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Hydrolytic Enzymes of the Ischemic Kidney (40630)

MARC CANTIN, MARIA ARTIZZU, LUCIANO MAMELI, AND ROBERTO GIANETTO

Départements de Pathologie et de Biochimie, Faculté de médecine, Université de Montréal, Montréal, Québec, Canada H3T 1J4

Partial ligation of the aorta between the renal arteries in the rat induces rapidly progressive malignant hypertension as well as severe atrophy of the ischemic kidney (1–4). The pathology of this kidney is characterized by cellular changes in virtually all renal structures: There is widespread metaplasia of arteriolar smooth muscle cells into juxtaglomerular cells (3), simple atrophy of the outer cortical tubular cells, and atrophy, hyperplasia, and polyploidy in the inner cortex (pars recta) (5). In the cortex, these morphological alterations are accompanied by an increase in DNA and RNA with a decrease in proteins (5).

The present study indicates that not only renin (6–8) but also certain hydrolytic enzyme activities, such as those of acid phosphatase, β-glucuronidase, and cathepsin D, are increased in the ischemic renal cortex. As shown by their distribution pattern following isopycnic centrifugation, these enzymes appear not to be localized, like renin, in juxtaglomerular cell granules but rather in cortical tubular cell lysosomes.

Materials and methods. Animals and surgery. Female Sprague–Dawley rats (Fermes et Laboratoires Canadiens d'Elevage Ltée, St-Constant, Québec, Canada) were used in two series of experiments. Surgery always was performed under ether anesthesia on groups of 10 animals, on the 1st day of the experiments, with the animals being sacrificed on the 10th day.

The first series was done on rats with an initial average body weight of 200 g (range: 190 to 210 g). Surgery consisted of partial ligation of the aorta between the renal arteries, using a silk thread and the style (diameter: 0.103 mm) of a No. 30 hypodermic injection needle, as described elsewhere (3). These animals, together with control, unoperated groups, were taken for histopathology, electron microscopy, and biochemical analysis of the left ischemic kidney: determination of renin, acid phosphatase, β-glucuronidase, cathepsin D, and protein content; isopycnic centrifugation of renal cortical homogenates; and electron microscopy of fractions obtained by centrifugation.

The second series of experiments was performed on rats with an average initial body weight of 215 g (range: 205 to 225 g) to compare the effects of styles of larger diameter on renal atrophy, induction of hypertension, and β-glucuronidase activity of the renal cortex. Since preliminary investigations had shown that a style larger than 0.740 mm does not induce any noticeable atrophy, the final study included styles ranging from 0.103 to 0.740 mm in diameter as well as an unoperated control group. All groups were made up of at least 20 animals. The heart and left kidney of 10 animals of each group were fixed in Bouin–Hollande fluid for 24 hr and then weighed. These kidneys were utilized for histopathological study. The left renal cortex of an additional 10 rats in each group was used for determination of β-glucuronidase activity.

Histopathology. After embedding in paraffin, the left kidney was cut longitudinally at 5 μm and stained according to the PAS technique.

Homogenization and ultracentrifugation. The left renal cortex was homogenized in an 0.25 *M* ice-cold sucrose solution, employing a Potter–Elvehjem apparatus at 800 rpm with three up-and-down strokes. The suspension was centrifuged at 1000*g* for 10 min. The sediment was resuspended in 0.25 *M* sucrose, homogenized with two strokes, and centrifuged for 10 min at 500*g*. The two supernatants were pooled and made up to volume with 0.25 *M* sucrose to form a 1:5 homogenate. Each homogenate was tested separately for protein and hydrolytic enzyme activity, while pooled homogenates were first tested

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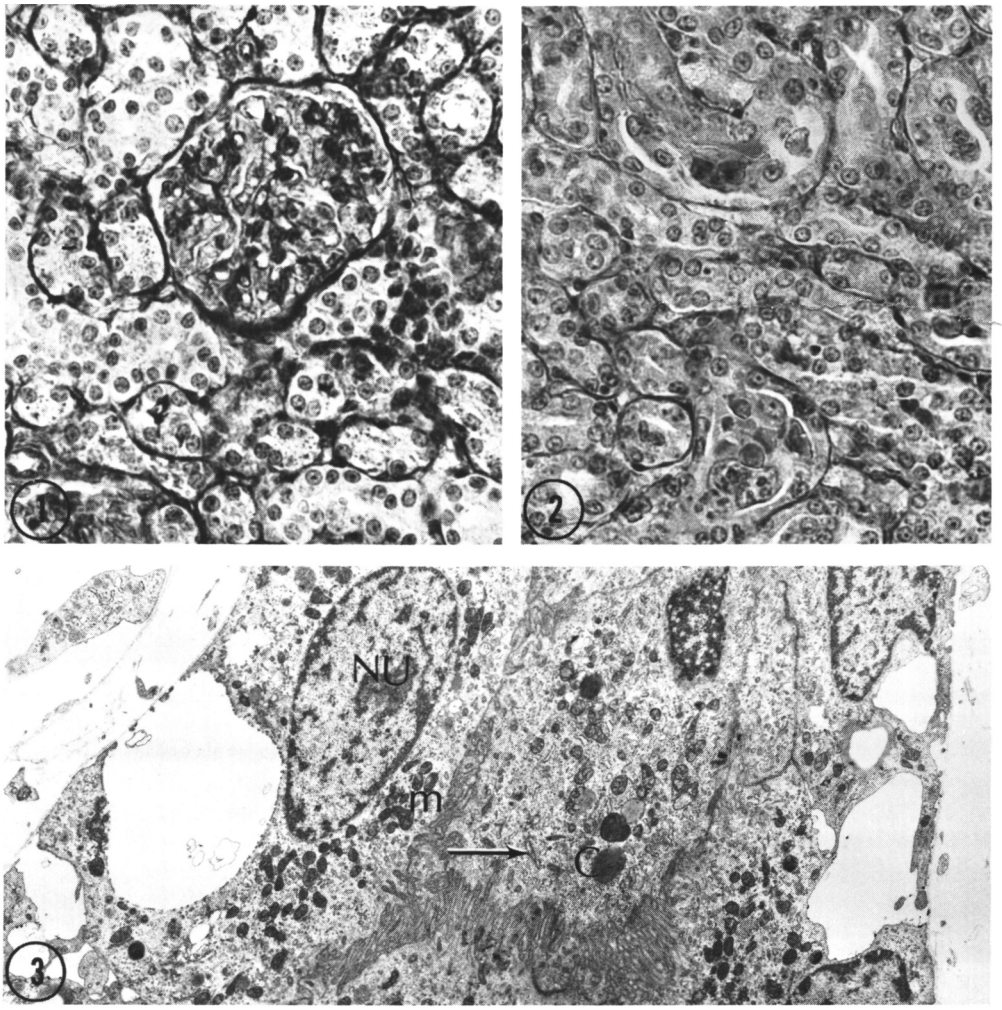

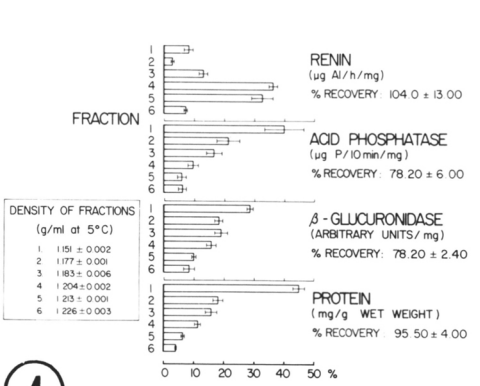

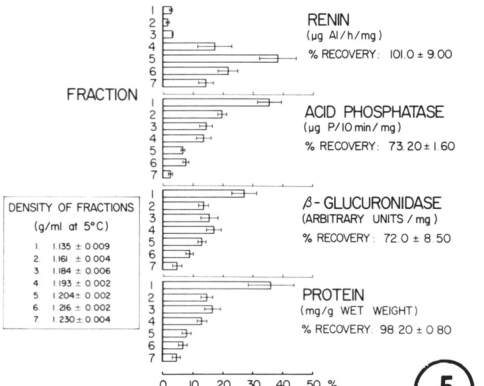

FIGS. 1–5. (1) Severely ischemic outer cortex (style diameter 0.103 mm). All the proximal convoluted tubules have lost their lumina and brush border. Note the regularity of size, shape, and staining intensity of tubular cell nuclei (PAS × 332.5).

for protein and enzymes (hydrolytic enzymes and renin) and then fractionated by isopycnic centrifugation in a sucrose density gradient according to the method of Morimoto *et al.* (9). Following centrifugation, the bands were removed by aspiration and assayed for protein, enzyme activity, and density (at 5°).

Enzyme assays. β-Glucuronidase and acid phosphatase activities were assessed by the methodology of Gianetto and DeDuve (10), and cathepsin D activity by the Barrett technique (11). Renin activity was measured with the radioimmunoassay method of Haber *et al.* (12) for the generation of angiotensin I, as modified by Gross and Barajas (13) for renal cortical homogenates and fractions. A commercial Angiotensin I Immutope Diagnostic Reagent Kit (E. R. Squibb and Sons, New York) was used. Plasma (substrate) for the reaction was obtained from 24-hr bilaterally nephrectomized, 200-g female, Sprague–Dawley rats. Incubation of homogenates and fractions with substrate was done at pH 7.4 for 2 hr. The reactions were linear.

Protein determinations. The protein content of the renal cortex was measured by the technique of Lowry *et al.* (14). In all cases, the Student's *t* test was used for statistical analysis.

Electron microscopy. The technique employed for electron microscopy of the ischemic renal cortex and fractions has been described earlier (3).

Results. Renal ischemia induced with a style of 0.103 mm in diameter. Histopathology and electron microscopy. Outer cortex. After 10 days of ischemia, atrophy was extremely marked, particularly of the proximal convoluted tubular cells and, to a much lesser degree, of those of the distal tubules and cortical collecting ducts (Fig. 1). In the proximal convoluted tubules, the lumina had collapsed and the brush border was often absent; the basement membranes were thickened, the basilar interdigitating processed had disappeared, and the intercellular spaces were enlarged. All the tubular cell nuclei were similar in size, contour, and staining intensity. The cytoplasm contained only a few mitochondria, prominent lipid vacuoles, and a few cytosomes.

Inner cortex. Many proximal tubular cells of the pars recta were filled with cell cords containing nuclei of irregular size, shape, and staining intensity (Fig. 2). The most striking characteristic of these cells was the hypertrophy of the protein-synthetizing apparatus: prominent nucleoli and numerous cisternae of rough endoplasmic reticulum, and free and polyribosomes. Apart from these changes, the cells were similar to those of the outer cortex. The number of cytosomes in particular was not increased (Fig. 3).

Arteries and arterioles. A great number of smooth muscle cells of arterioles, and to a lesser degree of some arteries, were replaced by cells having all the characteristics of hyperactive juxtaglomerular cells (3). There were no degenerative changes in arteries or arterioles.

Enzyme activity and protein content of the renal cortex. There was an increase in the activity of all three hydrolytic enzymes in the ischemic renal cortex, as compared with the left renal cortex from control rats, when expressed per milligram of protein (Table I). Acid phosphatase was, however, increased the least of all and was not significantly enhanced over control values, when expressed per gram of wet weight (not shown). Renin activity was elevated fivefold over corresponding values of the control renal cortex. The protein content of the ischemic renal cortex was much lower than that of the control left cortex.

Isopycnic centrifugation of renal cortical ho-

(2) Severely ischemic inner cortex (style diameter 0.103 mm). The tubules are filled with cell cords showing irregularity of size, shape, and staining intensity of nuclei (PAS × 332.5).

(3) Proximal convoluted tubular cells of severely ischemic inner cortex (style diameter 0.103 mm), showing absence of lumen, part of brush border, a nucleus with a prominent nucleolus (NU), and a markedly simplified cytoplasm containing mitochondria (m), a few cytosomes (C), cisternae of rough endoplasmic reticulum (arrow), and numerous ribosomes. Note the enlargement of the intercellular spaces (× 7847).

(4) Isopycnic centrifugation of control renal cortex. Each value represents the mean ± SE of five experiments using pools (five renal cortices) of homogenates.

(5) Isopycnic centrifugation of ischemic renal cortex (style diameter: 0.103 mm). Each value represents the mean ± SE of five experiments using pools (8 to 10 renal cortices) of homogenates.

TABLE I. RENIN ACTIVITY, HYDROLYTIC ENZYME ACTIVITY, AND PROTEIN CONTENT OF THE CORTEX FROM THE ISCHEMIC (ENDOCRINE) KIDNEY

	Protein (mg/g wet wt)	Renin activity[a] (μg AI/hr/mg protein)	β-Glucuronidase activity (AU[b]/mg protein/10 min)	Cathepsin D activity (BU[c]/mg protein/10 min)	Acid phosphatase activity (μg P/mg protein/10 min)
Control kidney	97.8 ± 2.7	7.5 ± 0.65	15.6 ± 0.7	0.382 ± 0.002	14.5 ± 0.9
Ischemic kidney	77.1 ± 2.5*	36.4 ± 7.02*	62.8* ± 2.4	0.886* ± 0.031	19.5** ± 0.8

[a] Renin activity was measured in homogenates (pool of 5 control renal cortices and 8 to 10 ischemic renal cortices). Each value represents the mean ± SE of five experiments. Hydrolytic enzyme activity was measured in homogenates of kidneys of single rats.

[b] Arbitrary units.

[c] Barrett units.

* $P < 0.005$.

** $P < 0.01$.

mogenates. Isopycnic centrifugation of control cortical homogenates yielded six bands (Fig. 4), and the ischemic kidney seven bands (Fig. 5). The greatest activity of acid phosphatase and β-glucuronidase and the greatest amount of protein were localized in band 1 of both normal and ischemic renal cortices. There was a decrease in activity of the hydrolytic enzymes and in protein content as the density increased. Renin activity was mostly localized in bands 4 and 5 of control and in bands 4, 5, and 6 of ischemic cortices.

Electron microscopy of fractions. Control renal cortex. Fraction 1 contained small vesicles, many of which exhibited attached ribosomes; it also showed other vesicles of larger size with a flocculent, slightly granular content corresponding to lysosomes. Fraction 2 displayed the latter type of vesicles with much less rough and smooth endoplasmic reticulum. Fraction 3 was made up mostly of mitochondria. Fraction 4 revealed dense granules and other granules with a finely dispersed content of lower electron density. Fraction 5 was made up of the same type of granules but in lesser number, many empty vesicles and vacuoles being present. Fraction 6 contained a few dense or pale granules and many empty vesicles and vacuoles.

Ischemic renal cortex. Fractions 1, 2, and 3 were identical to those of the control renal cortex. Fractions 4, 5, and 6 contained mainly granules of either high or low electron density. The granules were most abundant in fraction 5 (Fig. 6) and less so in fractions 4 and 6, the rest of the fractions being made up of empty vesicles and vacuoles. Fraction 7 still contained a few dense or less dense granules and many empty vacuoles and vesicles.

Renal ischemia induced with styles larger than 0.103 mm in diameter. As can be seen in Table II, partial ligation of the aorta produced renal atrophy which was roughly inversely proportional to the diameter of the style used. The decrease in protein content of the kidney was significant in all cases, compared with control values. Heart weight was increased when expressed as mg/100 g body wt in all groups with ligated aortas. The decrease in body weight was marked in all ligated animals.

Histopathological examination of the ischemic kidney showed an identical picture, irrespective of the diameter of the style: simple atrophy of the outer cortex and hyperplasia with polyploidy in the inner cortex. These changes were evident in all ligated rats except those operated on with the largest style: They were then visible in 80% of the animals. β-Glucuronidase activity was significantly increased above control values in the renal cortical homogenates of all ligated animals.

Discussion. The histo- and cytopathology of the renal cortex after 10 days of ischemia correspond to our previous observations (3, 5). The level of renin activity found in the control renal cortex by radioimmunoassay is within normal limits. With this technique, depending on the method of homogenization and the purity of the fraction, results have varied between 500 ng angiotensin I/mg protein/hr (15) to 530 μg angiotensin I/μg protein/hr (16). The fivefold increase in renin activity noted in the ischemic renal cortex is also analogous to previous values obtained by bioassay in the same type of kidney (6–8).

Since renin granules contain hydrolytic enzymes (acid phosphatase, β-glucuronidase,

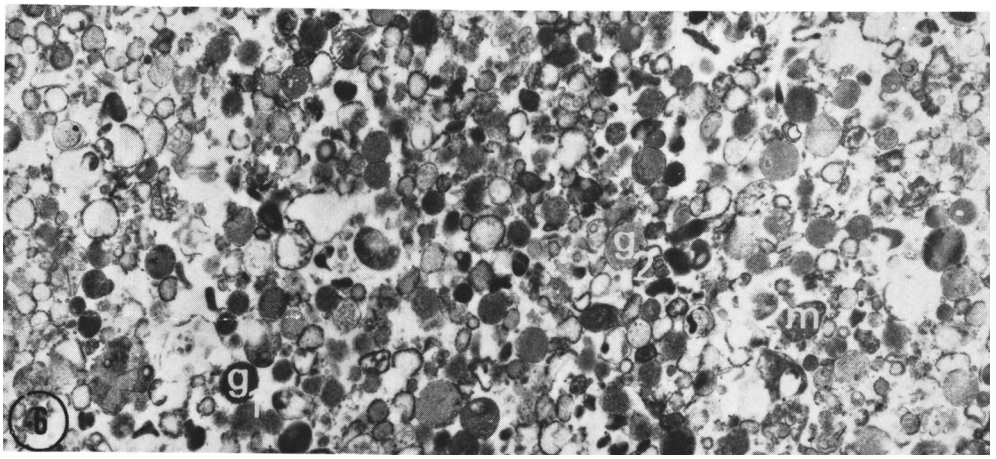

FIG. 6. Electron microscopic appearance of fraction 5 from ischemic renal cortex. Note the abundance of dense (g_1) and less electron-dense granules (g_2). Mitochondrion (m) (× 11305).

TABLE II. EFFECT OF VARIOUS DEGREES OF AORTIC CONSTRICTION ON THE PROTEIN CONTENT AND β-GLUCURONIDASE ACTIVITY OF RENAL CORTICAL HOMOGENATES AND ON KIDNEY WEIGHT, HEART WEIGHT, AND FINAL BODY WEIGHT

	Style[a]						
Group	No.	Diameter (mm)	Protein (mg/g wet wt)	β-Glucuronidase (arbitrary units/g wet wt)	Final body weight (g)	Left (ischemic) kidney weight (mg/100 g body wt)	Heart weight (mg/100 g body wt)
1	—	—	91.80 ±2.00	1220.0 ±119.0	236.00 ±8.57	396.00 ±81.20	319.00 ±42.30
2	26	0.180	72.70[b] ±3.60	1323.00[b] ±125.00	143.60[b] ±3.91	267.00[b] ±24.00	473.00[b] ±22.00
3	30	0.500	59.40[b] ±1.50	2950.00[b] ±146.00	131.70[b] ±3.12	244.00[b] ±10.00	481.00[b] ±20.00
4	18	0.740	62.10[b] ±1.40	2801.00[b] ±263.00	188.00[b] ±9.69	372.00 ±70.00	404.00 ±30.00

[a] Partial constriction of aorta using styles of various diameters was performed on the 1st day of the experiment. The animals were killed on the 10th day.

[b] Significant ($P < 0.001$) in comparison with the unoperated control.

and aryl sulfatase) (17) and in view of their enormously augmented number in the newly formed juxtaglomerular cells of the ischemic kidney (3, 5), the increase of β-glucuronidase, if not of cathepsin D, found in the ischemic renal cortex, could be localized either in juxtaglomerular cells or in renal cortical tubular cells. The first results obtained by differential centrifugation of the renal cortex indicated that renin granules were localized in the heavy mitochondrial fraction together with lysosomes (18–20) in rat, pig, and rabbit kidneys. Further work, particularly with the introduction of discontinuous sucrose density gradient centrifugation, showed that lysosomes and renin granules are in fact localized in fractions of different density in dogs (9), rats (15), and rabbits (13). It has also been demonstrated that renin granules, although they contain hydrolytic enzymes, do not behave like lysosomes (17). The present results confirm that renin granules and lysosomes of the control renal cortex behave differently as regards their localization in fractions of dissimilar density. With a technique identical to the present one, Morimoto *et al.* (9) showed that, in the dog, the top layer, as judged from the distribution pattern of enzymatic activity, was made up of the bulk of rough endoplasmic reticulum and of lysosomes, the middle layer was mostly comprised of mitochondria, and the bottom layer of renin granules. In the rat, isopycnic centrifugation of the heavy granule fraction (obtained by differential cen-

trifugation of the renal cortex) also yielded analogous results (15): Lysosomes were in the top layer (density = 1165), mitochondria in the middle (density = 1175), and renin granules at the bottom (density = 1200). We have also obtained analogous results with the ischemic renal cortex, although here the density of the top layer was lower and renin granules were more dispersed in fractions 4, 5, and 6. These differences are most probably due to the changes in shape, size, and density of lysosomes and renin granules, produced by severe ischemia (3, 5). Since juxtaglomerular cells contain only a very small number of lysosomes (21), our results indicate that the increased hydrolytic enzyme activity of the ischemic kidney is attributable to changes occurring in cortical tubular cell lysosomes, most probably of the proximal convoluted tubular cells because of their concentration of lysosomes (22). Whether these increased lysosomal enzyme activities are located either in the outer or inner cortical tubular cells or in both remains to be determined. Although we did not undertake any quantitative study, the impression was gained that there was no striking increase in the number of lysosomes in the atrophic cortical tubular cells after 10 days of ischemia. It must be emphasized that, at this time period, there are no autophagic vacuoles and no degenerating or necrotic cells in the cortical tubules.

From the data in Table II, it can be deduced that very slight renal ischemia leading to minimal atrophy still produces a significant increment in β-glucuronidase activity. This indicates that, at least in the rat, and probably in other species as well, one cannot induce even mild atrophy of the renal cortex without setting into motion a great rise in hydrolytic enzyme activity in cortical tubular cells. In view of the report indicating that injection of renal lysosomal products may induce angionecrosis and increased vascular permeability (23), experiments are now in progress to find out if, during renal hypertension, lysosomal enzymes of renal origin are present in the systemic circulation.

Severe starvation does not enhance the activity of renal lysosomal enzymes (24). Hence, the intense loss of weight occurring in rats bearing an ischemic kidney (3, 5) is not the cause of the presently found increment of renal lysosomal enzyme activity.

Since cathepsin D is able to split the leu–leu bound of angiotensinogen and thus produce angiotensin I during incubation at acid pH (25), the results of bioassay or radioimmunoassay of renin activity in ischemic kidneys must be interpreted with caution, if the incubation is done at low pH.

Numerous reports have revealed that the activity of certain hydrolytic enzymes is augmented in the urine in case of parenchymal renal damage, both in man (26–30) and in experimental animals (31–33). In controlled studies on the rat, the increased urinary excretion of hydrolytic enzymes has been correlated with degeneration leading to necrosis of cells from the proximal convoluted tubules (31, 33). Apart from the well-known effect of testosterone on mouse kidney where an increase in lysosomal enzyme activity can be detected not only in the urine but also in proximal convoluted tubular cells (34), enhanced hydrolytic enzyme activity (cathepsin and β-glucuronidase) has only been noted in nephrosclerotic rat kidneys following the administration of desoxycorticosterone and NaCl (35) and in spontaneously hypertensive rats with or without NaCl supplements, where an increase in the renal activities, not only of these two enzymes but also of those of RNAse, DNAse, and β-*N*-acetylglucosaminidase, has been observed (36). In both cases, the renin activity of the kidney was decreased. These increased hydrolytic enzyme activities have been tentatively ascribed to vascular changes produced in the kidney by hypertension (35, 36). In view of the present results, they are probably localized in renal cortical tubular cells where zones of atrophy alternating with zones of hyperplasia and hypertrophy are characteristic of nephrosclerosis.

Summary. In rats, severe but partial ligation of the aorta between the renal arteries induces striking changes in the left, ischemic renal cortex: simple atrophy of the outer cortex, and atrophy with hyperplasia and polyploidy of the inner cortical tubular cells. In the ischemic renal cortex, there is a significant increase not only of renin activity but also of β-glucuronidase, of cathepsin D and, to a much lesser degree, of acid phosphatase. The results of isopycnic centrifugation indicate

that these enhanced hydrolytic enzyme activities are localized in renal cortical tubular cell lysosomes. Since these changes occur even with minimal atrophy of the kidney, they must accompany all cases of hypertension of renal origin.

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