Alternate Angiotensin II-Forming Pathways and Their Importance in -Physiological or Physiopathological Conditions

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For a long time, the renin-angiotensin-aldosterone system was considered a component of the endocrine system because angiotensin II, the main effector of this system was thought to be generated exclusively in blood, thereafter being distributed to all organs and tissues by the blood flow and becoming active on those targets that possessed the appropriate receptors for this peptide. Recently though, this model had to be revised, because it was discovered that all the components of this system, particularly angiotensinogen, renin, and angiotensins I and II, can also be locally produced in several organs and tissues[1,2]. Since this discovery, angiotensin II has also been considered a peptide with paracrine, autocrine, or both paracrine and autocrine action in several places in an organism. Thus the concept was established of the existence of several renin-angiotensin systems distributed throughout different organs (heart, blood vessels, adrenal marrow, central nervous system, and others), whose actions complement those of the classical renin-angiotensin-aldosterone system. The importance of these local renin-angiotensin systems seems to be related to the fact that they may have a direct effect on local regulation mechanisms. This direct effect may contribute to a great number of slower progressing, yet longer lasting, tissue homeostasis mechanisms, such as cell growth, the formation of tissue matrix, vascular proliferation, endothelium function modulation, and the control of the apoptosis process, particularly during embryonic development.

The angiotensin-converting enzyme kininase II plays an important role in the generation of angiotensin II, whose action is systemic, because renin, which is produced in the juxtaglomerular system, is released into the blood to act there on its specific substrate, angiotensinogen. By this enzyme reaction, angiotensin I is formed. The conversion of angiotensin I into angiotensin II is carried out both by the angiotensin-converting enzyme-found in the vascular endothelium and by the plasma-soluble enzyme, which comes from the endothelial cell membrane. However, angiotensin II can also be generated from angiotensin I by a number of other peptidases capable of cleaving the $Phe^{8}His^{9}$ bond of the decapeptide (fig. 1). The formation of angiotensin II by peptidases, which are different from the angiotensin-converting enzyme, has been referred to in the literature as alternate angiotensin II-generating pathways. Several studies have shown that these alternate pathways might be more important for the formation of angiotensin II on a tissue level, ie, they might be more important where angiotensin II has paracrine, autocrine, intacrine effects, or effects of all three of these.

The first biochemical description of one of these pathways was made by Boucher et al [3], who showed that tonin obtained from rat salivary glands could cleave angiotensin I, thus generating angiotensin II. This initial finding was followed by Arakawa et al [4] for trypsin and kallikrein[5], Tonnesen et al [6] for cathepsin G, and Wintroub et al [7] for a chymotrypsin found in human and rat skin. So it can be seen that several proteases found in many parts of the body can generate angiotensin II from angiotensin I or from angiotensinogen itself.

More recently, a serine-proteinase that is also capable of cleaving angiotensin I into angiotensin II, called chymase, has been the object of major attention, because of its great importance as an alternate angiotensin II-generating pathway in several places, particularly in the heart and in the blood vessels. Schechter et al [8] were the first to identify chymase in human skin mast cells. Actually, in vitro studies had already shown the existence of a serine proteinase similar to chymotrypsin, able to form angiotensin II, in the hamster cheek pouch [9] and in the cat papillary cardiac muscle [10].Even though these authors did not identify this enzyme as being responsible for the formation of angiotensin II in those places, strong evidence exists of its actually being a chymase. Recent studies detected angiotensin II-forming activity by the chymase pathway in several tissues of different species. However, many doubts still exist concerning the physiological and physiopathological role of this enzyme. A more consistent mapping of these pathways is becoming increasingly important, mainly now that specific antagonists of angiotensin II AT_1 receptors, such as losartan, have been

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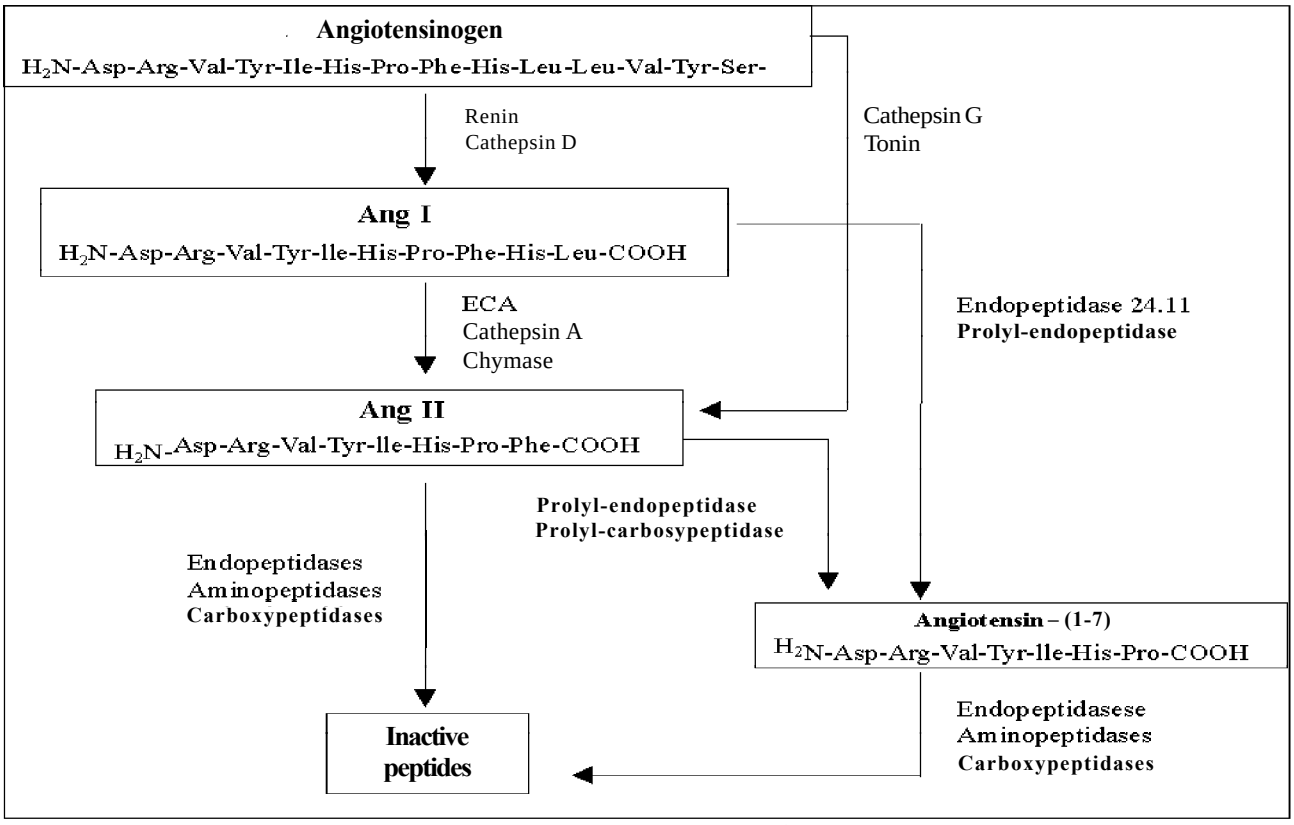

Fig. 1 – Angiotensin-converting-enzyme(ACE)-dependent and -independent pathways in the generation of angiotensin II. Observe that the angiotensin-converting enzyme and chymase can act on the same substrate (angiotensin I) to generate angiotensin II, which can also be formed directly from angiotensinogen through the cathepsin G and tonin pathway.

developed, because this new class of drugs has allowed broadening of the range of therapeutic interventions in the renin-angiotensin-aldosterone system. Even if the inactivating role of the angiotensin-converting enzyme bradykinin is disregarded, if angiotensin II were generated by the angiotensin-converting enzyme alone, a noteworthy overlapping of effects of the angiotensin-converting enzyme inhibitors and the AT_1 blockers would be expected. Otherwise these 2 classes of drugs would have to have more specific effects and not entirely superimposable therapeutic indications.

Chymase in the heart - Chymase was first identified in the human heart by Urata et al [11], who showed, with experiments carried out on human myocardium homogenate, that chymase was responsible for approximately 80% of the angiotensin II formation in the human heart. This finding brought about a certain degree of interest and controversy. In vitro studies carried out by Balcells et al [12] on fragments of dog hearts also showed chymase to be the main angiotensin II-forming enzyme in this species. Yet the measurements made in vivo on the same species showed the angiotensin-converting enzyme to be the main angiotensin II-forming cardiac enzyme. The authors attributed those differences to the distinct physical compartmentalization of the 2 enzymes, because this factor affects the access of the substrate (angiotensin I) to the enzymes. Chymase is preferentially located in cytoplasmatic mast cell granules and in the cardiac interstitium [13]. The angiotensin-converting enzyme, on the other hand, is located mainly in the endothelial cells, with the catalytic sites exposed to the vascular surface [14]. Therefore, in circumstances that are closer to the physiological ones, ie, in the in vivo experiments, the angiotensin-converting enzyme would have greater access to the angiotensin I present in plasma, ie, in the circulating medium. On the other hand, access to the plasmatic substrate would be more difficult for chymase, because of its predominantly interstitial location. When these data are analyzed, it is important to consider that, in the experiments done in vitro, the homogenizing process leads to the rupture of the compartments that exist under physiological conditions, ie, in vivo. Because the specific angiotensin I-hydrolyzing activity of chymase is greater than that of the angiotensin-converting enzyme [11], the experiments performed with a homogenized heart might tend to show a predominance of chymase over the angiotensin-converting enzyme with regard to its angiotensin II-generating capacity. These data show how difficult it is to transpose, without a more detailed analysis, in vitro findings to an in vivo situation. This argument seems logical. Yet, when Zisman et al [15] quantified the chymase and angiotensin-converting enzyme activities in a membrane fraction obtained from human myocardium homogenates, ie, using a preparation similar to that used by Urata [11], they obtained opposite results: the angiotensin-converting enzyme seemed to be the main angiotensin II-forming enzyme in the human heart, and not chymase. Later on, Wolny et al [16] discovered that the preparation of homoge-

nate with high concentrations of detergent, like the one used by Zisman et al [15], leads to a greater loss of chymase activity, and this might be an explanation for the low activity that was detected for this enzyme. The differences reported in the earlier papers still seem far from being finally settled because more recently Balcells et al [17], as they were trying to clarify the controversies created by those investigations, showed that chymase is likely to be the main angiotensin II-forming enzyme in the human heart, even when the homogenate is prepared with a high concentration of detergent. So it becomes evident that a small modification in the methodology used, or even different technical artifacts, can significantly alter the activity of these enzymes. This certainly makes it more difficult to understand the true contribution of the angiotensin II-forming pathways, whether dependent or independent from the angiotensin-converting enzyme, and how the specific activities of these pathways might change under physiopathological conditions. Unlike angiotensin-converting enzyme, no specific inhibitor for chymase exists, which makes it even more difficult to solve the doubts still left in this field.

The data available to date seem to indicate that the angiotensin-converting enzyme could doubtlessly be the enzyme with a predominant role in the processing of the substrate (angiotensin I) coming from plasma, given the fact that the highest concentration of this enzyme is found in the vascular endothelium. With regard to the angiotensin II locally generated in the heart, it is presently impossible to define this matter precisely. The modifications in the expression of enzymes that occur in physiopathological situations have to be taken into account. In an infarction, for example, a great increase occurs in the activity of the detected angiotensin-converting enzyme, both in the rat heart homogenate [18] and in the human heart [19]. This increase seems to be the result of the fact that, during the cicatrization process, a great amount of young myofibroblasts and fibroblasts accumulate in the lesion area and in the adjacent myocardium, and these cells express great amounts of the angiotensin-converting enzyme [20]. The prevalence of the angiotensin-converting enzyme activity in the generation of angiotensin II in a postinfarction heart is supported by the fact that the AT_1 antagonists, such as losartan, do not have better ventricular remodeling effects that those obtained by the angiotensin-converting enzyme inhibitors [19]. Recent multicenter studies, like ELITE phase II [21], support this impression.

Chymase in the blood vessels – The first studies in this field were conducted by Cornish et al [9], who discovered that the vasoconstriction induced by angiotensin I in the blood vessels of the hamster cheek pouch was partially inhibited by the angiotensin-converting enzyme inhibitors, and completely inhibited by an antagonist of the angiotensin receptor or by angiotensin II antibodies. The characteristics of the enzyme or enzymes responsible for the conversion of angiotensin I into angiotensin II were then totally unknown.

Between 1984 and 1990, Okunishi et al [22,23] carried out studies of fundamental importance, attempting to clarify the role played by the alternate angiotensin II-forming pathways in the blood vessels. In their first, biochemical studies, they observed the presence of an enzyme responsible for most of the conversion of angiotensin I into angiotensin II in the blood vessels of humans, monkeys, and dogs [22,23]. This enzyme was not inhibited by the angiotensin-converting enzyme inhibitors but by serine proteinase inhibitors, like chymostatin. This enzyme was called CAGE (chymostatin-sensitive angiotensin II-generating enzyme). Although the enzyme had not been identified, it was believed that it was a chymase because of its similarities to the chymase previously isolated from other tissues. Thus, just like in the heart, evidence was collected about a double angiotensin II-formation pathway in the blood vessels, where chymase seemed to contribute more than the angiotensin-converting enzyme to the conversion of angiotensin I into angiotensin II. These findings were later supported by studies made on isolated human blood vessels, in which Okunishi et al [24] showed that chymostatin blocked approximately 80% of the contractile response induced by angiotensin I, whereas captopril blocked only about 40% of this response. The association of the two inhibitors (captopril + chymostatin) determined the complete block of the contractile response. However, in a number of investigations conducted during this period, other researchers were unable to reproduce these findings. They were generally unable to detect alternate angiotensin II production pathways in the blood vessels, for the contractile response to angiotensin I was completely blocked by the angiotensin-converting enzyme inhibitors [25-27]. Considering the simplicity and the widespread use of such preparations, it seemed unlikely that the technique could be the responsible factor for these differences. Later, Okunishi et al made a careful review of the research works that had failed to confirm their original observations. They found then that all the investigations in which no alternate angiotensin II-production pathway was detected had been performed with rodent (rat or rabbit) blood vessels, differently from their own, where human and monkey blood vessels had been used. Confirming their original data, Okunishi et al [23] showed that captopril blocked only 30% of the conversion of angiotensin I into II in the human gastroepiploic artery, whereas chymostatin, a chymase inhibitor, blocked 80% of this conversion. On the other hand, in rabbit arteries, captopril determined an inhibition of over 90% in angiotensin II formation, whereas chymostatin had virtually no effect at all. The authors believed their observations justified the fact that the angiotensin-converting enzyme inhibitors were unable to prevent the arterial proliferation response secondary to the arterial lesion in primates [28], the opposite of what was observed in rats [29]. In this case, the therapeutic implications concerning the use of inhibitors of the alternate angiotensin II-formation pathways seem to be obvious.

More recent studies have shown how important the alternate angiotensin II-formation pathways may become in the development of vascular diseases. Ihara et al [30] reported a marked increase in the angiotensin II-forming activity in

atherosclerotic human aorta sections and in aortas with an aneurysm. Chymase was the main responsible enzyme for this increase (about 80 to 90%). Yet, although these data suggest a major contribution of chymase to the development of these diseases, the mechanisms of action of this enzyme are still not entirely clear, for they seem to depend not only on the increase in angiotensin II formation, but also on the increase in the proteolytic activity of chymase on the cell membrane [30,31].

Actually, other observations also support the hypothesis that chymase might play a relevant role in the development of vascular diseases. One of them is the fact that this enzyme lies preferentially in the vascular adventitia, whereas the angiotensin-converting enzyme is found in the endothelium and macrophages of the neointima [28]. The involvement of the adventitia in the progression of balloon or atherosclerosis-induced vascular lesions is currently known to be quite relevant [32,33]. In fact, recent studies show an increase in the expression of the chymase gene in atherosclerosis [34] and in balloon-injured arteries [35]. Both the activation of the chymase gene expression in injured arteries and the formation of the neointima and luminal stenosis are suppressed by drugs that prevent the degranulation of mast cells. In spite of the fact that these cells contain other mitogenic components, it seems valid enough to conclude, based on the collected experimental evidence, that chymase seems to contribute significantly to the vascular proliferation processes, both in atherosclerosis and after lesions caused by intraarterial devices.

Differences between species in the generation of angiotensin II – As seen earlier, a great debate is still under way concerning the quantification of the activity of the angiotensin II-forming enzymes in different tissues and species. Angiotensin II has a positive chronotropic and inotropic effect in the dog [36], hamster [37] and cat heart [38]. However, this effect is not seen in the adult rat and guinea pig [39]. In vitro studies of the myocardium of different species show that the angiotensin-converting enzyme is the main angiotensin II-forming pathway, the exceptions to this rule being the human [11,16,17], hamster [37] and dog hearts [40]. In view of this fact, caution has to be doubled when extending to the human heart data obtained from other species. It is worth pointing out, for example, that the rat has been the most commonly used animal model in investigations on the cardiac renin-angiotensin system. Yet, recent studies carried out with this animal prove that its angiotensin-converting enzyme and chymase activity profile is quite different from the one found in man [41]. Okunishi et al [23] had already mentioned this point in their studies on blood vessels. The chymase existing in the rat has to be considered, a priori, as an angiotensinase, for it hydrolyzes angiotensin I, producing inactive peptides [42]. The diversity of the enzyme with regard to its preferential substrate can be better understood starting from phylogenetic data [43]. Studies of that kind showed that chymase is expressed in mammals in 2 different forms named α and β, which differ in their substrate specificity. The α-chymases are angiotensin II-forming enzymes, because they hydrolyze the Phe ^{8}His ^{9} bond of angiotensin I. Chymase found in man and dog, mouse chymase-5, and gerbil and rat chymase-3 belong to the group of α-chymases. On the other hand, β-chymases are angiotensinases, for they hydrolyze the Tyr ^{4}Ile ^{5} bond of angiotensin I, producing inactive peptides. Rat chymases-1 and -2 and mouse chymases-1, -2, -3 and -L are β-chymases. The numbering system proposed for the chymases indicates different isoforms of this enzyme found in these species. Thus, the rat isoform, classified as chymase-3, forms predominantly angiotensin II, but isoforms -1 and -2 degrade angiotensin I to inactive peptides. This shows that the biochemical heterogeneity of this enzyme in mammals makes it difficult to choose the most adequate animal model for the study of the angiotensin II-generating pathways in man.

Recently, Akasu et al [41] showed that none of the species they studied (dog, hamster, rat, rabbit, and marmot) had in their lung, aorta, and heart a balance of chymase and angiotensin II-converting enzyme activities identical to that found in the respective human organs. However, when each organ is considered in isolation, an animal model exists whose activity has a greater similarity to the corresponding human organ. The differences detected among species may be due to the subclass, density, and heterogeneity of each cell, thus bringing about a differential enzyme distribution and, as a consequence, alterations in the tissue enzyme activity. The cellular distribution of chymase in the human heart was determined by immunoreactivity, showing that this enzyme is found in mast cells, endothelial cells, and mesenchymal cells [44]. Yet, no systematic study of the cellular distribution of chymase mRNA and immunoreactivity in animals exists to date. In rodents, chymase was detected only in mast cells [45]. It is therefore believed that the increased activity of chymase in a certain tissue or organ might be associated mainly with the local density of mast cells. These, in turn, also have considerable differences in different species and tissues [46]. Mast cells are classified into 2 groups: those containing tryptase (M_T) and those containing tryptase+chymase (M_{TC}) [47]. Under certain circumstances, changes in the proportion of these 2 types of mast cells may occur, and, consequently, in the metabolizing capacity of angiotensin I by the chymase pathway.

Studies in the intact kidney – To determine the role played by the angiotensin II-forming pathways in the dog kidney, Murakami et al [48] used a synthetic peptide that functions as a specific substrate for chymase. This substrate has 2 amino acids coupled to angiotensin I, preventing it from binding to the angiotensin-converting enzyme: proline at position 11 and D-alanine at position 12 (pro11, D-ala12-angio I). The renal vasoconstriction response to the artificial substrate was similar to that produced by angiotensin I, indicating that chymase activity also contributes in vivo to the formation of angiotensin II in the kidney. These data were supported by other studies performed in vitro, showing that approximately 80% of the angiotensin II-forming activity in the renal cortex depended on the angiotensin-

converting enzyme, and the other 20% on a chymase-dependent pathway. As can be observed, this proportion is different from the one measured, also in vitro, in the heart, where chymase activity is predominant[11]. Unfortunately, so far no comparative biochemical studies have been carried out in renal blood. Consequently, it is still unknown whether the same proportions as detected in vitro for the angiotensin I-converting activity apply to the in vivo situation or not.

Some evidence exists showing that, as it occurs in the heart and in the blood vessels, other enzymes besides the angiotensin-converting enzyme take part in the formation of angiotensin II in the human kidney. Cordero et al[49] performed a study on patients ingesting a low salt diet (10 mEq Na/day), aiming at activating the renin-angiotensin-aldosterone system. They used captopril or enalkiren, a renin inhibitor, to block the generation of angiotensin II. Surprisingly, the renal vasodilation response to enalkiren was superior to that produced by captopril. In fact, these results were unexpected, because the inhibition of the angiotensin-converting enzyme, in addition to reducing the circulating angiotensin II levels, also diminishes the breakdown of bradykinin and angiotensin 1-7, which are vasodilating peptides [50]. Renin inhibitors, instead, are highly specific and will therefore not alter the circulating levels of these peptides by inhibiting renin. To prove the participation of alternate angiotensin II-production pathways in the kidney, the same authors used two AT_1-receptor antagonists, eprosartan and irbesartan. This study defined the relation between the antagonist dose and the renal vasodilation response. It was observed that both antagonists induced a response that exceeded that seen after renin inhibition[51,52]. These results suggest the existence of renal angiotensin II-forming pathways that are independent from the angiotensin-converting enzyme, but nevertheless greatly dependent on renin. From comparative analyses, it seems likely that the renal enzyme responsible for the formation of angiotensin II might be chymase or enzymes similar to chymase. Based on this, it can be seen that renal lesions resulting from the interaction of angiotensin II could be better controlled by AT_1 blockers or by renin blockers than by angiotensin-converting enzyme inhibitors.

Chymase in cardiomyopathies - Angiotensin-converting enzyme inhibitors are widely used for treating hypertension and congestive heart failure. Although the mortality and morbidity rates of these patients, mainly of those with a myocardial infarction, have dropped considerably ever since these drugs were introduced to treat heart failure, they are still considered rather high. In fact, the studies show that the chronic use of angiotensin-converting enzyme inhibitors produces a rather modest reduction in angiotensin II plasma levels, the same thing being observed in other tissues[53,54]. After a certain time of using angiotensin-converting enzyme blockers, the circulating angiotensin II levels may even be higher than before the beginning of treatment, a phenomenon known as escape of angiotensin-converting enzyme inhibitors. The inadequate suppression of angiotensin II generation seems to be associated with a progressive worsening of heart failure [55]. Other enzymes, different from the angiotensin-converting enzyme, appear to be responsible for the maintained angiotensin II formation under such circumstances. When the previous data were analyzed, it was found that chymase might play a fundamental role in the progression of ventricular remodeling and of heart failure under these circumstances. In addition to this, the localization of chymase in the heart is suggestive. Immunolocalization studies show that this enzyme is more concentrated in the left ventricle, preferentially in mast cells and in the cardiac interstitium [44], which could explain its involvement in several pathogenic processes in the cardiovascular system, because an increased mast cell density occurs in different physiopathological conditions involving this system [56,57].

Noda et al [58] observed that the increase in angiotensin II concentrations in the coronary sinus of dogs, after ligation of the anterior descending coronary artery, was not prevented by the angiotensin-converting enzyme inhibitors, but by serine proteinase inhibitors like aprotinin and chymostatin, thus suggesting that these enzymes could be strongly involved in the acute formation of angiotensin II in the ischemic heart. Associating these results with others, several authors believe that, because it occurs with the expression of the angiotensin-converting enzyme, certain stimuli, such as ischemia or a mechanical injury, may be required for the expression or release of chymase in the heart and blood vessels [59-62]. In addition to this, the presence of mast cells around the coronary artery and in the coronary atheroma, especially in patients with ischemic heart disease [56], could explain the increased formation of angiotensin II during infarction. Daemen and Urata [63] showed in human heart tissue that the distribution of chymase changes during the infarction. Using immunoreactivity techniques, they observed that in the normal heart chymase is found in a higher concentration in cardiomyocytes and in endothelial cells. Six hours after infarction, a loss occurs in immunoreactivity in the ischemic cardiomyocytes, concomitantly with a great increase in the scar region. The increase of chymase in this region is certainly due to the migration of macrophages, mast cells, and myofibroblasts to this region, an event similar to that observed concerning the angiotensin-converting enzyme [18]. So, these data show that the activity of chymase, as one of the angiotensin-converting enzyme, may be enhanced in the postinfarction heart. The pH lowering occurring during the ischemic episode can also be one of the factors that contribute to enhancing the importance of chymase in the local generation of angiotensin II, because this enzyme operates well in the pH range going from 7.0 to 10.0, whereas the angiotensin-converting enzyme is active within a much narrower range, from 7.5 to 8.5 [11,64]. The combined use of the AT_1 antagonist and of the angiotensin-converting enzyme inhibitor could be justified in this condition, because many data exist suggesting that the increased angiotensin II generation in the postinfarction heart contributes to ventricular remodeling and worsens with the development of heart failure. In fact, some studies already show

the benefits of this combined therapy (captopril+losartan) in the early phase of infarction, as compared with the isolated treatment with angiotensin-converting enzyme inhibitor[65].

Chymase inhibition - This is currently one of the greatest problems facing the study of chymase activity, for no specific inhibitor of this enzyme exists so far. Consequently, the physiological or physiopathological function of chymase is difficult to distinguish.

Recent studies have shown that, in vivo, chymase is complexed with heparin, which makes the inhibition of the enzyme by exogenous agents more difficult. To bypass this problem, most of the in vitro studies related to chymase activity were made with purified enzyme, ie, after the removal of heparin. Consequently, the results obtained in vitro may also not reflect exactly the phenomena occurring in vivo. In fact, Kokkonen et al[66] observed in vitro studies that α-antitrypsin blocked the activity of purified cardiac chymase completely, and that this enzyme probably plays no relevant role in the intact organism. Parallel to that, Takai et al's[67] biochemical studies of human vascular tissue showed that, when chymase is bonded with heparin, α-antitrypsin practically does not inhibit enzyme activity. So, heparin seems to protect chymase against certain types of inhibitors, like α-antitrypsin, which leads us to suppose that, in vivo, heparin may play a fundamental role in the regulation of chymase inhibition.

Considering the data available so far, we conclude that it is still rather difficult to visualize in a reliable manner how the different angiotensin II-forming pathways interact in vivo. The availability of a specific chymase inhibitor with easy access to the enzyme in the intracellular environment is today an indispensable requirement for better clarification of such an important matter, so as to make it possible for the therapeutic interventions with angiotensin-converting enzyme inhibitors, angiotensin antagonists, or inhibitors of other enzymes (chymase, tonin, renin, etc.) to find a more precise therapeutic indication.

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