Increased serum apoA-IV concentrations in experimental uremic rats

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Abstract Normal histochemical analysis localizes apoA-IV within renal proximal tubules, which suggests that the kidney is a major catabolic site. In clinical renal failure and animal models of decreased renal function, low molecular weight proteins cannot be efficiently filtered through the glomerular basement membrane, and therefore they accumulate in plasma. In normal plasma, apoA-IV exists as both lipoprotein associated and lipoprotein-free, low molecular weight forms. To examine this further, uremic serum apolipoprotein and mRNA levels were examined in surgically 5/6 nephrectomized rats. Compared to sham-operated controls, uremic serum apoA-IV was elevated twofold and was distributed to a greater extent in the lipoprotein-free subfraction. Serum triglycerides were unchanged. Despite finding no correlation between serum apoA-IV and triglyceride levels (in either the d<1.006 g/ml or 1.006< d<1.019 g/ml fraction), serum apoA-IV was positively correlated with the renal function parameters of blood urea nitrogen ($r = 0.949$, $P < 0.001$), creatinine ($r = 0.952$, $P < 0.001$), and uric acid ($r = 0.903$, $P < 0.001$). In addition, the concentration of apoA-IV per milligram of renal homogenate protein in uremic rats was significantly higher than that of control rats, whereas there was no difference in the content of apoA-I between the two groups. ApoA-I, apoA-IV, and apoB mRNA levels in hepatic and in intestinal tissue were undistinguishable between the uremic and surgical sham rats. ■■ These data suggest that serum apoA-IV elevation in uremia is due to a decreased catabolism of "lipoprotein-free" apoA-IV by the kidney rather than an increased synthesis or decreased catabolism of apoA-IV associated with triglyceride-rich lipoproteins. — **Seishima, M., H. Torizawa, Y. Muto, and A. Noma.** Increased serum apoA-IV concentrations in experimental uremic rats. *J. Lipid Res.* 1992. **33:** 1441–1447.

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It is well known that apoA-IV in humans is exclusively synthesized in the small intestine (1–4). The bulk of apoA-IV appears to be secreted on intestinally derived chylomicrons; however, in plasma the protein appears mainly unassociated with lipoproteins and, to a much lesser extent, HDL (5, 6). Although recent studies have suggested that apoA-IV may play a role in LCAT activation (7), binding of HDL to cell surfaces (8), cholesterol efflux (9), modulation of lipoprotein lipase activity (10), and appetite suppression (11), its exact physiologic function remains unclear. During intestinal fat absorption, the synthesis, secretion, and plasma levels of apoA-IV markedly increase (12–15). In a variety of human pathological states of impaired fat absorption, including abetalipoproteinemia (5), chronic pancreatitis (16), and malabsorption syndrome (16), and in subjects receiving total parenteral nutrition (17, 18), diminished plasma apoA-IV levels have been observed. Overall, these observations suggest a direct relation between intestinal lipid absorption and apoA-IV secretion. In contrast, chronic renal failure, a condition unrelated to fat absorption, results in marked elevation of plasma apoA-IV (19, 20). Although Nestel, Fidge, and Tan (19) reported that the elevated apoA-IV observed in human chronic renal failure reflects the impaired clearance of triglyceride-rich lipoproteins, we have previously provided evidence suggesting that the elevated levels instead directly relate to renal function (20). The purpose of the present study was to confirm whether the rat model of uremia shows high serum apoA-IV concentration and to clarify, if so, the mechanism for the elevation. Although nephrotoxic agents can be used to induce renal failure (21), effects on other organ systems cannot be ruled out. Therefore, in the present studies we surgically induced 5/6 nephrectomy in rats to further explore the relation between apoA-IV clearance and renal function.

MATERIALS AND METHODS

Induction of uremia

Male Wistar rats (200–240 g) were individually housed in metabolic cages in a temperature-controlled room (20 ± 1°C) throughout the study. Renal failure (5/6 nephrectomy) was induced by a two-stage surgical procedure (22). Surgery was performed on rats under light diethyl ether anesthesia. Briefly, in the initial operation, a midline abdominal incision was made, and both poles and the lateral cortex of the left kidney were excised resulting in a 2/3 nephrectomy of this organ. Seven days later, a right total nephrectomy was performed. Animals were allowed standard rat chow and water ad libitum. Control rats were subjected to comparable sham surgical procedure and were pair-fed.

Determination of serum lipids and other chemical substances

Triglyceride (TG), total cholesterol (TC), phospholipid (PL), and free fatty acid (FFA) were enzymatically measured using an automatic analyzer (Hitachi 736 60E, Japan). In addition, TG concentration in the d < 1.006 g/ml fraction (TRL; chylomicron plus VLDL) and 1.006 < d < 1.019 g/ml fraction (IDL) was determined (23). TG, TC, and PL of HDL (1.063 < d < 1.21 g/ml) were determined as described above. Protein was assayed according to the method of Lowry et al. (24) using BSA as a standard. Blood urea nitrogen (BUN), creatinine (Cr), and uric acid (UA) were determined by the automatic analyzer.

Determination of serum apolipoprotein levels and the distribution of apolipoproteins

Serum apolipoproteins (apoA-I, apoA-IV, and apoB) were measured by rocket immunoelectrophoresis as previously reported (25). Apolipoprotein concentrations were compared to those of a pooled reference serum (from non-treated rats). Each apolipoprotein from this reference pool was assigned a concentration value of 100 arbitrary units (AU). Apolipoproteins (A-I and A-IV) associated with and without (i.e., free) lipoproteins were quantitated in the d < 1.21 g/ml and the d > 1.21 g/ml fractions, respectively. Although it is well known that ultracentrifugation can lead to apolipoprotein dissociation from lipoproteins (26–28), all samples were isolated and treated in an identical manner. Therefore, comparisons between the two experimental groups could be made, despite any artifacts introduced by the isolation procedure.

RNA extraction and analysis

Total RNA was extracted from livers thoroughly perfused with ice-cold PBS (pH 7.4). For small intestinal total RNA, the intestinal contents were flushed out with ice-cold buffer. The small intestine was then divided into jejunum and ileum segments and the mucosa was scraped off with a glass slide. The intestinal mucosa and the liver were homogenized in 6 M urea, 3 M LiCl solution containing SDS for 2 min with a Polytron. RNA was precipitated overnight at 4°C, pelleted by centrifugation, and extracted by phenol–chloroform. Apolipoproteins A-I, A-IV, and B mRNA relative to poly A mRNA levels were determined by slot-blot hybridization as previously described in detail (29). Briefly, filters were sequentially probed for apoA-I, apoA-IV, apoB, and polyadenylated RNA with ^{32}P-labeled oligonucleotide probes. Between hybridization steps, filters were washed twice at 75°C for 1 h in 5 mM Tris-HCl, pH 8.0, 2 mM Na$_2$EDTA, 0.1 × Denhardt's solution, and confirmed radioactivity-free prior to rehybridization with successive probes. Hybridizations and washings were carried out at 55°C for ^{32}P-A-I, 60°C for ^{32}P-A-IV, 50°C for ^{32}P-B, and room temperature for ^{32}P-oligo d(T)$_{18}$ (Sigma), respectively, under conditions described by Church and Gilbert (30). The relative signal intensities for apolipoproteins and polyadenylated mRNA were calculated by quantitative scanning densitometry using a ACD-25DX Computing Densitometer (ATTO, Tokyo).

Immunohistochemical analysis

Protein A affinity-purified IgG from control rabbits, and rabbit anti-rat apoA-I and rabbit anti-rat apoA-IV sera were used for immunohistochemical studies. To prepare tissue sections, normal kidneys (from the sham-operated animals) or the partially 2/3 nephrectomized left kidney were thoroughly perfused by with PBS via the renal artery and then promptly frozen in liquid nitrogen. Ethanol-fixed specimens were sliced (5 μm) and subsequently immunostained by the peroxidase–antiperoxidase technique using a Histoscan-SP Kit (Zymed Laboratories Inc., South San Francisco, CA). The staining was carried out according to instructions supplied with the kit.

Apolipoprotein quantitation in the renal tissue

ApoA-I and apoA-IV in the kidney from control and uremic rats were determined. The kidney was removed after extensive perfusion with PBS (pH 7.4) and the whole kidney was homogenized in 1 ml of PBS containing 1% Triton X-100, 1 mM PMSF, 1 mM trypsin inhibitor, 1 mM benzamidine, pH 7.4, for 20 sec with a Polytron, and a 105,000 g supernatant was prepared. Aliquots of homogenate were removed for protein assay by the method of Lowry et al. (24). Apolipoproteins were quantitated by rocket electroimmunoassay using our rat reference serum. Appropriate dilutions of the tissue extracts were made to fall within the linear portion of the assay. No additional apoA-I or apoA-IV were detected after re-extraction of the 105,000-g pellets.

TABLE 1. Body weight serum lipid and triglyceride levels in lipoprotein fractions

	Body Weight	TG	TC	PL	FFA	HDL	TRL-TG	IDL-TG
	g		*mg/dl*		*mEq/l*		*mg/dl*	
Control (n = 7)	222 ± 4.5	40.4 ± 14.3	68.9 ± 9.8	109 ± 12.1	0.64 ± 0.16	209 ± 25[a]	30.4 ± 11.7	0.74 ± 0.41
Uremic (n = 7)	228 ± 11.9	47.0 ± 18.2	75.0 ± 13.5	123 ± 15.4	0.85 ± 0.13[b]	222 ± 34[a]	38.3 ± 15.8	1.33 ± 0.70

Values are mean ± SD. Abbreviations: TG, triglyceride; TC, total cholesterol; PL, phospholipid; FFA, free fatty acid; TRL, triglyceride-rich lipoprotein (d<1.006 g/ml); IDL, intermediate density lipoprotein (1.006<d<1.019 g/ml); HDL, high density lipoprotein (1.063<d<1.21 g/ml).
[a]N = 5.
[b]$P < 0.05$ compared to control.

Statistical analyses

Data are expressed as mean ± SD. Statistical analyses were determined using Student's two-tailed t test. Linear regression analysis was used to determine the correlations between serum apoA-IV concentrations and the levels of BUN, Cr, and UA.

RESULTS

In preliminary experiments, BUN, Cr, and apoA-IV were monitored at 10-day intervals for up to 30 days after the two-stage operation in the 5/6 nephrectomized and control sham rats (three/group). Ten days postsurgically, BUN, Cr, and apoA-IV peaked at 61 ± 8.8 mg/dl, 1.0 ± 0.2 mg/dl, and 202 ± 23 AU, respectively, in the uremic rats. At 30 days, however, these parameters gradually decreased in parallel (BUN = 43 ± 5.8 mg/dl, Cr = 1.5 ± 0.2 mg/dl, and apoA-IV = 146 ± 18 AU). The gradual reduction in BUN and Cr levels may, in part, be explained by a compensatory response of the remaining uremic kidney. Similarly, Ormond and Miller (31) noted a gradual postsurgical reduction of BUN and Cr. On the basis of the values for BUN and Cr, our model corresponded to mild type uremia according to the criteria of Ormrod and Miller (31). On the other hand, apoA-IV, BUN, and Cr levels did not change during the observation period in control rats.

In additional preliminary experiments, acute renal failure was induced by total instead of 2/3 left nephrectomy during the first surgical procedure. Rats survived only a few days after the second surgical procedure. We observed an approximate 10-fold increase in BUN and Cr compared to presurgical levels (data not shown). In this model, apoA-IV was markedly decreased compared to presurgical values. However, caloric intake of these animals was severely reduced compared to the 5/6 nephrectomized rats. Although we did not study this acute model further, we suspect the near-starvation state decreased intestinal apoA-IV secretion which is thereby reflected in reduced serum apoA-IV. In the 5/6-nephrectomized rats, 5-day postsurgical food consumption became relatively constant at approximately 13 g/day. To minimize any effects absorption might have on intestinal apolipoprotein synthesis and secretion, we pair-fed equivalent calories to sham-operated controls. We observed no difference in body weight between the groups (**Table 1**).

Serum lipid levels and TG levels in the lipoprotein fractions are shown in Table 1. Although FFA was elevated in uremic rats ($P < 0.05$), no differences were found for other lipids. Furthermore, we observed no difference in TRL or IDL TG levels or HDL concentrations between groups. The chemical composition of HDL was also unchanged (data not shown). In uremic rats, serum apoA-IV concentrations were elevated approximately twofold ($P < 0.001$), whereas apoA-I and apoB levels were similar to controls (**Table 2**). As expected, BUN, Cr, and UA levels were significantly elevated in the uremic rats.

Apolipoprotein distribution between the d<1.21 g/ml and d>1.21 g/ml fractions is shown in **Table 3**. When represented as either absolute amounts (i.e., in AU) or percent distribution, apoA-IV was found predominantly in the lipoprotein-free fraction in uremic rat serum. No difference in the distribution of apoA-I was observed between the two groups.

TABLE 2. Serum apolipoprotein and parameter levels of renal function

	ApoA-I	ApoA IV	ApoB	BUN	Cr	UA
	AU			*mg/dl*		
Control (n = 7)	91.7 ± 5.9	91.4 ± 14.7	102 ± 17.1	15.2 ± 2.1	0.69 ± 0.07	2.19 ± 0.38
Uremic (n = 7)	94.5 ± 7.6	174 ± 27.5[a]	107 ± 7.9	56.1 ± 7.9[a]	1.46 ± 0.23[a]	3.81 ± 0.56[a]

Values are mean ± SD. Abbreviations: AU, arbitrary unit; BUN, blood urea nitrogen; Cr, creatinine; UA, uric acid.
[a]$P < 0.001$ compared to control.

TABLE 3. Distribution of apoA-I and apoA IV

	ApoA-I	ApoA IV
	%	
Control (n = 5)		
d<1.21 g/ml	92.5 ± 10.4	59.8 ± 4.6
d>1.21 g/ml	7.2 ± 2.0	40.6 ± 5.9
Uremic (n = 5)		
d<1.21 g/ml	89.4 ± 12.1	40.4 ± 6.5
d>1.21 g/ml	8.1 ± 3.2	62.4 ± 9.3[a]

Values are mean ± SD.

[a]$P < 0.001$ compared to control.

Liver and intestinal apolipoprotein mRNA levels are shown in **Table 4.** We found no differences between apoA-I, apoA-IV, or apoB mRNA levels in either organ between the groups. Correlation analysis between serum apoA-IV levels and BUN, Cr, or UA concentrations showed strong positive correlations (for each parameter $P < 0.001$) (**Fig. 1**). In contrast, serum apoA-IV levels did not correlate with either serum, TG or TRL- or IDL-TG ($r = 0.230, 0.383, 0.374$, respectively).

Histochemical analysis indicated that both apoA-I and apoA-IV are localized within proximal tubular cells and in the tubular lumen in both uremic and control kidneys. Hypertrophic changes were obvious in the remaining uremic kidney (**Fig. 2**). Renal apoA-IV content in the tissue homogenate (A-IV AU/mg protein) from uremic rats was significantly higher than that of controls (**Table 5**). No difference in renal apoA-I was observed between groups (Table 5).

DISCUSSION

In the present study we investigated the role renal tissue may play in the clearance of apolipoproteins. Our initial studies focused on development of an appropriate animal model representative of chronic renal failure. Total surgical nephrectomy and chemical-induced renal toxicity were considered to be inappropriate models since animals rejected caloric intake postsurgically and nonspecific chemical effects could not be excluded, respectively. Instead we investigated a near total model of renal damage, the 5/6-nephrectomized rat. These animals recovered postsurgically and the amount of food intake became constant. They also expressed classical serum parameters of chronic renal failure. However, since the compensation of renal function by the partially damaged organ was evident in our preliminary experiment, animals were killed 10 days after the second operation.

In the current study, we observed elevation of serum apoA-IV levels in the experimental uremic rats for the first time. However, this surgical model failed to demonstrate the elevation of serum TG levels or the accumulation of TG-rich lipoproteins. Previous reports (32–34) have shown high TG levels in uremic rats, possibly due to the decrease in lipoprotein lipase activity (35). In the present study, though FFA levels in uremic rats were significantly higher than in control rats, TG levels were undistinguishable between the two groups. This finding is consistent with the data of Heuck et al. (33) who showed that TG levels were unchanged in the first week after the operation and afterwards gradually increased, reaching a maximum in the fourth week. Therefore, serum TG levels may depend on the duration of uremia.

A striking and unexpected finding in the uremic animals was a strong positive correlation between a markedly elevated serum apoA-IV and parameters suggestive of renal failure (BUN, Cr, and UA). Determinations of hepatic and intestinal apoA-IV mRNA levels were undistinguishable between uremic and control rats. Nestel et al. (19) have suggested that the accumulation of triglyceride-rich lipoproteins in patients with chronic renal failure causes a significant increase in apoA-IV levels. However, our prior work (20) suggests that apoA-IV elevation in chronic renal failure is directly related to the renal function rather than the impairment of TG-rich lipoprotein catabolism. The current data confirm our previous clinical findings and our apoA-IV mRNA data suggest that the apoA-IV elevation observed in chronic renal failure is likely a result of decreased renal

TABLE 4. Apolipoprotein mRNA levels in the liver and small intestine

	ApoA-I			ApoA IV			ApoB		
	Liver	Jejunum	Ileum	Liver	Jejunum	Ileum	Liver	Jejunum	Ileum
	RAU								
Control (n = 5)	108 ± 22	78.8 ± 15	40.4 ± 13	87.6 ± 22	55.8 ± 15	29.4 ± 8.3	175 ± 44	36.8 ± 14	26.9 ± 11
Uremic (n = 5)	125 ± 33	67.3 ± 23	35.9 ± 9.8	63.3 ± 23	64.3 ± 23	21.9 ± 6.8	159 ± 38	33.0 ± 12	28.6 ± 9.0

Values are mean ± SD. Abbreviations: RAU, relative absorbance unit. No significant difference was observed in apolipoprotein mRNA levels between control and uremic rats.
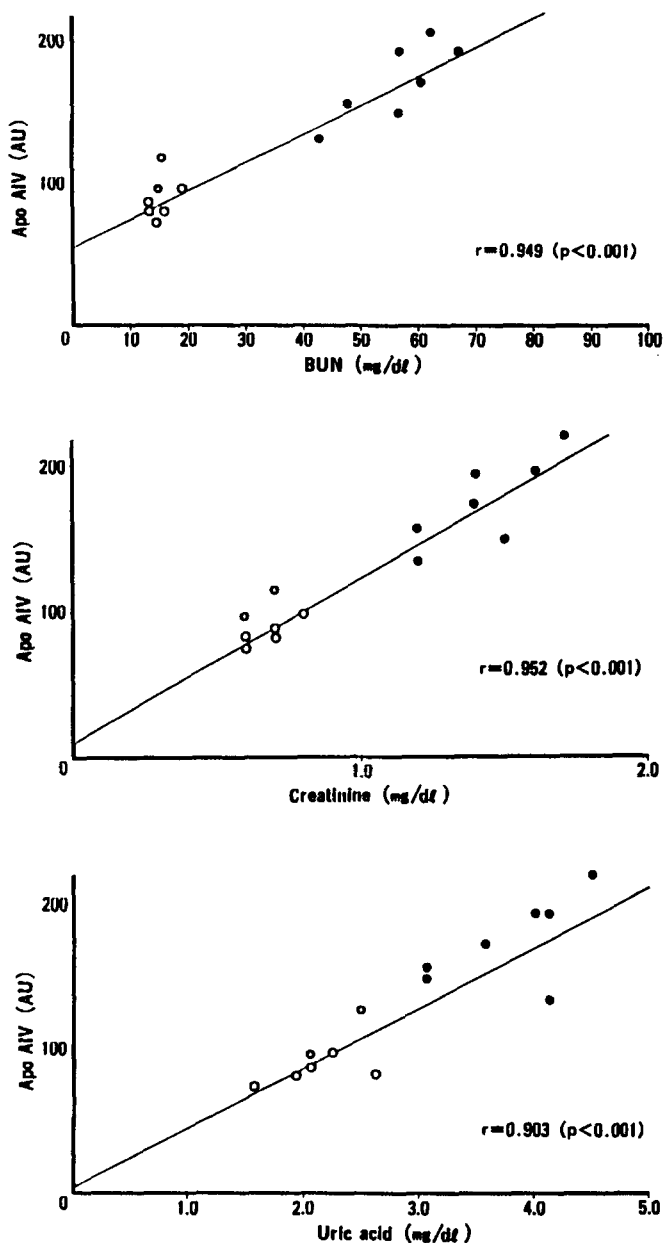
Fig. 1. Correlation of serum apoA-IV concentrations with BUN (upper panel), creatinine (middle panel), and uric acid (lower panel) levels. Significant positive correlations were observed in all cases. (O) control rats (n = 7); (●) uremic rats (n = 7).

catabolism rather than increased synthesis. However, we cannot exclude the possibility that the accumulation of TG-rich lipoproteins affected the serum apoA-IV level. Further experiments will be necessary to clarify this, using a long-term uremia model showing higher TG level as described above.

Our immunohistochemical analysis of renal apoA-I and apoA-IV demonstrates localization within renal proximal tubular cells of both the normal and remaining uremic kidney. In this regard, it has been considered that kidneys play a role in the degradation of apolipoproteins (36, 37). Dallinga-Thie, van't Hooft, and van Tol (38) have shown that both apoA-I and apoA-IV accumulate in the liver and kidney of leupeptin-treated rats. Other studies have reported urinary HDL in normal subjects (39), and HDL reabsorption by renal tubules (40). However, it is not yet completely understood as to whether HDL can pass through glomeruli.

Considering the molecular weight size (41), at least some of free apoA-IV (mol wt 46,000) (42) can be filtered by glomeruli. It could be taken up afterwards, by proximal tubular cells in the same fashion as other low molecular weight proteins and eventually degraded there. From this point of view, the reduction in glomeruli can decrease the amount of free apoA-IV to be filtered. Additionally, a significant amount of apoA-IV was found in the $d > 1.21$ g/ml fraction in the uremic rat, leading to a much higher ratio of free apoA-IV to the number of glomeruli. The content of apoA-IV in renal tissues may reflect the increase in the net amount of free apoA-IV to be filtered by glomeruli and taken up by proximal tubular cells in uremic rats. However, it will be necessary to quantify apoA-IV within tubular cells only in order to ascertain this point. On the other hand, with respect to the molecular weight size, apoA-I (mol wt 28,400) (43) should be considered to be more readily filtered by glomeruli than apoA-IV. In fact, apoA-I was also detected within tubular cells in both control and uremic rat kidneys. However, we failed to find a significant difference in serum apoA-I levels, in its distribution in serum, and also in the content of apoA-I in the renal tissues between control and uremic rats. This may be associated with the different distribution in serum between these two apolipoproteins. That is, apoA-I is largely associated with HDL (200,000–3000,000 mol wt particles), while approximately half the serum apoA-IV in the rat is unassociated with lipoproteins (i.e., free apoA-IV) (28). Therefore, the net amount of apoA-I to be filtered by glomeruli might be less than that of A-IV. Alternatively, the liver may be more responsible for the degradation of apoA-I than kidney. Finally, apoA-I results may be due to the susceptibility to self-association of apoA-I in the lipoprotein-free fraction (44).

Since the remaining kidney from 5/6 nephrectomized rats is considered to be intact and its function would be gradually compensated, the current animal model may not correspond exactly to a model of stable chronic renal failure. However, in terms of the reduction in the degradation site for apoA-IV, this model is available for studying the metabolic behavior of apoA-IV. In patients with chronic renal failure, especially on hemodialysis, the kidneys have almost no function and even show an atrophic change. From the view point of the blood circulation system, there exists the arterio-venous shunt leading to the
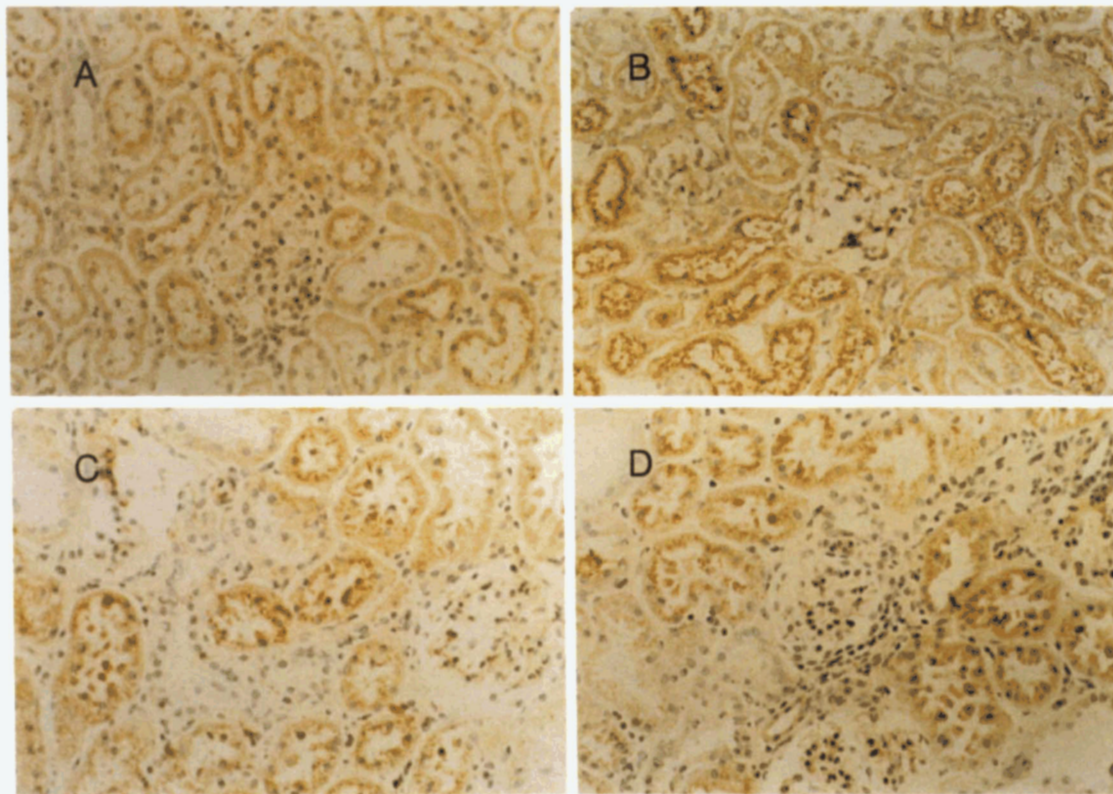
Fig. 2. Immunoperoxidase staining for apoA-I and apoA-IV in kidneys from control and uremic rats. A, B: apoA-I and apoA-IV in the kidney from a control rat, respectively. C, D: apoA-I and apoA-IV in the remaining uremic kidney. Both apoA-I and apoA-IV are localized within proximal tubular cells and are not seen in glomeruli. Note the hypertrophic change in the uremic kidney (original magnification: ×200).

reduction in the blood flow through the glomeruli in this disease. Consequently, the amount of free apoA IV filtered by glomeruli could be decreased and the degradation of apoA-IV reduced in tubular cells. This will eventually cause the high apoA-IV levels in serum. Taken together, the elevation of serum apoA-IV levels in uremia is considered to be due mainly to the decrease in its catabolic sites. ▣

TABLE 5. Quantitation of apoA-I and apoA-IV in the kidney

	ApoA-I (AU × 10⁻³)	ApoA-IV (AU × 10⁻³)
Control (n = 5)	8.9 ± 1.1	31.6 ± 4.6
Uremic (n = 5)	9.4 ± 1.9	55.1 ± 9.0[a]

Values are expressed per milligram of kidney homogenate protein ± SD.
[a]$P < 0.001$ compared to control.

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