DETERMINATION OF RENIN ACTIVITY IN SINGLE RENAL GLOMERULI AND THEIR FRAGMENTS

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UDC 616.612-008.931 :577.152.344]-074

A modification of the method of determination of renin activity in a single glomerulus and its fragments, based on the use of cold EDTA-treated whole plasma of nephrectomized animals as the renin substrate, is suggested to replace the use of a substrate obtained from plasma by the previous complicated method. A number of simplifications are made to the biological method of renin determination. Soviet equipment is used for all the procedures. The principle of the modifications is suitable for clinical purposes.
KEY WORDS: renal glomerulus; renin; juxtaglomerular apparatus.

Determination of the renin concentration in single glomeruli is a very valuable procedure for the investigation of many problems in the physiology and pathology of the kidney, especially in conjunction with morphological or micropuncture studies. Several methods of determining renin activity are known [13], but they are either insensitive or they require complex, precise, and multistage biochemical manipulation in order to obtain the substrate for renin from the blood plasma. The simpler method [12] proved unsuccessful in the writer's hands. It was therefore decided to use whole blood plasma from nephrectomized rats as the source of the substrate, as is widely used for the investigation of renin in different objects, including different parts of the nephron [2, 3]. In the latter case, the test material was obtained from a pool of different nephrons. Having tried several modifications, a simple method of determination of renin in the single glomerulus, perfectly satisfactory for our purposes, was obtained.

PREPARATION OF THE PLASMA

Wistar rats were nephrectomized 48 h before the blood was taken. Blood was collected under pentobarbital anesthesia (60 mg/kg) through a polyethylene cannula from the carotid artery. The rats were first heparinized (500 units/kg). Plasma was separated by centrifugation at 4°C (5000 rpm, 15 min), collected in a common receiver, and kept at −20°C. Such plasma, as the writer has shown [5], does not contain renin. To increase the sensitivity of the method, the modification described by Osmond et al. [10] was used. The plasma was thawed, mixed in the ratio of 4 :1 with cold 3.8% Na-EDTA solution, pH 6.5, and kept at 4°C. The yield of angiotensin during subsequent incubation is increased, depending on the keeping time, after addition of the same quantity of renin. After keeping for 7 days, the yield of angiotensin per hour of incubation was shown to be increased by 2.8 times compared with a keeping time of 1 day [5] (Fig. 1). This time is to be preferred, for it is convenient for preparation of the plasma. Moreover, a further increase in the time ought not to increase sensitivity significantly, for after keeping for 5 days the angiotensin yield does not increase significantly.

CONDITIONS OF INCUBATION

The effect of incubation time, of the volume of plasma, and of the angiotensinase inhibitor contrykal [8] on the angiotensin yield was determined experimentally. For this purpose a homogenate of the kidney cortex of a Wistar rat was used (as the source of renin). This was prepared in the proportion of 1 ml 0.15 M phosphate buffer, pH 6.5, to 1 mg tissue, and 0.01 ml of the product was added to each sample of plasma. The samples were incubated for 30, 60, and 90 min. During the first hour of incubation the angiotensin yield rises significantly, so that after 60 min it is 1.5 times higher than after 30 min, but after 90 min it is significantly lower (Fig. 2a). One possible reason for this could be the gradual recovery of angiotensinase activity, inhibited in the cold in the presence of EDTA, during incubation at 37°C. This problem is discussed in more detail in

Laboratory of Endocrinology, Institute of Cytology and Genetics, Siberian Branch, Academy of Sciences of the USSR, Novosibirsk. (Presented by Academician of the Academy of Medical Sciences of the USSR V. P. Kaznacheev.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 84, No. 11, pp. 632-635, November, 1977. Original article submitted March 1, 1977.

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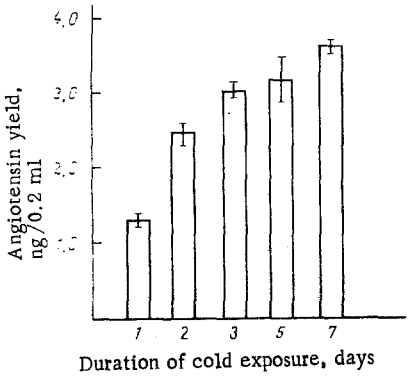

Fig. 1

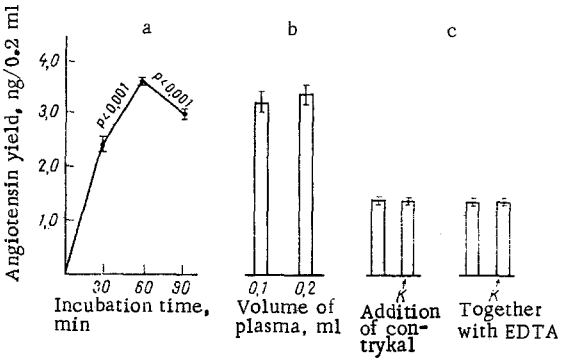

Fig. 2

Fig. 1. Angiotensin yield during incubation for 1 h depending on duration of cold incubation.

Fig. 2. Dependence of angiotensin yield on conditions of incubation. a) Incubation time (cold exposure 7 days, volume 0.1 ml); b) volume of plasma (cold exposure 5 days, incubation for 1 h); c) addition of contrykal (K, cold exposure 1 day, volume 0.1 ml, incubation for 1 h).

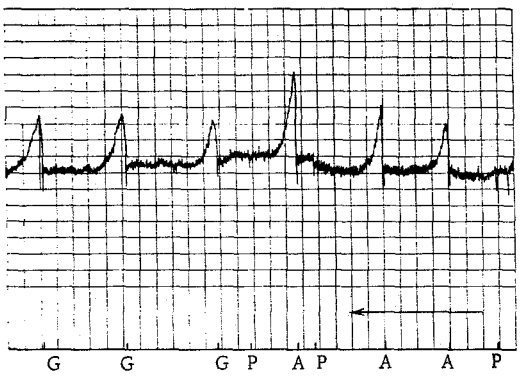

Fig. 3. Response of blood pressure of test rats to physiological saline (P), synthetic angiotensin (A), and samples from individual glomeruli (G). Order of injection indicated by arrow. Angiotensin injected in increasing doses; tape winding speed of automatic writer 4 mm/min.

[5]. An essential condition of incubation is an excess of substrate. For this purpose, volumes of 0.1 and 0.2 ml were investigated. In the latter case the angiotensin yield was higher, although not significantly (Fig. 2b). Contrykal was added at the rate of 2500 k.i.u./ml [8]. Under these conditions this did not affect the yield of angiotensin (Fig. 2c). It is thus preferable to incubate for 1 h in a volume of 0.2 ml without the addition of contrykal.

ISOLATION OF GLOMERULI

The method described by Dahlheim et al. [7] was used. Experimental rats were anesthetized with pentobarbital (60 mg/kg, intraperitoneally) and 0.3-0.4 ml of a 3% solution of Evans' blue dye, made up in physiological saline, was injected into the jugular vein (the 2% solution recommended by these workers did not give a sufficiently contrasted picture under our conditions under high power). Before injection, the solution was thoroughly shaken. Laparotomy was performed and a clamp applied to the pedicle of the kidney to be examined. If infusion has been good, the surface of the kidney under these circumstances turns blue. The kidney was excised, freed from adipose tissue, and rinsed in physiological saline. It was then transferred into a 30% solution of glycerol in physiological saline and the middle part was cut into segments 1- to 2-mm thick with a razor blade. The individual segments were transferred to a second flask containing the same solution. All manipulations were carried out on an ice bath. The individual segments were placed on a tin plate covered with white enamel. Drains were made in the plate which emptied into the chamber of a TLM microrefrigerator, filled with a mixture of ethyl alcohol and water in the ratio of 3 : 2. In this way a subzero temperature of the preparation was obtained. The glomeruli were isolated under the MBS-2 microscope under a magnification of 87.5×, using the interlobular artery as a guide. Two dissection needles were used, one shaped like a scalpel, the other like

TABLE 1. Results of Investigation of Renin Activity in Single Glomeruli Isolated from the Intracortical Zone of the Cortex of the Left Kidney in Rats

Nature of material	Renin activity, ng angiotensin/0.2 ml plasma
Whole single glomerulus (intact animals)	0.37 ± 0.04
Arteriolar fragments (intact animals)	0.4 ± 0.02
Actual capillary fragment (intact animals)	0*
Whole single glomerulus (constriction of abdominal aorta between mouths of renal arteries for 1 week)	0.62 ± 0.12
Whole single glomerulus (same operation for 2 weeks)	1.14 ± 0.16

*A similar absence of renin activity was reported by other workers [3, 7].

a hook. The glomeruli were dissected with an attached arteriole equal in length to the diameter of the glomerulus. To investigate the renin in the individual parts of a glomerulus, the capillary bundle was taken up by the hook and carefully separated from the arteriole, which was fixed by the second needle. The surrounding tubular tissue and the injected dye did not affect the results of determination of renin [7]. Dahlheim et al. [7] used ultrasonic homogenization. In our experience, the manual method is quite satisfactory. The glomerus (or its corresponding components) was placed by means of a needle in a glass microhomogenizer filled with 0.04 ml of the prepared plasma. Homogenization was carried out by rotation of a Teflon pestle (up to 30 revolutions). This procedure was carried out in the cold. The homogenate was then drawn off by means of a capillary tube and transferred to the incubation tube with 0.16 ml plasma.

INCUBATION SYSTEMS

Tubes 24-mm high were made from glass tubing with a bore of 4 mm. The base for the thermostats was a magnetic mixer equipped with a heating device. The electric heating coil of the latter was led out of the general electric circuit and connected to an autotransformer, connected in series with a relay and contact thermometer. A stand measuring 40 × 35 × 20 mm, with 20 holes for the tubes and a projection measuring 10 × 10 mm with a hole for the contact thermometer was made from an aluminum block. A second similar stand (but without the projection) was used for the manipulations in the cold. The mixers were made from a nail 1.5 mm in diameter and soldered into polyethylene sheaths.

INCUBATION

The system was first heated to 40°C, for the tubes had previously been at zero temperature. The temperature fell to 37°C during the first minute after insertion of the tubes into the incubation stand, and the required voltage was set by the transformer. Under our conditions the temperature in the system varied between 36.5 and 37.4°C, which is perfectly satisfactory. The samples were incubated for 1 h. The reaction was stopped by boiling for 3 min and the tubes were cooled in an ice bath. The contents were then mixed for 5 min at 4°C. After this the samples were frozen. These procedures ensure better separation of the supernatant during subsequent centrifugation, which was carried out on the day of the test. The samples were thawed and centrifuged at 4°C on the TsLN-2 centrifuge for 30 min at 8000 rpm. The supernatant was drawn off by a syringe, made up to 0.4 ml with sterile physiological saline, and the renin activity was determined.

DETERMINATION OF RENIN ACTIVITY

The amount of angiotensin formed during incubation serves as the criterion of activity. Its content in the tubes was determined in an acute experiment on a test rat by the increase in arterial pressure, compared with the response to known doses of angiotensin. The results were expressed in nanograms angiotensin per 0.2 ml plasma. Details of the preparation of the test rats are described elsewhere [4, 6, 11]. Bilateral nephrectomy was performed on noninbred rats weighing 170-240 g 16-24 h before the investigation. The experiments (Fig. 3) showed that this ensures satisfactory sensitivity of the rats to angiotensin, low background fluctuations of blood pressure, and absence of a pressor response to injection of physiological saline without additional premedication or other interference. This cannot be done with Wistar rats. In addition, they are less tolerant than

noninbred rats. Testing was carried out under pentobarbital anesthesia (60 mg/kg, intraperitoneally). The jugular vein (for injection of the samples) and the common carotid artery (for measuring the pressure) were cannulated. A preliminary injection of heparin (500 units/kg, intravenously) was given. The arterial cannula was connected to a strain gauge (on silicon crystals), developed at the Institute of Theoretical and Applied Mechanics, Siberian Branch, Academy of Sciences of the USSR [1]. The strain gauge was powered from a VSP-33 rectifier operating at 3 V. The pressure was recorded by a G_1B_1 automatic writer (Carl Zeiss, East Germany). Each sample was determined between 2 doses of standard angiotensin solution, one of which gave a greater, the other a smaller pressor effect, for the sensitivity of the animal to angiotensin can vary in the course of one experiment, as other workers also have observed [4]. After injection of the samples or standards in doses amounting to 6-8 ng the animal requires a rest (40-50 min) to allow the vessels to recover their sensitivity. During prolonged determinations, the animal was given one quarter of the original dose of pentobarbital periodically (every 1.5-2 h) intraperitoneally.

PREPARATION OF THE ANGIOTENSIN STANDARDS

The method recommended in the literature [4], in the writer's opinion, is not very convenient because a new sample has to be weighed each time, for the solution is active for only a short period. The following method* was used. A glass capillary tube with a tip 0.04-0.06 mm in diameter, giving drops of uniform volume, was used. The angiotensin (valyl-5-angiotensin-2-amide, from Ciba, Switzerland) solution was applied as one drop (15 ng per drop) to half-rings made from Capron tube, and kept at −20°C in a bottle. On the day of the determination, one half-ring was placed in a polystyrene tube into which sterile physiological saline was poured. By this method, having weighed one sample, it is possible to obtain standard solutions of different concentrations operatively. A range from 0.2 to 2 ng angiotensin per 0.1 ml physiological saline was used. Before injection into the animal the standard sample was made up to 0.4 ml in the syringe with physiological saline. In the course of the determination the tube containing the standard was kept in the cold. Standard solution not completely used up can be kept at −20°C.

SPECIFICITY OF THE METHOD

During the investigation of plasma to which no homogenate was added, no pressor response was obtained, whether the plasma was incubated for 1 h or not incubated [5]. This suggests that the quantity of pressor substance formed in the plasma during its incubation with homogenate reflects the renin activity in the sample. The character of the pressor responses to injection of these samples was identical to the response to angiotensin (Fig. 3).

REPRODUCIBILITY OF RESULTS

This can be judged from parallel samples during the investigation of the incubation conditions. With accurate treatment of the samples and constant control of the sensitivity of the rats to angiotensin the differences did not exceed 0.1 ng/0.2 ml. For exzmple: 3.65, 3.7, and 3.75 ng per 0.2 ml (plasma, standing in the cold for 7 days, incubation for 1 h) (Fig. 2a).

SENSITIVITY OF THE METHOD

The smallest quantity of angiotensin detectable under these conditions was 0.25 ng per 0.2 ml, compared with 1 ng per 0.2 ml reported by other workers [4]. The difference was evidently due to the advantages of the strain gauge over the mercury manometer which they used.

Results of the determination of renin activity in single glomeruli of control and experimental rats by this method are given in Table 1.

Dahlheim et al. [7] considered that the renin content in a single glomerulus becomes determinable if a highly purified substrate free from angiotensinases is used. More recent investigations [9] showed that satisfactory results can also be obtained by the use of partially purified or even of a heterologous substrate, and the results of biological and radioimmune methods of analysis showed very high positive correlation. The method of treatment of whole plasma used in the present experiments gave results not much less than those given by Dahlheim et al. [7] (for intact rats): 0.25-0.6 and 0.5-1.5 ng respectively (calculated for absolute quantities of angiotensin). The difference is explained by the absence of angiotensinases in the solution of the substrate obtained by the method used by the authors cited. With the use of EDTA in our own modification, activity of angio-

*The idea of this method belongs to N. A. Kolchanov.

tensinases is inhibited by only 50% [8]. Cold preincubation, however, evidently neutralizes activity of the unbound part of the angiotensinases, although reversibly [5]. In the method of determination of renin in the blood adopted in the USSR [4], only EDTA is also used to inhibit angiotensinase activity, and this is no obstacle to the work. The modifications now described can therefore be regarded as completely satisfactory, especially for comparative studies. Its advantage lies in the several essential simplifications of the techniques of analysis, the most important of which is overcoming the difficulty of obtaining the substrate. During the choice of appropriate conditions, this modification, it seems, may prove to be suitable for the investigation of single glomeruli in biopsy specimens of human kidneys under clinical laboratory conditions.

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METHOD OF SONICATION OF CELL SUSPENSIONS

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UDC 612.014.2.014.45

A simple chamber is suggested for the sonication of small quantities of a cell suspension. It consists of a transparent plastic cylinder, which fits on the top of a UTP-1 ultrasonic generator, the top of which serves as the floor of the chamber. Losses of ultrasonic energy are thus eliminated and it is possible to determine the intensity of the ultrasound acting on the cells of the suspension with fair accuracy.

KEY WORDS: ultrasound; cells; suspension.

Progress in the most rational use of ultrasound in medicine is hampered by the lack of study of its action on cells and tissues [6]. The most accessible form for the study of the direct action of ultrasound on cells is in suspension, for in that case both free-living cells (such as blood cells or microorganisms) and cells forming organs and tissues of animals or plants can be treated in this way.

Attempts to study the action of ultrasound on cell suspensions has been undertaken for a long time [1, 5, 10], but the methods used for these purposes have had many disadvantages. The most important of these was inability to determine with any degree of accuracy the intensity of the ultrasonic energy acting on the cells. The reason for this was usually that the suspension was kept in glass flasks, immersed in an oil fountain formed by the action of powerful ultrasound [4, 7-10]. The presence of intermediate media between the ultra-

Kiev Research Institute of Orthopedics. (Presented by Academician of the Academy of Medical Sciences of the USSR A. D. Ado.)

Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 84, No. 11, pp. 635-637, November, 1977. Original article submitted June 6, 1977.