angiotensin system was not excluded (n=5, 9, 8 for A2, A3, and A4, respectively). Results are expressed as percent vasoconstriction (mean±SEM). n, Number of arterioles studied.

**Figure 7.** Bar graph shows vasoconstrictor responses of second- to fourth-order arterioles (A2 to A4) to topical administration of 1.2 µg/mL renin. Black columns show results found in muscle from which circulating renin-angiotensin system was not excluded (n=5, 9, 8 for A2, A3, and A4, respectively). Because the mean value was zero, columns corresponding to results found in muscle from which circulating renin-angiotensin system was excluded do not appear (n=5, 9, 8 for A2, A3, and A4, respectively). Results are expressed as percent vasoconstriction (mean±SEM). n, Number of arterioles studied.
higher than that of the local renin. Consequently, it is likely that in the series of experiments with TDP, enzymes, other than active renin, were responsible for the vasoactive effects observed. It may be stressed that the present results found for arterioles with a diameter of less than 120 μm cannot be extrapolated to the other parts of the vascular system, because qualitative or quantitative differences between renin formation in the small arterioles and in the arteries are very possible.

As regards angiotensinogen, no vasoconstriction was found when renin was administered to the microvascular preparation isolated from the circulating RAS (Figure 7). In contrast, the same dose of renin induced significant constriction of all vessels when blood, and consequently circulating angiotensinogen, was allowed to perfuse the preparation. These results showed that at the microvascular level, the potential effect of local angiotensinogen was considerably less important than that of circulating angiotensinogen. Because renin has been found to produce an effective increase in pressure when perfused in an isolated rat hindlimb,9 it may be hypothesized that the local angiotensinogen is unequally distributed in the vascular network and the pressure effect observed by these authors was due to Ang I production from angiotensinogen in large vessels. Part of this Ang I might then be converted to Ang II in the distal part of the network (i.e., the arterioles) and induce vasoconstriction in the microcirculation.

In conclusion, in the present work, we found very effective local conversion of Ang I to Ang II in the microvascular network of skeletal muscle. This local conversion was markedly inhibited by quinapril, showing that the present results found for arterioles with a diameter of less than 120 μm cannot be extrapolated to the other parts of the vascular system, because qualitative or quantitative differences between renin formation in the small arterioles and in the arteries are very possible.

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