Direct Evidence for the Presence of a Different Converting Enzyme in the Hamster Cheek Pouch

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Summary Kininase II (angiotensin I-converting enzyme) is generally accepted to be the enzyme responsible for the conversion of angiotensin I (A I) to angiotensin II (A II). This study examined the response of the microvasculature of the hamster cheek pouch to the local application of A I, A II, and the renin substrate, tetradecapeptide (TDP). A I and TDP caused a localized vasoconstriction that was not blocked by converting enzyme inhibitors (CEI; BPFJ and BPF6) and the nonapeptide inhibitor for TDP. However, both the A II antagonist [Sar1, Ala8]angiotensin II and the antiserum to A II blocked completely the A I- and TDP-induced vasoconstriction. Sixty-eight percent of the applied A I was converted to A II in the presence of CEI as well as in its absence. It is concluded that the vasculature of the hamster cheek pouch converts significant amounts of A I to A II by a route that does not involve kininase II.

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Nearly all aspects of the renin-angiotensin system have been extensively investigated (for reviews, see Peach, 1977; Davis and Freeman, 1976; Regoli et al., 1974), yet in spite of the effort that has been expended in elucidating the system, there still remain many areas of uncertainty. However, it is generally accepted that this system is controlled by the kidney through the release of renin, which initiates the formation of angiotensin I (A I) from angiotensinogen, a plasma globulin produced by the liver. Subsequently, A I is converted to angiotensin II (A II) by converting enzyme [kininase II (CE)] which is found in many tissues of the body (Erdös, 1975). Some of the actions of A II are to alter vascular resistance, stimulate thirst, and release aldosterone (Peach, 1977).

Recent work in this laboratory has been directed toward the study of the conversion of A I to A II by the microvasculature of cardiac tissue transplanted into the hamster cheek pouch (Cornish et al., 1978). During the course of this study, it was observed that the cheek pouch arteriole exhibited a rapid and prolonged tachyphylaxis to A II, whereas the vessels of transplanted tissue did not (for review of transplant procedure, see Greenblatt et al., 1969, 1971; Oestermeyer and Block, 1977). Thus, this study was designed to characterize the response of the hamster cheek pouch microcirculation to angiotensin and to determine the degree to which A I is converted to A II.

Methods

Twenty-five female hamsters weighing 120-200 g were anesthetized with chloral hydrate (40 mg/100 g body weight given ip), a tracheostomy was performed, and polyethylene catheters were inserted into a femoral artery and vein. Additional anesthetic was administered through the venous catheter as needed, and the arterial catheter was used to measure mean arterial blood pressure. A transparent plastic plate was inserted through the mouth into the cheek pouch, and the skin over the cheek pouch was incised and retracted to expose the vasculature of the cheek pouch membrane. The retracted skin was attached to a platform that surrounded the base plate, forming a reservoir 3-4 mm in depth over the cheek pouch membrane for subsequent suffusion. The connective tissue over the microvasculature was dissected away to permit better visualization and to allow closer approximation of the micropipettes to the vessels. The preparation was then suffused with Ringer's bicarbonate solution containing 2% gelatin (pH 7.28-7.47). A plexiglass rod with a polished, beveled tip (45°) was inserted below the plastic plate and illuminated at the distal end with a 100-Wt halogen lamp to permit transillumination of the membrane. A Carl Zeiss optics system (Collins Microscope Co.) permitted visualization of the microvasculature of the cheek pouch membrane. After this preparation was completed, suffusion was continued for 10-20 minutes to allow the vascular bed to equilibrate. Since previous work in this laboratory has shown that a single application of angiotensin to a cheek pouch artery causes immediate tachyphylaxis lasting 1-2 hours, a vascular diagram was sketched for each preparation. Thus, as a drug test was completed on an arteriole, its location was indicated on the diagram so that another site on the same or a different
arteriole could be used for the next drug test. This procedure was continued throughout the experiment.

A I, A II, and renin substrate tetradecapeptide (TDP) were dissolved in Ringer's bicarbonate solution at concentrations of 0.1, 0.5, 1, and 5 ng/2.25 µl and applied to the vessels with a micropipette (tip diameter, 10-20 µm) in a delivery volume of 2.25 µl with a syringe micrometer. When converting enzyme inhibitor [BPF, Schwarz/Mann (CEI)], the nonapeptide [Beckman (CEI)], or the A II antagonist [Sar¹, Ala⁸]angiotensin II (SAR) were applied, suffusion was discontinued, the suffusion solution aspirated from the reservoir, and the reservoir refilled with 1.5 ml of Ringer's bicarbonate solution containing the inhibitor (0.44 mg/ml) that was being tested. A similar procedure was followed for the antiserum to A II (Ab-A II) except that the reservoir was filled with 1.5 ml of the undiluted antiserum. The cross-reactivity of the A II antiserum for A I previously had been determined to be 3% (Garcia et al., 1978). Vessel diameters were measured continuously before, during, and after each drug application with a Vicker's shearing eye piece.

The protocol for drug testing was: (1) application of A I, A II, or TDP to a vessel; (2) application of A I or TDP to a vessel with CEI in the reservoir; (3) a control test for A I or TDP after CEI had been flushed from the reservoir (2 and 3 were repeated several times for each experiment); (4) application of either A I, TDP, or A II with SAR or Ab-A II in the reservoir. Because of the long-lasting effects of SAR, it was the last inhibitor used. A I or A II was applied at least 2 minutes after the inhibitors had been added to the reservoir. Since there is the possibility that the nonapeptide CEI is a more effective inhibitor than the pentapeptide, both inhibitors were used in the TDP studies.

The percent amount of A I converted to A II was calculated with the formula proposed by Gerlings and Gilmore (1973):

\[
\text{% conversion} = \frac{\text{response/dose A I}}{\text{response/dose A II}} \times 125.
\]

The figure 125 takes into account the fact that, if 1 ng of A I were converted to A II, only 0.8 ng of A II would be produced. The 0.1-ng dose was used to calculate conversion, since 1.0 ng of A I caused maximal constriction with cessation of flow.

The percent of vasoconstriction resulting from angiotensin application was calculated by the formula:

\[
\text{% constriction} = 100 \times \frac{\text{vessel diameter at maximal response}}{\text{control vessel diameter}}.
\]

All data were analyzed for statistical significance by Student's t-test (unpaired), with P values less than 0.05 considered significant.

Results

Figure 1 is from a representative experiment and shows the vasoconstriction elicited by applying 1 ng of A II (A), 1 ng of A I (B), or 5 ng of TDP (C) to cheek pouch arterioles (most of the studies done with TDP used the 5-ng dose, as this gave a response most comparable to that caused by the 1-ng doses of A I and A II). Figure 2 shows results of a typical experiment in which 1 ng A I was applied to three different arterioles before, with, and after CEI. Figure 3 shows a plot of all the data from this series of experiments. As there was no statistically significant difference between the pre- and post-CEI controls, these data were considered together. CEI had no significant influence on the vasoconstrictor response to either 0.1 or 1.0 ng of A I. However, as shown in Figure 3, both Ab-A II and SAR effectively blocked the vasoconstriction caused by 0.1 (P < 0.001) and 1.0 ng of A I (P < 0.001), respectively. Figure 4 shows the blocking effect of Ab-A II and Figure 5 the blocking effect of SAR on the vasoconstriction induced by A I. Figure 6 shows results of a typical experiment, and Figure 3 summarizes the data showing the efficacy of Ab-A II in blocking the vasoconstrictor effects of A II. As shown in Figure 3, SAR also inhibited A II-induced vasoconstriction.

The vasoconstriction caused by TDP was unaffected by either CEI or CEI but was effectively blocked by both Ab-A II and SAR (Fig. 7).
FIGURE 2  Vessel diameter changes induced in three cheek pouch arterioles in response to 1.0 ng of A I before CEIs, with CEIs in the reservoir, and after flushing CEIs from reservoir. C: close suffusion; O: open, begin suffusion.

By applying the formula of Gerlings and Gilmore (1973) to the data obtained for 0.1 ng of A I and A II, it was determined that the cheek pouch vessels convert 68% of the applied A I to A II. This conversion was not altered by adding CEIs to the reservoir.

Discussion

When A I was applied to the cheek pouch arterioles in the presence of CEIs, the vasoconstrictor response was still present and was similar to the response obtained without CEI (Figs. 2 and 3). These results were quite unexpected and raised several questions. One major concern was the activity of the CEIs used in these studies. Initially, stock supplies of CEIs that were not outdated, but also not recently ordered, were used to block CE activity. Then new supplies of CEIs were ordered and tested. Both batches of CEIs failed to inhibit A I activity. Thus, the inability of CEIs to attenuate the response to A I was not due to inactive CEIs. Our next concern related to the concentrations of CEIs used in this study. Concurrently, we were investigating the conversion of A I to A II by the microcirculation of the cardiac transplant (Cornish et al., 1978). In this coronary circulation, the vasoconstriction produced by A I was inhibited by the same concentrations and stock supplies of CEIs that were being used in this study. Also, others (Erdős, 1975; Ferreira et al., 1970) have found that concentrations of CEIs required to inhibit the conversion of A I to A II in vitro by CE were $10^{-7}$-10^{-9} M as compared to a 7.2 x 10^{-4} M concentration of CEI used in this study. Furthermore, Gerlings and Gilmore (1973) used CEIs, 2.48 µg/ml, to inhibit CE activity in vivo. Even if the concentrations of CEIs were not adequate to block the A I response, there should have been an attenuation of the response. Figure 3 shows no such attenuation by CEIs. Thus our concentrations of CEIs (440 µg/ml) appear to have been more than adequate. Finally, the only alternative explanation for the lack of activity for the CEIs might be that CEIs did not reach the site where CE was located. However, this seems unlikely since the molecular weight and size of CEIs are much less than those of A I (five amino acids compared to 10 amino acids, respectively). In addition, CEIs inhibited CE activity in the cardiac transplant (Cornish et al., 1978). Therefore, it became evident that the potent vasoconstriction produced by A I in the presence of CEIs was not due to the conversion of A I to A II by A I CE (kinase II).

The failure of CEIs to block A I-induced vasoconstriction could have been explained by (1) an intrinsic vasoconstrictor action of A I in this preparation or (2) the conversion of A I to A II by another enzyme. It has been suggested (Ackerly et al., 1977) that A I may have intrinsic activity. However, caution has been advised in reaching such a conclusion, since other enzymes besides CE have been found that convert A I to A II (Boucher et al., 1974, 1977; Grandino and Paiva, 1974). The inhibition of the A I- and A II-induced vasoconstriction by SAR in this study implied that A I was acting at the receptor site for A II. However, this did not preclude the possibility that A I has intrinsic activ-

FIGURE 3  Vasoconstriction caused by varying doses of A II (C), A II with Ab-A II ( ), A I ( ), A I with CEIs ( ), A I with Ab-A II ( ), and A I with SAR ( ). Points represent the mean with standard error bars. The numbers in parentheses are the number of vessels tested.

FIGURE 4  Vessel diameter changes induced by 0.1 ng of A I before and with Ab-A II in the reservoir. C: closed suffusion; O: open, begin suffusion.

FIGURE 5  Vessel diameter changes induced by 1.0 ng of A I before SAR and with SAR in the reservoir (two separate vessels). C: close suffusion; O: open, begin suffusion.
Angiotensin I conversion/Cornish et al.

Figure 6 Vessel diameter changes induced by 0.1 ng of A II before Ab-A II and with Ab-A II in the reservoir. C: close suffusion; O: open, begin suffusion.

Figure 7 Vasoconstriction caused by TDP (○), TDP with CEI's. (▲), TDP with CEIs. (▲), TDP with SAR (■), and TDP with A-A II (◆).

Angiotensin conversion, as depicted for A II. To demonstrate that A I was being converted to A II, and that it was A II that SAR was blocking, it was necessary to attenuate or abolish the vasoconstrictor response to A II with a substance specific for A II. This was done with Ab-A II. When A II or A I was applied to the cheek pouch vessels in the presence of Ab-A II, the vasoconstriction was blocked (Figs. 3, 4, and 6). If A I possessed intrinsic activity in this preparation, the application of 0.1 ng of A I to a cheek pouch vessel in the presence of the A II antiserum should have caused vasoconstriction of a magnitude similar to that observed when 0.1 ng of A I was applied without the antiserum. Since A I-induced vasoconstriction was completely blocked by Ab-A II (Fig. 3), it can be concluded that A I has no measurable intrinsic activity in this preparation and that the A I-induced vasoconstriction results from the conversion of A I to A II.

When it was observed that CEIs. was an ineffective inhibitor of the vascular response to A I, we began to consider the possibility of another enzymatic system for the conversion of A I to A II. Boucher and others in several studies have demonstrated the presence of tonin in the rat (Boucher et al., 1974, 1977; Garcia et al., 1978). This enzyme rapidly converts A I, TDP, or angiotensinojen to A II. Although CE (kininase II) also converts TDP to A II, it does so at an extremely slow rate (Dorer et al., 1975). For this reason, and because TDP itself is a poor vasoconstrictor (Montague et al., 1966a, b), TDP was used in this study. A rapid vascular response to TDP would be indicative of an enzyme other than CE, possibly tonin, mediating the production of A II, whereas no response or one that was greatly delayed would tend to rule out tonin as a possible mediator. As can be seen in Figure 1, there is no difference in the time course of vasoconstriction resulting from the application of A I, A II, or TDP.

The addition of CEI's or CEIs had no significant effect on the vascular response to TDP, whereas the specific A II blockers, SAR and Ab-A II, almost completely abolished the TDP response. Since CE is significantly inhibited by CEI's and CEIs at these concentrations (Erdos, 1975; Cornish et al., 1978; Gerlings and Gilmore, 1973; Ferreira et al., 1970), and since CE is very ineffective in converting TDP to A II (Dorer et al., 1975), CE (kininase II) is not the enzyme responsible for converting A I or TDP to A II in this preparation.

Recently, various studies (Boucher et al., 1974; Grandino and Paiva, 1974; Garcia et al., 1978) have demonstrated the existence of enzymes in addition to CE that naturally convert A I to A II. The results of this study clearly demonstrate the presence of an additional pathway for the conversion of A I to A II. One way to gain additional insight into the role of this pathway in converting A I to A II is to calculate the percent of A I converted to A II. Using the vascular responses to the application of 0.1 ng of A I and A II, we found that 68% of the applied A I is converted to A II and, more importantly, that CEI's does not alter this conversion. Even if CE were being only partly inhibited by CEI's, it would be expected that there would be some diminution of the A I response or of the percent of A I converted to A II. That this was not observed indicates that the usual CE plays little or no role in the conversion of A I or A II in this tissue, and that there is another enzymatic pathway involved.

Discoveries of other enzymes that convert A I to A II (Boucher et al., 1974, 1977; Grandino and Paiva, 1974) give credence to the hypothesis that there are other enzymes that convert A I or TDP to A II. In our preparation, this enzymatic pathway may act in concert with the usual CE or as the sole enzyme for the generation of A II. These enzymes are known to readily convert A I, TDP, or angiotensinojen to A II as described by the Michaelis constant (K_m) for CE, which is 10^{-5} M, and for tonin, which is 3.9 × 10^{-3} M (Erdos, 1975; Boucher et al., 1977). Also, tonin is not inhibited by CEI (Boucher et al., 1974). Thus, it appears that these enzymes are specifically related to the angiotensin system, since they are involved only in the generation of A II, whereas CE is a nonspecific dipeptidylcarboxypeptidase.

It is thought that the circulating levels of angiotensin are under the control of the kidney, since it, through renin, controls the conversion of angiotensinojen to A I. Due to the major role that renin is
presumed to play in regulating angiotensin levels, it has been used as an indicator of angiotensin activity. In hypertensive states in which renin levels are normal, angiotensin has been eliminated as a possible etiologic factor. The demonstration that renin is not the only enzyme capable of initiating A II production dictates that the role of angiotensin in low-renin hypertensive states be reevaluated.

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