Laboratory Studies

Plasma Prorenin in Humans and Dogs
Species Differences and Further Evidence
of a Systemic Activation Cascade

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SUMMARY We have studied the dog as a potential model for the human plasma prorenin-renin system. On a regular sodium intake, healthy conscious dogs apparently have a much lower plasma renin activity (PRA) than healthy human volunteers. Cryoactivation of prorenin is virtually absent in dogs, in contrast to that in humans, but becomes more effective after preacidification of the plasma. The concentration of trypsin required for optimal activation of prorenin is 6 to 10 times higher for dog plasma, revealing a prorenin:renin ratio about 10 times greater than in humans. Dialysis of posttrypsinic plasma decreases the PRA, but it remains 5 times higher than in pretryptic plasma, indicating that activation is not totally dependent on any renin system component that has been rendered dialyzable by trypsin, e.g., substrate converted to tetradecapeptide (TDP). This argues against the view that trypsinic activation is attributable to angiotensin production from TDP by the action of cathepsin D, rather than from new renin converted from prorenin. The posttryptic increase in PRA is evident whether plasma incubation is carried out at pH 6.0 or at 7.4, and can be largely blocked by pepstatin, which also implicates a prorenin-renin mechanism rather than TDP-cathepsin. The low PRA in dogs, the negligible cryoactivation and its improvement by preacidification, and the requirement and tolerance of high trypsin concentrations, all point to greater protease inhibition in dog plasma and/or departures from the enzyme(s) responsible for human prorenin activation. Moreover, the trypsinic activation of prorenin is not completed quickly as in human plasma, but carries over into the posttrypsinic stage of angiotensin generation, even in the presence of excess soybean trypsin inhibitor (SBTI), and other potent inhibitors. Such ongoing prorenin activation cannot be attributed only to trypsin itself, nor to kallikrein (both are inhibited by SBTI), but rather to some other enzyme(s) derived by the action of trypsin. This new prorenin convertase activity (possibly renin itself) can be effectively transferred from trypsinized to control dog plasma, in which it greatly accelerates prorenin activation. Thus, contrary to other reports, dog plasma has a high content of activatable prorenin, and with appropriate methodological changes, the dog can be used as an animal model for physiological and biochemical studies of the prorenin-renin system. (Hypertension 5:277-285, 1983)

KEY WORDS • trypsin-activation • cryoactivation • pepstatin • non-serine proteases • inhibitors • autocatalysis

There is now abundant evidence in favor of an inactive renin precursor, often called "prorenin". In normal human plasma, prorenin has been activated in vitro by cryoactivation, cryoactivation, or acid treatment. The mechanism by which activation occurs in vivo is not yet understood, but it is likely that enzymes of other blood cascades are involved. There is a paucity of corresponding data on animal plasmas. The earliest study, using techniques based on human plasma, revealed modest prorenin levels in dog plasma, even less impressive amounts in rabbits and rats. Subsequently, Gallagher et al. reported that they were unable to demonstrate prorenin in dog plasma. Potter et al. detected activatable renin in dog plasma fractions but could not demonstrate it clearly in whole plasma, probably because they used an inadequate concentration of trypsin. On the other hand, using higher concentrations of trypsin than previously, we were able to demonstrate even greater proportions of plasma prorenin in dogs than in humans. The reported presence of prorenin in dog kidneys also makes its presence in plasma probable. Moreover, it is becoming increasingly obvious that different activation techniques are required in plasmas of different species.
Plasma Preparation

Six healthy male beagles, 13-16 kg, aged 12-20 months, and on an unrestricted diet were trained to require only minimal restraint during venipuncture. Blood samples were collected from the conscious dogs into EDTA (ethylene diamine tetaacetic acid, ammonium form, 15% solution in water, 0.3 ml/10 ml blood), and chilled immediately. The blood was centrifuged at 1500 x g, 4°C, for 20 minutes, and the plasmas separated and stored at −70°C until further assay. The human plasma pool was prepared in the same way from six healthy men on an unrestricted diet.

Materials and Methods

Plasma Renin Activity

Plasma renin activity (PRA) was determined as described previously, using an angiotensin I radioimmunoassay kit (RIA, New England Nuclear Medical Diagnostics Division, North Billerica, Massachusetts). Plasma samples were incubated for up to 30 minutes at 37°C, pH 6, in the presence of angiotensin I values were expressed in terms of nanograms (ng) generated per hour.

Active, preformed renin in the dog and human plasmas was measured by direct PRA assay of control, untreated plasmas. 'Prorenin' was determined by subtracting the PRA measured in untreated plasma from the PRA of activated plasma. This arithmetic difference does not represent the total prorenin, but rather that proportion of the total that had been unmasked under the described experimental conditions.

Cryoadvitation

Dog and human plasmas were incubated in the presence of neomycin sulfate, with no pH adjustment, for up to 42 days at 0°C. Some dog plasma samples were first exposed for 3 hours to acid dialysis at pH 3.3, then 3 hours of dialysis at pH 7.4, followed by cold incubation for up to 7 days at 0°C. Control nonacidified samples were dialyzed against buffer at pH 7.4 for 6 hours, and then incubated at 0°C.

Tryptic Activation

Titration of Plasma with Trypsin

In the presence of various concentrations of trypsin (T-8253, Sigma, St. Louis, Missouri) dog and human plasma samples were incubated for 10 minutes at 23°C, followed by the addition of an excess concentration of lima bean trypsin inhibitor (LBTI, Sigma, T-9378). We refer to this trypsinization step as "Stage 1 incubation," and the subsequent PRA incubation at 37°C as "Stage 2." For dog plasma, 1 to 6 mg of trypsin per milliliter (ml) was used, and for human plasma, 0.5 to 3 mg/ml. The "total renin" (or total PRA) was then determined as described above.

Time Course of Tryptic Activation

An 'optimal' concentration of trypsin was selected for dog and human plasmas, i.e., the trypsin level found to be effective in all cases, with no destructive effects. For dog plasma this was 3 mg trypsin/ml plasma, and for human plasma, 0.5 mg/ml. The effect of exposing plasma to these 'optimal' concentrations of trypsin for up to 60 minutes at 23°C was studied. In each case, any remaining action of trypsin was stopped by the addition of excess LBTI, and renin and prorenin were determined by PRA assay.

Effect of Pepstatin on Tryptic Activation in Dog Plasma

To determine whether it is renin and not some other angiotensin-producing enzyme that results from trypsin activation of dog plasma, trypsinized plasmas (3 mg/ml, 10 minutes, 23°C) were incubated in the presence of the renin inhibitor pepstatin (pepstatin A, Sigma, P-4265). The pepstatin solution was prepared according to Guyene et al., (13.7 mg pepstatin dissolved in 10 ml of acetic acid:methanol, 1:19, vol/vol; brought to a concentration of 10⁻³ M with 10 ml 1 N NaOH:methanol, 1:2, vol/vol), and added to plasma to give a final concentration of 10⁻³ M. The same volume of vehicle was added to the control plasma.

Following incubation of the plasmas at 37°C for 15 and 30 minutes, with, and without pepstatin, PRA was determined by RIA.

Effect of pH on the PRA Incubation Step of Posttrypptic Plasma

To further determine whether trypsin-induced angiotensin I generation in dog plasma is related to an acid protease other than renin, the PRA incubation step at 37°C (Stage 2) was carried out both at pH 6.0 and pH 7.4, comparing the effects in human and dog plasma. Dog plasma (pool from two males) was incubated with trypsin, 3 mg/ml plasma (10 minutes, 23°C), and human plasma (pool from three men), with 0.5 mg/ml (10 min, 23°C). In each case, excess LBTI was added at the end of the 10 minutes of incubation. Activated plasmas, as well as nontryptsinized controls, were then incubated at 37°C for up to 30 minutes at pH 6.0 and pH 7.4, and PRA was determined by RIA.

Effect of Dialysis on Posttrypptic PRA in Dog Plasma

Control and trypsin-activated dog plasma (3 mg/ml, 10 minutes, 23°C, followed by LBTI addition) were subjected to 24 hours of dialysis, pH 7.4 at 4°C. All
samples, including nondialyzed controls contained DFP (diisopropylfluorophosphate, Sigma, D-0879).

Determination of Involvement of Nonserine Proteases in Dog Plasma

*Use of Inhibitor Cocktail*

In human plasma that had been pretrypsinized for 10 minutes at 23°C (Stage 1 incubation), the subsequent rate of angiotensin generation at 37°C in the PRA step (Stage 2 incubation) was relatively constant over time. In contrast, dog plasma gave greater hourly angiotensin generation rates as Stage 2 incubation time extended beyond 15 minutes. This occurred in spite of the presence of excess LBTI added at the end of Stage 1, which should have completely neutralized all continuing tryptic activity in Stage 2. To determine whether such continuing posttrypsic activity was susceptible to protease inhibitors, we added a "cocktail" of inhibitors at the end of Stage 1, consisting of: LBTI (3 mg/ml), soybean trypsin inhibitor (SBTI, 3 mg/ml, Sigma, T-9003), benzamidine (8 mM, Sigma, B-6506), and polybrene (150 µg/ml, Sigma, P-4515). The control plasma samples were treated with trypsin plus all these inhibitors, added simultaneously, allowing no time for the trypsin to act.

Transfer of the Developed Posttrypsic Prorenin Activators

Small aliquots (50 µl) of plasma samples that had been treated with trypsin (3 mg/ml) for either 5 or 30 minutes before the addition of LBTI were transferred to untreated control plasma samples (300 µl), and incubated at 37°C for 60 minutes without further pH adjustment. The PRA of all samples was determined using suitable corrections for the transferred renin activity. In this manner all newly developed renin activity attributable to "seeding" with the 50 µl posttrypsic aliquot could be accounted for.

In some cases, the posttrypsic aliquot was boiled for 10 minutes prior to incubation (60 minutes, 37°C) with fresh plasma.

**Results**

**Active Renin**

The active renin in dog plasma was considerably lower than that found typically in normal human plasma (fig. 1). The difference seems great enough not to be attributable to any subtle differences in sodium and fluid volume status due to diet.

**Cryoactivation**

Cryoactivation has been found to be most effective in human plasma at a temperature as close to the freezing point as possible — around −5°C. Generally, a temperature of −4°C has been used in human studies to avoid freezing of the plasmas. In dog plasmas we noted a tendency to freeze solid at −4°C, and therefore used 0°C for cryoactivation. The effect of up to 42 days of cold incubation of human and dog plasmas is shown in figure 2. Human PRA increased stead-

![Figure 1. Active renin in human and dog plasmas. Average PRA values (±SEM) in terms of angiotensin I, ng/ml/hr, in untreated plasmas from six healthy men and six healthy male dogs. Numbers in parentheses represent the number of determinations.](http://hyper.ahajournals.org/)

![Figure 2. Effect of prolonged cryoactivation on human and dog PRA. Average PRA values (±SEM) in plasmas from six healthy men and six healthy male dogs after incubation at 0°C for up to 42 days. All the renin indicated above the horizontal broken line is "new renin" (prorenin) due to cryoactivation of human plasma. In dog plasma, cryoactivation had very little effect, and no prorenin is singled out.](http://hyper.ahajournals.org/)
Preacidification of dog plasma for 3 hours followed by pH restoration to 7.4 and then 7 days of cold incubation resulted in some cryoactivation, but this was accompanied by a loss of active renin (fig. 3). Such loss can apparently be attributed to dialysis itself because it also occurred in samples dialyzed at pH 7.4. Overall, the effect of preacidification for 3 hours on the efficacy of cryoactivation was only modest.

Tryptic Activation

Titration of Plasma with Trypsin

Activation of prorenin in human plasma appeared maximal with 1 mg of trypsin per ml of plasma which suggests that this concentration is optimal or close to it (fig. 4). Higher trypsin concentrations tended to become destructive, and PRA decreased. In dog plasma, however, trypsin was relatively ineffective at 1 mg/ml, and maximal activation occurred at closer to 5 mg/ml, revealing a much larger prorenin component than in human plasma (50 vs 15 ng/ml/hr, fig. 4).

Time Course of Tryptic Activation

In this experiment, plasma samples were not incubated with trypsin for the usual 10 minutes but, instead, for 1 to 60 minutes at 23°C; excess LBTI was added to stop the reaction at the end of each selected interval (fig. 5). It can be seen that about 90% of prorenin activation in human plasma occurred within the first minute, as observed previously.5 Thereafter, PRA remained relatively constant for 30 minutes, before destructive effects of trypsin became evident. In contrast, the PRA continued to rise in trypsinized dog plasma for up to 30 minutes. Only about 15% of tryptic activation occurred within the first minute.

FIGURE 3. Effect of preacidification on cryoactivation of dog plasma prorenin. Prorenin, renin (shown as average PRA values ± SEM) in normal dog plasma (six male pool) resulting from 7 days of incubation at 0°C, with and without preceding acidification of plasma (dialysis at pH 3.3 for 3 hours, then at pH 7.4 for 3 hours).

FIGURE 4. Effect of varying trypsin concentrations on prorenin activation in human and dog plasmas. Average PRA values (± SEM) after activation of human plasma (six male pool) and dog plasma (six male dogs) with different concentrations of trypsin. The interval of exposure to trypsin was the same for all concentrations (10 minutes, 23°C, terminated with excess LBTI). Numbers in parentheses represent the number of determinations.

FIGURE 5. Effect of varying Stage 1 incubation — the length of exposure to trypsin at 23°C — upon the subsequent hourly rate of angiotensin generation. The plasmas were from six healthy men (pool, two determinations) and six healthy male dogs (two determinations on each plasma). In human plasma, 90% to 100% of the tryptic effect was manifest within 1 minute of Stage 1 incubation. In dog plasma, trypsin continued its influence for at least 30 minutes.
Effect of Pepstatin on Tryptic Activation in Dog Plasma

The renin inhibitor pepstatin, added to trypsin-activated dog plasma virtually eliminated the active renin component and drastically reduced the total PRA (fig. 6). Since trypsin was already neutralized by endogenous inhibitors and excess LBTI, these data imply that posttryptic increases in angiotensin I generation are truly renin-mediated, and are not the product of direct tryp tic activity on renin substrate, or some other substrate.

Effect of pH on the PRA Incubation Step of Posttryptic Plasma

When plasmas were incubated at pH 7.4 rather than the usual pH 6.0 in the 37°C PRA incubation step (Stage 2), prorenin revealed by tryptic activation was reduced to a similar extent in both human and dog plasmas (41.6% and 41.2%, respectively, table 1). There was still a marked increase in dog PRA after trypsin even when Stage 2 incubation was carried out at pH 7.4. This indicates that the enzyme responsible is active at a physiological pH, and is therefore unlikely to be an acid protease with a pH optimum as low as that of the proposed plasma cathepsins.11

Effect of Dialysis on Posttryptic PRA in Dog Plasma

Dialysis of pre- and posttryptic dog plasmas for 24 hours, pH 7.4, 4°C resulted in a decrease in PRA when compared to their nondialyzed counterparts (table 2), as also reported by Gallagher et al.11 The PRA in nontrypsinized plasmas fell from 2.5 ± 0.2 to 1.2 ± 0.1 ng angiotensin I/ml plasma/hr, while in posttrypsin samples, the PRA decreased from 28.2 ± 0.4 to 6.7 ± 0.1 ng/ml/hr. The volume of all dialysis samples increased by approximately 25% during the 24-hour period. This change was accounted for in the calculations of PRA.

Involvement of Nonserine Proteases on Prorenin Activation in Dog Plasma

Effects of Inhibitor Cocktail

After 10 minutes of incubation with trypsin at 23°C and the addition of excess LBTI, plasmas were incubated at 37°C, pH 6, to allow angiotensin I generation for various time intervals. In human plasma the computed hourly rate of angiotensin I generation turned out to be constant whether incubation proceeded for 15, 30, 45 or 60 minutes (fig. 7). In the dog, however, the hourly rate of angiotensin I generation increased with the length of incubation time. Thus, even though trypsin must have been neutralized by excess LBTI, there was continuation of some after-effect of trypsin, and the rate of angiotensin generation continued to increase, as though there was also an ongoing increase in the concentration of renin.

Such enhancement of the hourly generation rate could not be prevented by several broad-spectrum serine, or other protease inhibitors, i.e., SBTI,22 benzamidine,23,24 and polybrene,25 used individually or in combination.
Transfer of the Developed Nontryptic Activator of Prorenin

Transfer of small aliquots of pretrypsinized plasma to control untreated dog plasma provoked a 300% to 400% increase in PRA (fig. 8). It appeared that some active prorenin "convertase(s)" resulting from trypsin was being transferred to the normal plasma. Samples in which formation of this convertase had been prevented by the simultaneous addition of LBTI exhibited no such activation. It would appear, then, that this convertase activity results from the action of trypsin, but is not itself completely inhibited by LBTI or by endogenous inhibitors of dog plasma.

When the 50 μl posttrypsin aliquot had been held 10 minutes in boiling water before transfer to 300 μl control plasma, no "new" renin activity was generated. In other words, boiling destroyed the prorenin "convertase" activity.

Discussion

Research on human plasma "prorenin" has progressed rapidly in recent years. The availability of animal models now undoubtedly facilitates further advances, but unexpected hindrances have been encountered. Using conventional techniques, in our earliest work we showed that dogs appeared to have an unimpressive concentration of plasma prorenin, while rabbits and rats had even lower concentrations. It was not clear whether these findings represented true species differences or merely experimental variables such as stress, anesthesia, rapid loss by spontaneous conversion of prorenin after blood collection, or a different efficacy of trypsin as a prorenin activator among the species. Accordingly, it became necessary to investigate the activation of plasma prorenin in dogs and other animals in greater detail.

We consistently observe that the PRA of healthy, conscious resting dogs on a regular sodium diet is far lower than the PRA values of normal human volunteers. With our assay procedure, employing minimal plasma dilution and short incubation periods, normal human PRA values frequently average 5 ng/ml/hr, as is the case here (fig. 1). In comparison, normal conscious dog PRA values are about 1 ng/ml/hr. This difference could represent a true species difference, or some idiosyncrasy of fluid and electrolyte status. In addition, dog plasma may be concentrated in protease inhibi-
tors, short or of a convertase enzyme, such that prorenin conversion to renin in vivo as well as in vitro (i.e., after blood collection) is damped, resulting in a characteristically low PRA.

Whereas cryoactivation of prorenin is highly effective in human plasma, it is virtually undemonstrable in dog plasma (fig. 2). Cryoactivation at 0°C for up to 42 days produced only a trivial increase in PRA, indicating a virtual absence of conventional prorenin activation in dogs, in contrast to the 36% increase observed under comparable conditions in humans. Such a lack of cryoactivation has also been reported by Gallagher et al. after incubation of dog plasma at −4°C for 4 days.

We therefore attempted to cryoactivate dog plasma that had been previously acidified. Such brief acidification does not necessarily inactivate dog renin substrate, but it probably destroys some of the protease inhibitors in plasma, and therefore should promote cryoactivation. This was, in fact, observed experimentally (fig. 3), indicating that cryoactivation can be facilitated, but presumably not to full potential under present conditions, as compared to trypsinic activation.

It is unclear whether more prolonged acidification would have promoted additional cryoactivation without destroying substrate, but, certainly dog plasma does not lend itself to simple cryoactivation. Its content of protease inhibitors may be high, and/or one or more of the cooperating proteases may be lacking. Trypsin, used in the low concentrations that are effective in human plasma, failed to activate dog plasma prorenin significantly. Whereas trypsin concentrations under 1 mg/ml are highly effective in human plasma, and 1 to 2 mg/ml begin to be destructive in some cases, such concentrations barely activate plasma prorenin in dogs (fig. 4). The highest degree of activation in most dogs was observed with 5 mg/ml. These results are in line with those of Gallagher et al. who showed a higher PRA resulting from trypsin at 2 mg as compared with 0.6 mg/ml plasma, using a trypsin preparation with a specific activity about 30% higher than the one we used.

Gallagher et al. attributed such a raised PRA not to the activation of prorenin, but to the formation of angiotensin I by a new pathway. They suggested that trypsin cleaved native globulin renin substrate to a tetradecapeptide (TDP) substrate, which became susceptible to endogenous cathepsin D. It was from this newly formed TDP and cathepsin D that extra angiotensin was formed, raising the PRA value, and creating a false impression of prorenin activation. The TDP, being a relatively small peptide, could be dialyzed out so that lower PRA and apparent substrate values were registered after dialysis of trypticized plasmas.

Several counterarguments to this interpretation can be brought forward. First, the original demonstration of TDP production by trypsin was in horse plasma under different, more drastic, conditions, the results of which are not necessarily applicable to the study of Gallagher et al., or the present one. Second, the evidence adduced by Gallagher et al. is inferential, not actual. They did not actually demonstrate the existence of higher TDP levels, or its cleavage by cathepsin D. They only showed that dialysis depressed the PRA, for which other plausible explanations than the removal of TDP are also possible. Third, Cooper et al. showed that in human plasma, at least, TDP plays only a trivial role in posttrypsinic activation. It is unlikely that in dogs TDP would account for all such activation. Fourth, Gallagher et al. reported that "trypsin shifted the pH optimum curve towards the alkaline range" in normal (nephric) dog plasma. They did not mention that cathepsin D would be essentially inoperative at alkaline pH, and therefore was not likely to be the enzyme mediating the observed trypsinic activation. On the other hand, renin formed from prorenin would be operative at alkaline pH and is therefore the more likely mediator of trypsinic activation.

Our experimental data further weaken the TDP-cathepsin D hypothesis. We determined PRA values at pH 6.0 and 7.4 in human and dog plasmas, with and without pretryanization (table 1). As expected, PRAs in both species were higher at pH 6, which is generally regarded as a near-optimal pH for renin. However, the PRAs at pH 7.4 were also quite high, and the differences between pH 6 and 7.4 values were comparable in humans and dogs. If the operative angiotensin-generating system had been TDP-cathepsin, virtually no activity should have been detectable at pH 7.4, and the differences between pH 6 and 7.4 values should have been much more pronounced. They should also have been far greater in dogs than in humans because Gallagher et al. attribute the TDP-cathepsin D mechanism only to dogs. The fact that dogs and humans had a similar pH 6 and 7.4 differential argues against a TDP-cathepsin mechanism only in dogs.

Moreover, we have found (table 2) that, while dialysis of posttrypsinic plasma lowered the subsequent PRA by 75%, a comparable lowering of about 50% was observed in nontrypsinized plasma. In other words, at least 50% of the decline in PRA is not attributable to any postulated trypsinic alteration of substrate that renders it dialyzable. Plasma dilution (affecting substrate concentration and therefore renin-substrate kinetics), or some other unknown consequence of dialysis, may contribute to subsequent PRA reduction. Further, Gallagher et al. trypsinized plasma at 4°C for 60 minutes, as compared with 10 minutes at 23°C in our experiments. Our method of prorenin activation yields much lower 4°C RIA blank values than theirs, implying less destructive activity by trypsin, with less formation of "unphysiological" cross-reactive peptides that may, or may not, include TDP. Indeed, the apparently more destructive conditions of Gallagher et al. may have produced effects that are not at all applicable to our own experiments.

We have demonstrated (table 2) that dog plasma dialysis after trypsin depresses PRA from what it would have been if dialysis had not been carried out. Nevertheless, the postdialysis, posttrypsin PRA is 500% higher than its postdialysis nontrypsinized con-
trol. In other words, tryptic activation survives dialysis, and cannot be attributed only to TDP, which would have been removed by dialysis. Even if TDP was part of the activation process, it certainly does not account for all of it.

To further implicate renin rather than TDP-cathepsin as the enzyme that results from tryptic activation of dog plasma prorenin, we added the potent acid protease inhibitor pepstatin to plasma, just before the PRA incubation step (Stage 2), but after trypsin had been operative for 10 minutes at 23°C. LBTI, maleate buffer, angiotensinase inhibitors, and pepstatin were added concurrently (Methods, fig. 7). The results show a substantial reduction in posttrypsin PRA, which may be interpreted as an inhibition of renin.

Pepstatin inhibits hog cathepsin D at Ki < 5 × 10^{-10} M, and rat renin at Ki approximately 6 × 10^{-7} M at pH 5.5, 29, 30 and could therefore be expected to inhibit dog renin at our much higher concentration of 1 × 10^{-5} M at pH 6.0 (see Methods). Pepstatin is known to be effective against dog renin 31 which has a similar amino-acid composition to that of hog renin, 32 to which pepstatin has an even greater affinity than to rat renin. 32

As indicated earlier, the enzyme activity produced by tryptic activation of dog plasma prorenin expressed itself strongly in terms of angiotensin I generation at a pH of 6.0, with 60% of the activity remaining at the pH of 7.4 (Table 1). Such activity is well beyond the effective pH range for cathepsin D. 29, 30 In addition, cathepsin D would probably have been more powerfully inhibited than renin by endogenous inhibitors in a plasma incubation mixture. 29, 30 This combination of experimental observations favors renin rather than cathepsin D as the main effective derivative of tryptic activation in dog plasma.

Evidence of a distinctive prorenin activation cascade in dog plasma is derived by contrast with human plasma. In humans, the action of trypsin is very rapid in Stage 1 at 23°C (fig. 5). 4 Delaying the addition of the trypsin inhibitor LBTI at intervals beyond 1 minute does not add to the activation process, as deduced from the hourly rate of angiotensin generation in Stage 2 at 37°C. Thus, essentially all the activation at a given concentration of trypsin has been completed within 1 minute of adding trypsin in Stage 1. This activation is expressed, but not further amplified, in Stage 2.

In contrast, the length of preincubation of dog plasma with trypsin (Stage 1) greatly influences the subsequent PRA values in Stage 2. Specifically, incubation with trypsin for 5 minutes (Stage 1) results in a subsequent hourly PRA of 32 ng, while a Stage 1 incubation of 30 minutes almost doubles the subsequent hourly PRA to a value of 55 ng (fig. 5). Also, the activating effect of trypsin is not restricted to Stage 1. Its action continues during Stage 2, and is not preventable by the inhibitors LBTI, SBTI, benzamidine, and polybrene (Results — "inhibitor cocktail"). Since both trypsin and kallikrein would have been blocked by these inhibitors, it follows that some other enzyme(s) was activating prorenin. In other words, trypsin had initiated a cascade effect, which continued even after trypsin or trypsin-generated kallikrein (from prekallikrein) had ceased to act directly. One or more different enzymes, not entirely inhibited by LBTI or the cocktail, were continuing to activate prorenin.

The nontryptic enzyme(s) responsible for ongoing activation of prorenin is obviously potent, and once formed, apparently resistant to the endogenous protease inhibitors of normal plasma. Aliquots as small as 50 µl of posttrypsin plasma incorporated into 300 µl of normal plasma provoked a marked increase in PRA (fig. 8). The greatest increase in PRA was provoked by the aliquot of transferred plasma that had been previously exposed to trypsin for the longest time (fig. 8, right bar). In other words, longer exposure to trypsin results in an increased formation of the prorenin activating enzyme(s) or convertase(s), which is therefore transferred in greater concentration to the normal plasma, causing it to have a higher PRA. We found that the "convertase(s)" is denatured by heat, i.e., boiling for 10 minutes, implicating an enzymic system.

The identity of the posttrypsin "prorenin convertase" is unknown. Enzymes of the coagulation and fibrinolytic cascades may be involved. 9 However, autocatalysis by renin itself in a manner analogous to another acid protease, i.e., pepsin acting on pepsinogen, 34, 35 cannot be ruled out. High renin concentrations are present in the reaction vessel, the enzyme is operative at the pH levels favoring renin, and is inhibited by pepstatin. But there may also be other activators. Barrett et al. 36 have suggested a prorenin activator in rat plasma that is neither a serine protease nor renin. They showed that, after acid dialysis to pH 1.5 while renin and renin substrate were inactivated, an "acid-stable factor" retained activity and, when the dialysate was added to normal rat plasma at pH 7.4, it activated prorenin. Thus, even if renin itself is a prorenin activator, some other activator(s) must also be considered for rat, and probably also dog plasma.

In conclusion, we have shown that there is a large prorenin component in dog plasma that can be activated by trypsin, but not by cold alone. The activation by trypsin does not appear to be direct, but is mediated by some other agent(s). Despite differences between the plasma prorenin-renin systems of dog and humans, our study suggests that the dog can be used as a model for studies of prorenin-renin. In addition, the distinctives of the dog prorenin-renin system can be exploited to define mechanisms of activation, which are obviously more complex and species-specific than previously expected.

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PLASMA PRORENIN IN HUMANS AND DOGS/Wilczynski and Osmond 285

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