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Renin and Aldosterone

Measurements of Renin-Angiotensin-Aldosterone*

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FROM a physiological point of view the renin-angiotensin-aldosterone system seems to be part of a chain of interrelations between kidneys, blood vessels, adrenals, and the central nervous system. The system influences the salt and water balance of the organism and has a possible role in the maintenance of normal blood pressure (51).

Since angiotensin is the most potent pressor substance present in the organism, it is of further interest to determine whether this system has a pathogenic role in renal and essential hypertension. Although the present knowledge of the renin system and its relation to arterial hypertension has been developing since 1930, the first sufficiently sensitive and precise methods for measurements of renin in plasma were not designed until 1963 (37).

The renin system outlined in figure 1 consists of a series of consecutive enzymatic reactions in which angiotensin II, the biologically active substance, is present as an intermediate product. The renin system is complex, and the concentrations of the component enzymes and substrates vary over narrow ranges, thus it is necessary to measure the parameters accurately, simultaneously, and independently in order to study the system.

Angiotensin

Since angiotensin is the only pure component of the system, radioimmunoassay can only be directed against this. The first antibodies against angiotensin were elicited by Deodhar (21) in 1960 by coupling angiotensin to γ-globulin with a diazo linkage. Radioimmunoassay, however, was first described by Haber *et al.* (36) in 1965.

The specificity of the assay depends on the antibody chosen but generally anti-angiotensin does not react with peptides other than angiotensin. Antibodies against angiotensin II also react with angiotensin I and anti-angiotensin I reacts with angiotensin II. In both instances, however, it is not difficult to find antisera which cross react less than 1% which means that one can in practice obtain assays specific for either angiotensin I or II (14, 38). When measuring the biologically active compound of the system, angiotensin II, in plasma one is faced with the problem that angiotensin II is an intermediate product rapidly degraded by enzymes. There will therefore always be metabolites of angiotensin II present in plasma, and unfortunately the antibody cross reacts with these to a considerable extent. The important cross reaction is to the heptapeptide $Arg^2 \ldots . Phe^8$ and the hexapeptide $Val^3 \ldots . Phe^8$ (10–12, 14, 17, 22–24, 70). The important question is therefore how large a fraction of the total immunoreactive material is angiotensin II. Cain *et al.* (10, 12) find that the fraction of the total immunoreactive material which is angiotensin II is 85% in human arterial blood and 96% in sheep arterial blood (14) as compared with only 28% in venous blood. The remaining fraction is metabolites of angiotensin II. In spite of this, a good correlation is found in both these studies

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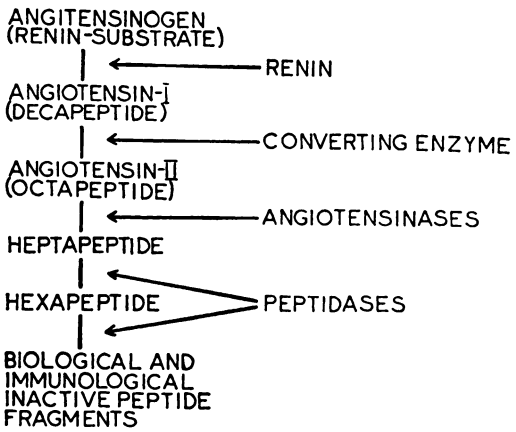

FIG. 1. Diagram of the renin-angiotensin system.

between the total immunoreactive material in arterial and venous blood; the venous concentration is 60 to 75% that of the arterial. This probably results because metabolites have a very short half-life in blood and thus are not accumulated. A determination in arterial blood should, therefore, be a good measure of the actual angiotensin concentration. A determination of the total amount of immunoreactive material in venous plasma (angiotensin II plus metabolites) might be a useful approximation of the arterial concentration of angiotensin II (12, 25). In addition to Cain *et al.*, Pernollet *et al.* (52) have described a thin-layer chromatographic method for the separation of angiotensin I from its biologically inactive but immunoreactive split products.

Methods for determination of angiotensin II in plasma by radioimmunoassay are described by several authors (7, 8, 13, 15, 16, 25, 28, 29, 44, 65, 69).

Plasma Renin Activity

From figure 1 it can be seen that several other parameters are of interest, but all must be quantitated from angiotensin I or II concentrations, the only parameters directly measurable.

All the components are present in plasma, and renin conversion of its substrate to angiotensin I is the rate limiting step. If we now block the consecutive reaction after the formation of angiotensin I, this product will accumulate. Such a blockage can be performed by adsorption of the angiotensin I formed to a resin, as in the method of Boucher *et al.* (5) or by adding a mixture of enzyme inhibitors which block the angiotensin I to angiotensin II converting enzyme and angiotensinases, as in the method of Haber *et al.* (35), the two most widely used methods. The most used inhibitors of angiotensinases in plasma are ethylenediaminetetraacetic acid (EDTA), diisopropylflourphosphate (DFP), dimercaprol, BAL, and 8-OH-quinoline and usually a mixture of several of these (41). Methods for determination of plasma renin activity have been published by many authors (5, 6, 9, 19, 35, 37, 39, 53, 63, 66). The parameter which is measured is the rate of angiotensin I generation, the so-called plasma renin activity. This is unfortunate terminology since the plasma renin activity is often misunderstood to be the enzymatic activity of renin. This is not the case since the reaction rate also depends on the plasma renin substrate concentration.

The Importance of the Substrate Concentration

The substrate concentration influences the reaction rate in two ways, which in the literature are often found mixed. 1) The angiotensin generation rate is dependent on the initial concentration of the renin substrate, in addition to the renin concentration. At very low concentrations of renin substrate, the reaction rate of angiotensin formation is proportional to the substrate concentration. By increasing the concentration of substrate, the angiotensin generation rate increases and reaches a maximum at very high renin substrate concentrations. The reason for this is that renin will be saturated with substrate and the reaction proceeds with zero order kinetics with respect to substrate. The initial velocity at so high a concentration of substrate (the maximum velocity) is only dependent on the enzyme concentra-

tion. 2) Furthermore, the substrate concentration influences the reaction rate because of the decrease of the substrate concentration with time during the reaction caused by the enzymatic splitting of the substrate. This means that the reaction rate decreases with time. If the reaction time is short, the consumption will be small in relation to the initial substrate concentrations and the initial velocities are measured.

The endogeneous concentration of substrates in plasma is not nearly high enough to give zero order kinetics with respect to substrate concentrations (30, 32, 54, 61). This means that the velocity by which a certain plasma forms angiotensin (plasma renin activity) is a parameter determined by the concentration of renin as well as by the substrate concentration in this plasma. However, the influence of the substrate concentration on the generation rate of angiotensin is not the same in all species. In normal human beings, for example, the endogenous substrate concentration approaches the substrate concentration required for zero order kinetics with respect to substrate (30, 32, 54, 61). This means that a decrease in the substrate concentration to half, with unchanged renin concentration, results in a decreased angiotensin generation rate, which is greater than half of that observed with the higher concentration. In pathological conditions, as in certain liver diseases, the substrate concentration can be extremely low, while the renin concentration in the plasma is increased (31). In the rat, for example, the substrate concentration is equally as important as the renin concentration in determining the rate of angiotensin formation (first order kinetics with respect to substrate concentration) for all normal and some pathologically increased concentrations of substrate (58).

Renin Concentration

If the renin concentration is the parameter to be measured, the influence of the variations in substrate concentration must be eliminated. This is in practice done in three different ways. 1) Addition of excess substrate to the plasma so that the total renin substrate concentration is far higher than that required to saturate renin. The reaction rate thus becomes independent (zero order) of variations in substrate concentration while still proportional to the concentration of renin (4, 31, 42, 43, 47, 62–64). 2) By determining the renin activity (angiotensin generation rate) of the plasma with and without a series of known concentrations of renin added (fig. 5B). From the increase in reaction rate caused by the added renin the endogenous renin concentration can be calculated and expressed in terms of international units. This principle has been introduced by Haas *et al.* (34) and has been found very useful in comparing different assays in different laboratories (27, 33, 34, 57). International standards for renin and angiotensin I and II are now available from the National Institute for Medical Research, Division for Biological Standards, Mill Hill, London. 3) A third principle is to determine the substrate concentration and its change with time and from this to calculate the renin concentration (54–56). This principle is especially valuable in animal experiments where the renin K_M is relatively high, and it gives three parameters simultaneously: the renin substrate concentration, the renin activity, and the renin concentrations.

Renin Inhibitors

One of the most discussed issues concerning the generation rate of angiotensin I is whether or not renin and renin substrate are the only parameters which determine this rate. In other words, do physiological *inhibitors* or *activators* exist? Bumpus and co-workers (50) have found evidence for such an inhibitor of renal origin in plasma treated in order to eliminate plasma angiotensinases. If, however, the reaction rate is studied in non-pretreated plasma (58) with all the enzyme systems intact including the angiotensinases, no evidence for active

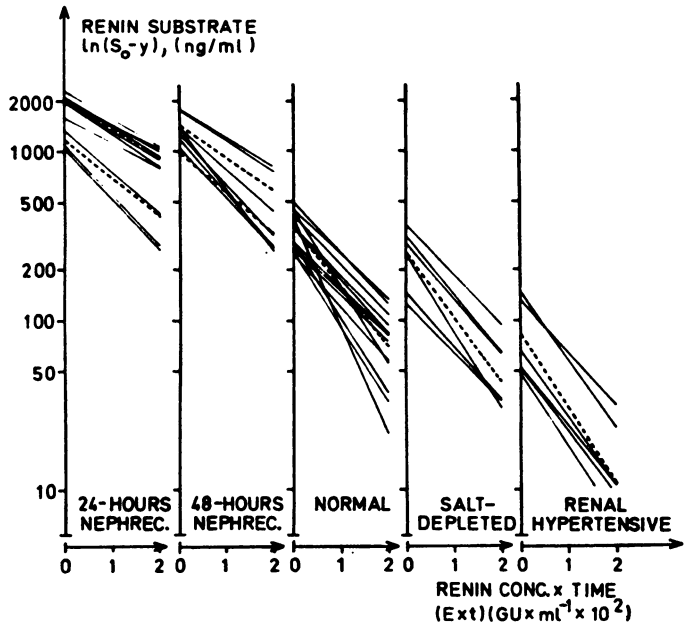

FIG. 2. No active inhibitors are present in non-pretreated plasma, since the measured reaction rates (slope of solid lines) are identical with the expected reaction rates (slope of dotted lines) calculated according to Michaelian kinetics. (From K. Pouslen: No evidence of active renin inhibitors in plasma. The kinetics of the reaction between renin and substrate in nonpretreated plasma. Scand. J. Clin. Lab. Invest. 27: 37–46, 1971.)

inhibitors are found. It is possible to measure the reaction rate in untreated plasma if it is determined with the method of substrate consumption, since renin and renin substrate are stable in untreated plasma at physiological pH. If the kinetics of the system have been determined, known amounts of standard renin can be added to individual plasma samples, from which the reaction rate can be determined. Deviations from expected values calculated according to Michaelian kinetics would indicate the presence of inhibitors. In figure 2 the measured reaction rates are shown for individual plasma samples as the slope of the solid lines and compared to the theoretical rates calculated according to Michaelian kinetics (dotted lines). There is no sign of inhibitors but there is the expected variation in reaction rate with the substrate concentration (probably one of the reasons for the postulated presence of inhibitors in plasma). (For details and literature see reference 58.)

Converting Enzyme Activity

From figure 1 it can be seen that we now have been dealing with measurements of circulating concentrations of angiotensin II, with determination of renin concentration, and with the angiotensin 1 generation rate, the so-called renin activity. These are the more or less routinely determined parameters in the renin system. However, from figure 1 we see that we are interested not only in the circulating concentrations of angiotensin II but also in the rate of angiotensin II formation. This rate is dependent on the converting enzyme concentration. Ng and Vane (48) have shown that angiotensin I is converted to angiotensin II by a single passage through the pulmonary circulation. This has been confirmed by Biron *et al.* (3) and Oparil *et al.* (49) and appears to be due to a membrane-bound enzyme in the lung tissue. Converting enzyme is also found in plasma but in a much lower concentration than in the lung (49). We have

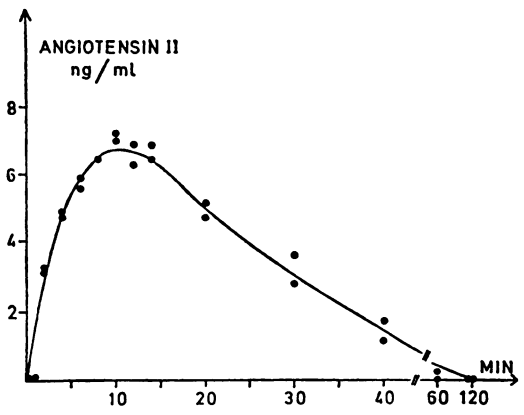

FIG. 3. Angiotensin I was added to untreated plasma. The angiotensin II formed with time was measured by radioimmunoassay. The solid line is a computer fit of experimentally determined values to a theoretical equation which takes into account the simultaneous presence of converting enzyme and angiotensinase activity. From the determined constants, the converting enzyme activity can be calculated. (From K. Poulsen and L. L. Poulsen: Simultaneous determination of plasma converting enzyme and angiotensinase activity by radioimmunoassay. Clin. Sci. 40: 443–449, 1971.)

no way to inhibit selectively the angiotensinases without also inhibiting converting enzyme. One way of measuring the plasma converting enzyme activity shown in figure 3 is to add angiotensin I to untreated plasma and measure the concentration of angiotensin II formed with time by radioimmunoassay. The increase in angiotensin II is due to converting enzyme, but since angiotensinases are simultaneously present, the curve reaches a maximum and eventually all angiotensin II is removed from the system. When angiotensin concentrations are low, both reactions are of first order. In figure 3 the solid line is a computer fit of experimental values to the relevant kinetics. From such data the converting enzyme and angiotensinase activity can be calculated. With low angiotensin I concentrations, the half-life for angiotensin I was determined to be about 2 min (59). However, the plasma converting enzyme showed little variation in activity in different individuals and is therefore a constant factor in the system.

The Generation Rate of Angiotensin II

The last parameter to be discussed briefly is the generation rate of angiotensin II. Here again we cannot inhibit the angiotensinases without also inhibiting the converting enzyme. The principle, which might be useful in other systems also, uses radioimmunoassay but not in the usual manner. From figure 4 it is seen that if antibody against angiotensin II is present during the enzymatic reaction, it will compete with the angiotensinases and if present in sufficiently high concentration, it will trap nearly all the generated angiotensin II. If the incubation mixture is cooled after the incubation, the enzyme reactions are stopped. Almost all the angiotensin II is bound to the antibody, since this is present in high concentration. When the reaction mixture is diluted, however, the angiotensin-antibody complex dissociates, so that a measurable fraction is free. If labeled angiotensin II is added, we have now the usual radioimmunoassay mixture in which the captured angiotensin II can be quantitated. The general idea in this principle is that as long as the angiotensin II is bound to the antibody it is not susceptible to enzymatic attack. That a specific antibody is capable of trapping an intermediate in a series of

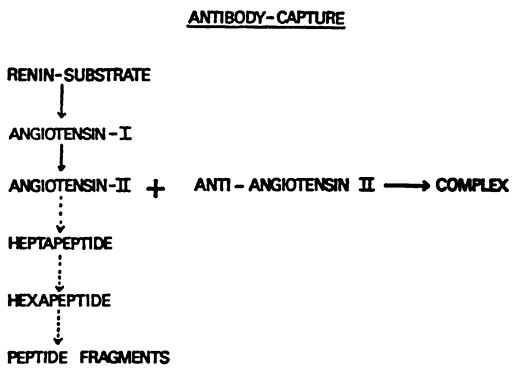

FIG. 4. The principle of the "antibody-capture" method. An antibody to an intermediate in an enzymatic reaction can capture this intermediate and thereby prevent it from enzymatic degradation.

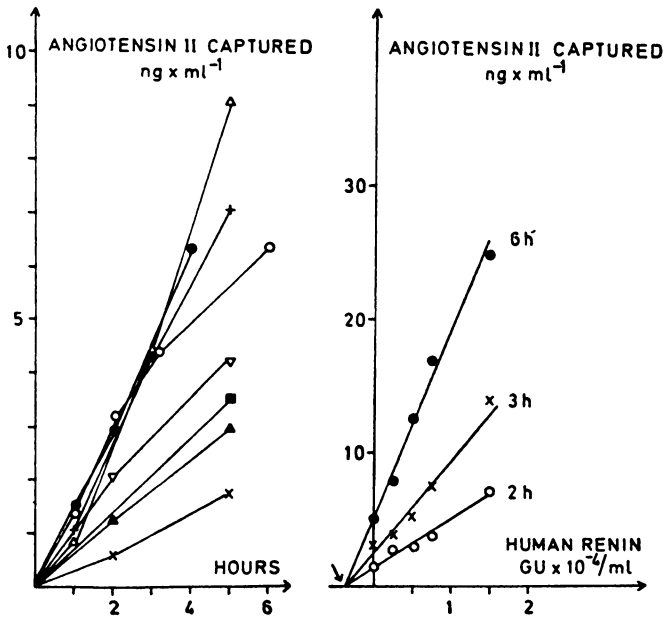

FIG. 5. A, measuring *angiotensin II generation rate* by the antibody capture method in human plasma. The figure shows the amount of angiotensin which is captured with time for eight normal plasma samples. B, measuring *renin concentration* by the angiotensin-capture method. A normal human plasma is incubated with and without four different concentrations of standard renin. The amount of angiotensin II which is captured after 2, 3, and 6 hr of incubation is measured. The arrow indicates the endogenous renin concentration. (From K. Poulsen: Simplified method for radioimmunoassay of enzyme systems. Application on the human renin-angiotensin system. J. Lab. Clin. Med. 78: 309–315, 1971.)

enzymatic reactions is shown in figure 5. It shows the rate of angiotensin II formation as measured by the "antibody capture method" (57). A detailed account on the kinetics of the renin system and its significance for determination of the components of the system is given elsewhere (60).

Which Parameter Should Be Measured?

In *clinical diagnostics*, the interest is in evaluation of conditions in which abnormal degrees of activity of the whole renin-angiotensin system are suspected. Since angiotension II is responsible for all known effects of this system, measurement of local circulating concentrations of angiotensin II would be the most logical choice. Such a determination is possible but still too complex for routine use. In its place a determination of plasma renin activity (generation rate of angiotensin I) is the method of choice. This method probably gives a good measure of the overall activity of the renin-angiotensin system.

If, however, the attention is directed toward the mechanism behind the pathological and physiological changes of the system the different components must be measured independently of each other. It should especially be remembered that plasma renin activity is not a measure for renin concentration.

Plasma Aldosterone

Aldosterone is the major mineralocorticoid secreted by the adrenal gland. Its production is stimulated by angiotensin II, adrenocorticotrophic hormone (ACTH), increased potassium and decreased sodium concentration in plasma. The measurement of plasma aldosterone is therefore an important parameter to measure together with parameters in the renin-angiotensin system.

The first methods available for the deter-

mination of aldosterone in plasma depended on the principle of double isotope dilution (3a). All the methods in use are complex and time consuming. For an extensive review of these methods see Coghlan and Blair-West (18).

Recently antibodies to aldosterone have been produced and radioimmunoassays described (2, 20, 26, 40, 45, 67). This substantially simplified the determination in plasma of aldosterone. The antibodies are all very specific but unfortunately not specific enough. This is due to the fact that corticosterone and cortisol are found in plasma in concentrations 10^3 to 10^4 times higher than that of aldosterone. Even a slight cross reaction of the aldosterone antibody with cortisol and corticosterone will, therefore, be crucial. A chromatographic separation of cortisol and corticosterone from aldosterone is a necessary step in all the described radioimmunoassays. These have major advantages over the isotope dilution methods, but they are not so simple as a widespread clinical application would require.

An even more specific antibody against aldosterone would seem to be the answer. Haber and his group have studied the antigenicity and specificity of antibodies elicited against different aldosterone protein conjugation (1).[1] On the basis of these studies they can conclude that aldosterone-dioxime-bovine-γ-globulin (BGG) used as antigen gives the most specific antibodies. A prerequisite seems to be that extreme basic conditions are avoided during the attachment of aldosterone to BGG.[1] Some of the elicited antisera have a unique specificity since they do not react with corticosterone and cortisol in concentrations of 10^7 pg per assay tube under conditions where the detection limit for aldosterone is 5 pg. Consequently, with such an antibody in a radioimmunoassay for aldosterone excludes cortisol and corticosterone from influencing the assay, and a chromatographic separation of aldosterone from corticosterone and cortisol is, therefore, not needed.

Aldosterone differs only from corticosterone by having an aldehyde group in position C-18 rather than a methyl group. The next question is therefore whether or not the antibody reacts with other steroids possessing related functional groups at position C-18.

18-Hydroxy-corticosterone and 18-hydroxy-11-deoxycorticosterone are found in plasma, the former being the accepted precursor for aldosterone. These steroids do cross react with the antibody but with an affinity 10^4 times lower than that of aldosterone. The plasma concentrations of these two steroids have been studied with other methods (46, 68) and found to be of the same order of magnitude as aldosterone and are thus unable to influence the assay.

With this antibody, a direct, simple radioimmunoassay for aldosterone has now been developed, characterized by the same sensitivity and specificity as this unique antibody.

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