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Hydrocarbon-Induced Hyaline Droplet Nephropathy in Male Rats during Senescence

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Hydrocarbon-Induced Hyaline Droplet Nephropathy in Male Rats during Senescence. MURTY, C. V. R., OLSON, M. J., GARG, B. D., AND ROY, A. K. (1988). *Toxicol. Appl. Pharmacol.* **96**, 380–392. Male rats administered unleaded gasoline rapidly develop nephropathy characterized by accumulation of hyaline droplets in cells of the proximal convoluted tubules (PCT). This acute response is implicated in development of renal carcinoma in male rats exposed chronically to wholly volatilized gasoline. A major constituent of hyaline droplets is α_{2u}-globulin, a protein of hepatic origin for which the rate of synthesis declines during aging. Little information, however, is presently available on possible age-dependent susceptibility of male rats to hydrocarbon-induced nephropathy. In kidneys of untreated male Fischer 344 rats the number of constitutive hyaline droplets declined progressively with increasing age. Electrophoresis of renal cortical homogenates revealed a protein with M_r about 18×10^3, probably α_{2u}-globulin, in young (3.5 months old) male rats and total absence of this protein in aged (26 months old) males. RIA confirmed that constitutive levels of renal and hepatic α_{2u}-globulin in old rats were less than 1.5% of those in young adults. Unleaded gasoline (0.4 ml/kg/day, po, 5 days) caused accumulation of hyaline droplets in renal PCT of 3.5-month-old males accompanied by a marked increase (about twofold) in the renal content of α_{2u}-globulin, whereas the same treatment was without effect in 26-month-old rats. Finally, in the renal cortex of young rats the activities of the lysosomal proteases cathepsin B and D were increased following gasoline administration, presumably in response to protein accumulation. However, in 26-month-old rats cathepsin B activity was unaffected, while cathepsin D was increased by gasoline administration. Thus, we conclude that animal age is an important determinant in the development of hydrocarbon-induced nephropathy and only rats which produce large amounts of α_{2u}-globulin are susceptible to development of this pathology.

INTRODUCTION

Acute or subchronic exposure of rats to a variety of pure hydrocarbons and mixtures such as unleaded gasoline has been reported to induce hyaline droplet nephropathy, a condition characterized by excessive accumulation of proteinaceous material in cells lining the renal proximal convoluted tubules (PCT), with minimal abnormality in renal function (Table 1). Such alterations in renal morphology and function are largely specific to male

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TABLE I

CHEMICALS REPORTED TO CAUSE HYALINE DROPLET NEPHROPATHY IN MALE RATS

Chemical and usage classification	Reference
Food constituents	
D-Limonene	Webb and Alden, 1987
Fuels and fuel components	
Decahydronaphthalene (Decalin)	Alden *et al.*, 1984; Kanerva *et al.*, 1987
Jet fuels (RJ-5, JP-4, JP-5, JP-10)	Bruner, 1984
Naphthas (various)	Halder *et al.*, 1984
Tetralin	Serve *et al.*, 1988
2,2,4-Trimethylpentane	Stonard *et al.*, 1986; Short *et al.*, 1987
Unleaded gasoline	Halder *et al.*, 1984; Olson *et al.*, 1987
Solvents	
60 Solvent, 70 solvent	Phillips and Cockrell, 1984
C_{10}-C_{12} Isoparaffinic solvent	Viau *et al.*, 1986
Diisobutyl ketone	Dodd *et al.*, 1987
Methyl isobutyl ketone	Phillips *et al.*, 1987
Napthenic aromatic solvent	Phillips and Cockrell, 1984
Pentachloroethane	Goldsworthy *et al.*, 1987
Perchloroethylene	Goldsworthy *et al.*, 1987
Stoddard solvent	Phillips and Cockrell, 1984
VM & P naphtha	Phillips and Cockrell, 1984
Miscellaneous	
p-Dichlorobenzene (deoderant)	Bomhard *et al.*, 1988
Isophorone (wood preservative)	Strasser *et al.*, 1988

rats less than 12 months of age when treated with these hydrocarbon toxicants.

A protein unique to the male rat, α_{2u}-globulin, is a primary constituent of the hyaline droplets accumulated during hydrocarbon exposure (Stonard *et al.*, 1986; Garg *et al.*, 1987; Kanerva *et al.*, 1987; Olson *et al.*, 1987). The presence of α_{2u}-globulin has been suggested, although not proven, to be the critical factor which renders male rats susceptible to chemically induced hyaline droplet nephropathy (Alden *et al.*, 1984; Gibson and Bus, 1988; Olson *et al.*, 1987, 1988). α_{2u}-Globulin is synthesized in the liver and the expression of this protein is strongly age dependent as shown by decreasing hepatic synthesis and urinary excretion of α_{2u}-globulin with increasing age (Roy *et al.*, 1983). The decline, and eventual cessation, of α_{2u}-globulin production is due to development of hepatic androgen insensitivity during senescence (Roy *et al.*, 1983; Sarkar *et al.*, 1987). However, the toxicity studies conducted with chemicals shown to cause hyaline droplet nephropathy in male rats have been performed with rats at ages when the synthesis and excretion of α_{2u}-globulin is near maximal (Table 1). Since α_{2u}-globulin synthesis declines during aging, we sought to determine whether a relationship existed between levels of α_{2u}-globulin and susceptibility to hydrocarbon-induced nephropathy. Furthermore, since alterations of lysosomal morphology are known to accompany hydrocarbon-induced α_{2u}-globulin accumulation (Phillips and Cockrell, 1984; Garg *et al.*, 1987; Kanerva *et al.*, 1987; Short *et al.*, 1987), we characterized the response of several key renal lysosomal enzymes involved in proteolysis to determine whether changes in the activity of these enzymes might be associated with gasoline-induced hyaline droplet nephropathy and how such changes might vary with animal age. The results show that, unlike the toxicity of certain therapeutic agents which is increased in aged rats (reviewed by Goldstein *et*

al., 1988), the nephrotoxicity associated with hydrocarbon exposure is decreased with age, probably due to the decline in α_{2u}-globulin production. Thus, when male rats are used in the evaluation of nephrotoxic potential of chemicals, especially those agents which cause hyaline droplet nephropathy, animal age is an important consideration in experimental design and interpretation of data.

MATERIALS AND METHODS

Animals and treatment protocol. Male Fischer 344 rats (CDF (F-344)Crl BR, VAF/Plus) obtained from Charles River Laboratories, Kingston, New York or the National Institute of Aging (strain F344/NHsd, maintained by Sprague–Dawley, Inc. Indianapolis, IN), were housed in the GMR Biomedical Science Department vivarium in conditions conforming to AAALAC recommendations with tap water and Purina 5012 chow freely available. Untreated rats of ages 3.5 (n = 6), 18 (n = 3), or 26 (n = 10) months were used for appraisal of renal morphology as a function of age. Other male rats, ages 3.5 or 26 months received either commercially available unleaded gasoline (0.4 ml/kg/day, 5 days; six 3.5-month-old and six 26-month-old rats) or 0.9% sodium chloride solution (0.4 ml/kg/day, 5 days; four young and four old rats) by intragastric intubation and were euthanized 24 hr after the final dose. Tissues from each of these rats was used for light and electron microscopy of kidney, RIA determination of renal and hepatic α_{2u}-globulin content, electrophoretic characterization of soluble renal proteins, and renal cathepsin activity measurements as described below. The chemical composition of the test material has been published previously (Olson *et al.*, 1987). Except as specified, all chemicals and reagents were supplied by Sigma Chemical Co. (St. Louis, MO).

Light and electron microscopic histology. While under pentobarbital anesthesia, whole body transcardiac perfusion was performed with 0.9% sodium chloride solution, 4°C. Samples of renal cortex were removed and fixed by immersion in 10% neutral buffered formalin. Tissues were processed for light microscopy by routine methods, embedded in glycol methacrylate, and stained with Lee's methylene blue–basic fuchsin method (Bennet *et al.*, 1976) after sectioning. Alternatively, samples of renal cortex were fixed in 2.5% glutaraldehyde, 0.1 M sodium cacodylate (pH 7.4). These tissues were postfixed in 1% osmium tetroxide in 0.1 M *sym*-collidine buffer (pH 7.4), embedded in Spurr's medium, sectioned to 1-μm thickness, mounted and stained with 1% toluidine blue (Richardson *et al.*, 1960) for light microscopy. Thin (ca. 60 nm) sections were cut and stained with uranyl acetate/lead citrate prior to inspection by transmission electron microscopy.

Electrophoresis of renal cortical proteins. Electrophoretic separation of soluble renal cortical proteins was accomplished with SDS-containing polyacrylamide gels (SDS–PAGE) using a 15% resolving gel with a 3% acrylamide stacking gel (Laemmli, 1970). Kidneys, frozen in liquid N_2 following whole body perfusion with 0.9% sodium chloride solution, were rapidly thawed and the medulla was removed and discarded. A 12.5% homogenate of the cortex was prepared in 0.3 M sucrose buffer (pH 7.0) with a Teflon–glass tissue grinder. A supernatant fraction (150g, 10 min, 4°C) of homogenized renal cortex was prepared and a portion added to 0.0625 M Tris–HCl (pH 6.8), 2% 2-mercaptoethanol, 2% SDS, 10% sucrose buffer and denatured at 100°C for 3 min. Samples (100 μg of supernatant protein) and heat-denatured marker proteins of known molecular weight were subjected to electrophoresis for about 5 hr at a constant current of 50 mA. Proteins were fixed in a methanol–acetic acid–water solution (5:5:2) and visualized by staining in 0.1% Coomassie blue made in the fixative solution (Sinclair and Rickwood, 1981).

Radioimmunoassay of α_{2u}-globulin. Cytosolic fractions were prepared from kidney or liver homogenates and assayed for α_{2u}-globulin by double-antibody radioimmunoassay (RIA) as described by Roy (1977). α_{2u}-Globulin in the cytosolic fraction was expressed relative to total protein (Lowry *et al.*, 1951).

Lysosomal enzyme activity determinations. Lysosomal enzyme activities were assayed in samples of the same supernatant fraction (PNS) prepared for electrophoresis. Determination of protein quantities used in activity measurements of lysosomal enzymes was performed using the BCA protein assay reagent kit (Pierce Chemical Co., Rockford, IL).

Acid phosphatase activities were determined from the amount of inorganic phosphate (P_i) liberated by the hydrolysis of β-glycerophosphate (Trouet, 1974). Reagent blanks containing no PNS protein were used for correction of nonenzymatic phosphate production. The amount of P_i released was established in absolute terms by comparison to an inorganic phosphate standard curve. Enzyme activity was expressed as nanomoles P_i released from β-glycerophosphate per minute per milligram PNS protein.

Cathepsin B activities were determined by fluorometric measurement of free β-naphthylamine released from *N*-carboxybenzoyl-L-alanyl-L-arginyl-L-arginyl-4-methoxy-β-napthylamide·2 HCl (Kugler and Vornberger, 1986). Verification that hydrolysis was catalyzed enzymatically was obtained by analyzing random homogenate samples in the presence of leupeptin (propionyl-L-leucyl-L-leucyl-L-argininaldehyde; 5 μM), an inhibitor of cathepsin B (Barrett, 1973). In all cases, addition of leupeptin resulted in greater than 90% inhibition of release of β-napthylamine from the peptide–napthylamine substrate. Cathepsin B activity was expressed relative to fluorescence values derived from standard concentrations of free β-naphthylamine and expressed as nanomoles product formed per minute per milligram PNS protein.

Cathepsin D activity was assayed by determining enzymatically catalyzed degradation of hemoglobin to acid-soluble peptides (Turk *et al.*, 1984). After incubation, protein content of acidified and filtered samples was determined relative to a standard curve prepared from L-tyrosine (Turk *et al.*, 1984). Enzyme activity was expressed as micromoles L-tyrosine released per minute per milligram PNS protein. Random PNS samples were incubated with pepstatin (isovaleryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid; 5 μM), a specific inhibitor of cathepsin D activity (Turk *et al.*, 1984), which caused near total loss of hemoglobin hydrolysis.

RESULTS

Renal Hyaline Droplets during Aging in Untreated Rats

Methylene blue–basic fuchsin staining. In renal tissue of untreated 3.5-month-old rats, the methylene blue–basic fuchsin staining method identified numerous small (≤0.5μm diam) hyaline droplets in epithelial cells of the PCT (Fig. 1A). These droplets were round or oval in cross section, uniform in size, and heterogeneously distributed within the cytoplasm of individual cells as described previously by Short *et al.* (1986, 1987). Infrequently, a single cell of the epithelial layer was observed which contained droplets with angular profiles or which was engorged with hyaline droplets. Macrodistribution of hyaline droplets was observed with cords of droplet-containing PCT cells extending from the renal capsule to the outer stripe of the medulla.

In contrast to tissues from 3.5-month-old rats, examination of kidney sections from untreated 18-month-old male rats revealed very few droplets which stained positively with the methylene blue–basic fuchsin method (Fig. 1B). The absolute number of droplets both on a per cell and a tissue area basis appeared to be reduced when comparing 18-month-old to 3.5-month-old rats. In general, the droplets visible in 18-month-old rats were distributed randomly within the cytoplasm of individual PCT epithelial cells but were smaller in diameter (ca. 0.2 μm) than the droplets observed in 3.5-month-old rats. Macrodistribution of PCT containing hyaline droplets within the kidney cortex was not apparent in 18-month-old animals. The number of droplets in animals aged 26-months was reduced greatly compared to rats at either 3.5 or 18 months. In viewing many microscopic fields of renal cortex from untreated 26-month-old rats it was impossible to identify any hyaline droplets with the methylene blue–basic fuchsin stain (Fig. 1C).

Toluidine blue staining. To obtain enhanced resolution at light microscopic magnification, 1-μm-thick sections of kidney cortex embedded in Spurr's medium were examined after preparation with toluidine blue stain. Hyaline droplets observed in the renal tissue of 3.5-month-old rats prepared with this method were nearly identical in number, size, shape, and distribution to those described in glycol methacrylate-embedded tissues prepared with methylene blue–basic fuchsin staining (*vide supra*) (Fig. 2A).

Furthermore, in renal tissues from saline-treated 26-month-old rats prepared in this manner, hyaline droplets were clearly visible in some PCT epithelial cells (Fig. 2C) although the quantity of these droplets was reduced from that observed in young male rats. Unlike the droplets in young rats, those in old rats appeared to be heterogeneous in size and shape with numerous irregular, enlarged droplets apparent (Fig. 2C). In addition, the distribution of hyaline droplets throughout the cortex of old rats was more variable than in young rats; areas were visible in which numerous small droplets of uniform size existed, other areas contained only a few large droplets of varying size.

Effect of Unleaded Gasoline on Renal PCT Morphology and Ultrastructure

Young rats. Renal PCT epithelial cells of saline-treated young rats contained numerous phagolysosomes (secondary lysosomes) distinguishable by electron microscopy (Fig. 3A). These organelles were mem-

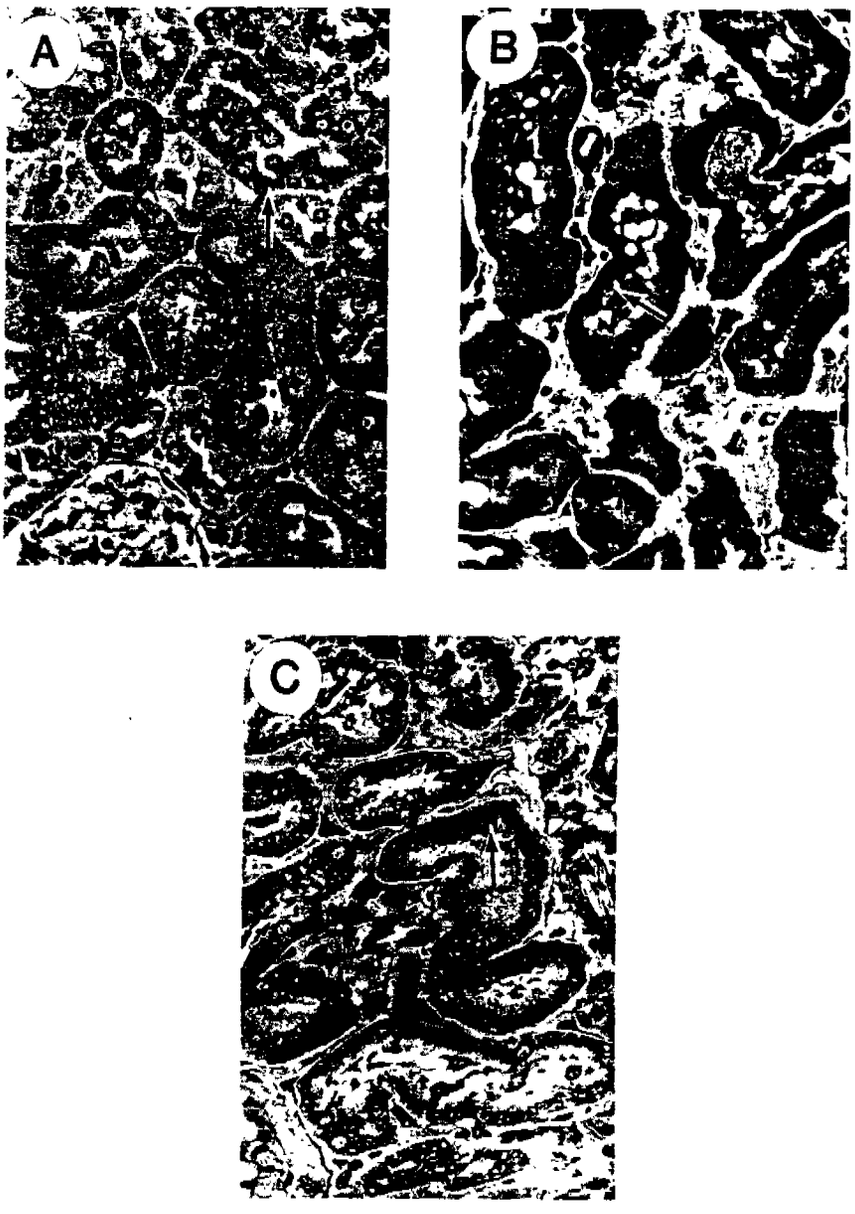

FIG. 1. Age-dependent decrease in renal cortical hyaline droplet number in untreated male rats. (A) Tissue from a 3.5-month-old rat showing profiles of proximal convoluted tubules with prominent hyaline droplets (→). (B) Male rat, 18 months old; (C) 26-month-old rat. Photomicrographs are typical of tissues examined. Methylene blue–basic fuchsin stain, glycol methacrylate embeddment, 50×.

brane bound, round in cross section, larger than primary lysosomes, and possessed variable electron density. Only rarely was an irregularly shaped phagolysosome observed in the kidney of saline-treated rats.

As described in previous reports from this laboratory (Garg *et al.*, 1987; Olson *et al.*, 1987), both the number and the size of hyaline droplets in epithelial cells of the renal PCT were markedly increased in young rats

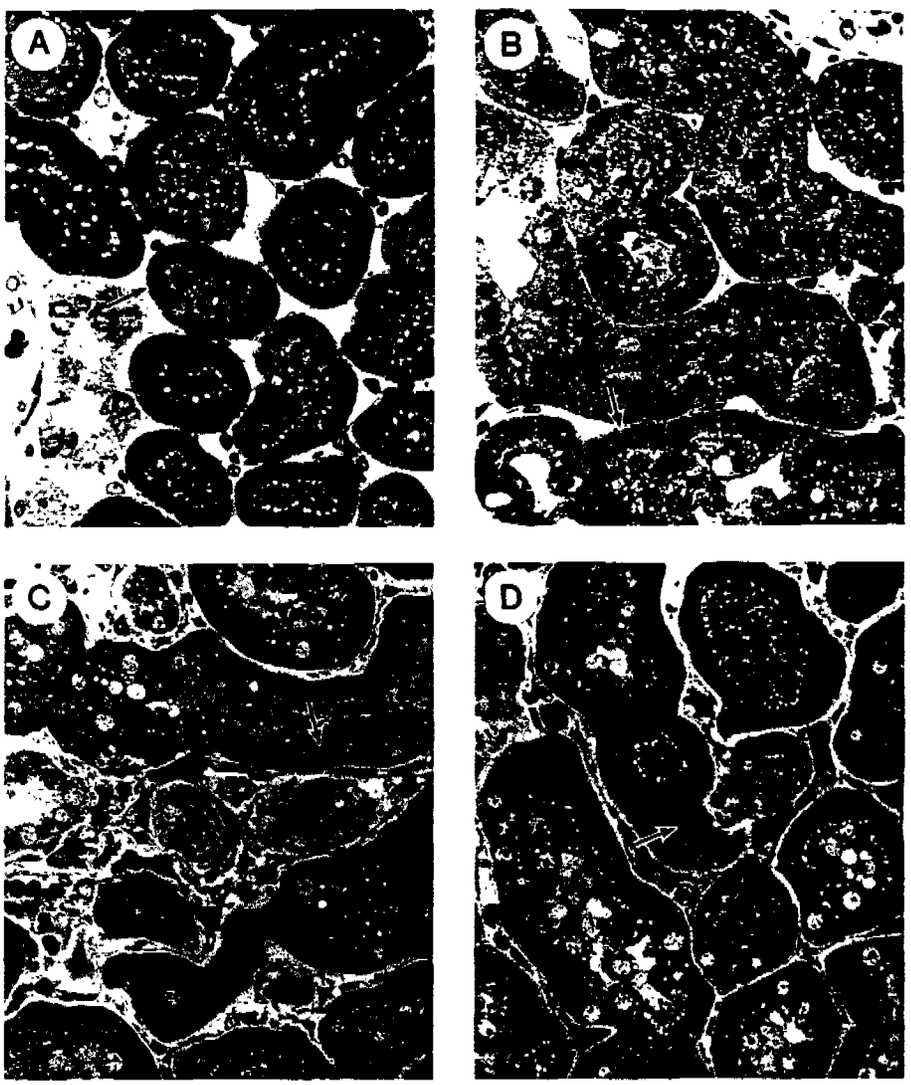

FIG. 2. Effect of gasoline administration on renal cortical morphology in young adult and senescent male rats. (A) Saline-treated 3.5-month-old rat; note uniform size and distribution of hyaline droplets (→). (B) Gasoline-treated 3.5-month-old rat; hyaline droplet number is increased and droplet shape and size are altered. (C) Saline-treated 26-month-old rat. Note variability in number, size, and distribution of hyaline droplets in saline-treated senescent rats compared to those of young rats. (D) Gasoline-treated 26-month-old rat; failure of gasoline administration to alter hyaline droplet number or size. Treatment protocol described under Materials and Methods. Photomicrographs are typical of tissues examined. Toluidine blue stain, tissue in Spurr's medium, 50×.

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treated with gasoline (0.4 ml/kg/day, po, 5 days) (Fig. 2B). In severely affected tubules, apical blebs were apparent and some cells undergoing lysis were observed (not shown). Consistent with earlier reports, hyaline droplets were seen to be phagolysosomes when examined by electron microscopy (Fig. 3B). Following repeated gasoline administration some phagolysosomes were still morphologically similar to those of controls (Fig. 3B).

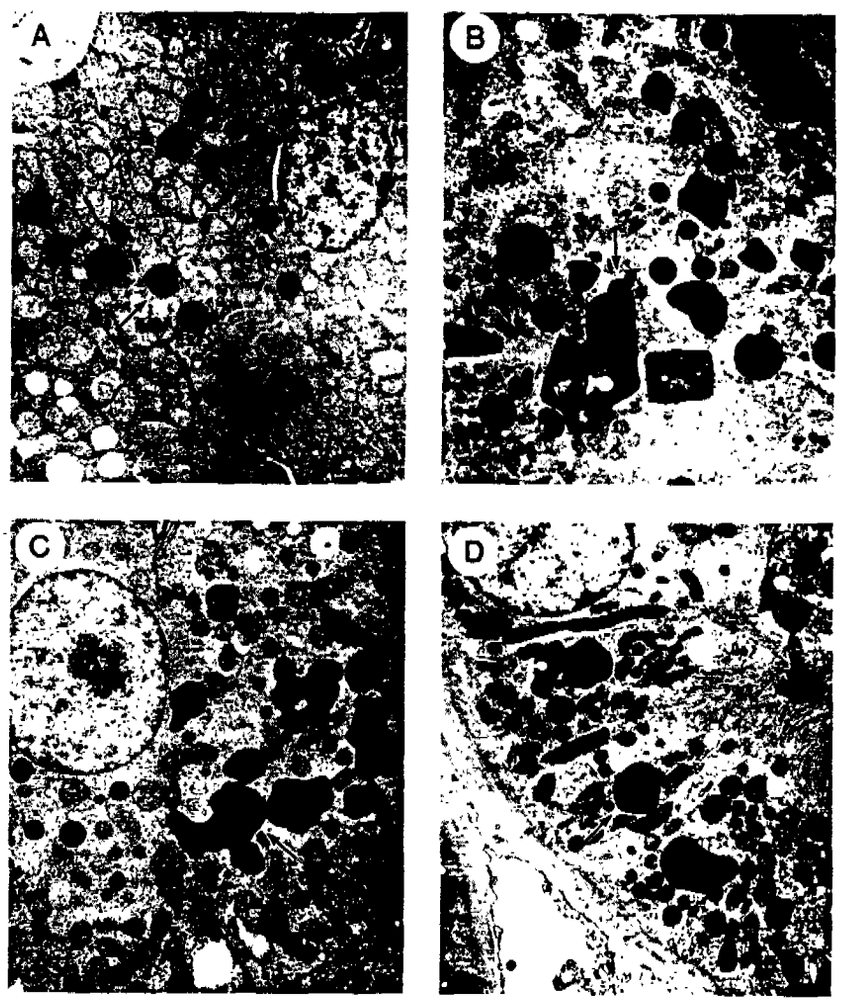

FIG. 3. Representative transmission electron micrographs of renal proximal tubules from 3.5-month-old (A,B) and 26-month-old (C,D) male rats. Electron-dense phagolysosomes (→) appear in the cytoplasm of animals at both ages. (A,C) Taken from rats treated with saline and illustrate the variable size and shape of phagolysosomes of aged control rats compared to those in young animals. (B,D) Gasoline-treated rats. Gasoline causes no change in phagolysosome number or conformation in 26-month-old male rats relative to age-matched saline-treated controls. 2400×.

However, most phagolysosomes were angular in profile with conspicuous electron-dense inclusions, some with crystalline structure, in their matrices (Fig. 3B). Infrequently, clear areas of cytoplasm without obvious delimiting membranes and containing phagolysosomes, electron-dense bodies and cellular debris were seen indicating the occurrence of autolysis (not shown). Small phagolysosomes were occasionally seen abutting each other, suggesting fusion to form the large phagolysosomes characteristic of gasoline intoxication. There appeared to be a slight increase in the number and size of endocytotic vesicles in gasoline-treated rats. However, other portions of the endocytotic apparatus (e.g., microvilli) appeared unaffected by administration of gasoline. Alterations in other cellular

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organelles were not apparent in comparison to rats receiving saline treatment only.

Senescent rats. As suggested by light microscopy (Fig. 2C), the number of phagolysosomes visible with electron microscopy in untreated or saline-treated aged male rats was diminished (Fig. 3C). The constitutive population of phagolysosomes in old animals was highly variable in size and shape with many large, irregular phagolysosomes obvious (Fig. 3C). Frequently these large phagolysosomes contained flocculent electron-dense inclusions (Fig. 3C) of a type not observed in young control or gasoline-treated rats. Aside from the age-related alteration in phagolysosomes, PCT of 26-month-old rats were marked by thickening and reduplication of the basal laminae. Age-related changes in other organelles were unremarkable. Gasoline administration (0.4 ml/kg/day po, 5 days) to 26-month-old rats caused no obvious alteration in number or morphology of phagolysosomes in PCT epithelial cells at either light microscopic (Fig. 2D) or electron microscopic magnification (Fig. 3D).

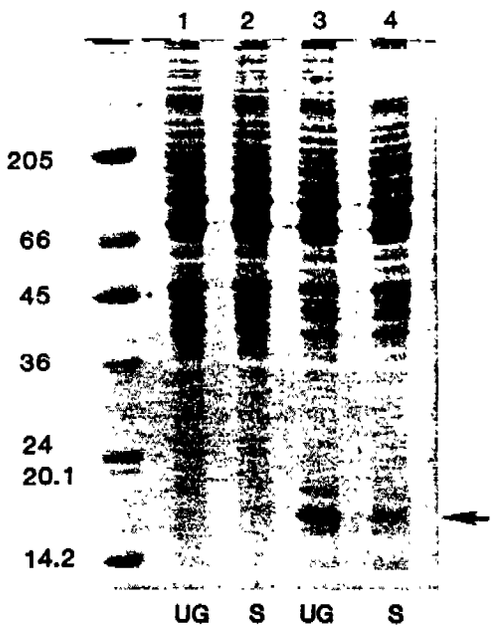

FIG. 4. SDS–PAGE of renal cortical proteins from male rats treated with saline (S) or gasoline (UG) demonstrating gasoline-induced accumulation of low-molecular-weight protein only in young adult rats. Lanes 1 and 2, 26-month-old rats; lanes 3 and 4, 3.5-month-old rats. Molecular weight markers are identified by numbers corresponding to $M_r \times 10^{-3}$. The position of α_{2u}-globulin is indicated (→).

Biochemical Characteristics of Hyaline Droplets in Young and Old Male Rats

Electrophoretic separation of renal cortical proteins. Polyacrylamide gel electrophoresis (SDS–PAGE) of soluble proteins in homogenized renal cortices from saline-treated 3.5-month-old rats showed a prominent component of M_r approximately 18×10^3 (Fig. 4). On the basis of the dramatic intensification of this protein band in renal cortical homogenates from gasoline-treated young males (Fig. 4) and apparent molecular weight, this protein was presumed to be α_{2u}-globulin. In 26-month-old rats no band corresponding to the prominent low-molecular-weight protein of young rats was observed in either saline- or gasoline-treated animals. (Fig. 4).

Renal content of α_{2u}-globulin. RIA quantitation of α_{2u}-globulin content in the kidney of young and aged male rats (Table 2) corroborated the observations made by SDS–PAGE of renal cortical proteins (Fig. 4). Specifically, gasoline administration (0.4 ml/kg/day, 5 days) to 3.5-month-old male rats increased renal α_{2u}-globulin content by 173% when determined 24 hr after the final dose (Table 2). Larger increases in the content of this protein are possible in young rats at higher gasoline doses (Olson *et al.*, 1987), however, the dose administered was chosen to ensure survival of the old rats. The constitutive level of α_{2u}-globulin in the kidney of 26-month-old rats was only 1.4% of that in young rats (Table 2). The low level of renal α_{2u}-globulin was likely due to the greatly diminished hepatic synthesis of this protein in aged rats; hepatic α_{2u}-globulin content in 26-month-old rats was 0.4% of that in 3.5-month-old rats. Administration of gasoline to old rats was without effect on the renal or hepatic content of α_{2u}-globulin (Table 2).

TABLE 2

EFFECT OF OLD AGE ON GASOLINE-INDUCED RENAL ACCUMULATION OF α_{2u}-GLOBULIN IN MALE RATS

Treatment	n	α_{2u}-Globulin content (μg/mg total protein)	
		Kidney	Liver
Young adult males (3.5 month)			
Saline	2	36.5 (38.0, 35.1)	1.3 (1.2, 1.4)
Gasoline	2	99.5 (83.0, 115.9)	1.5 (1.7, 1.3)
Senescent males (26 month)			
Saline	4	0.5 ± 0.2	0.005 ± 0.002
Gasoline	6	0.5 ± 0.1	0.003 ± 0.000

Note. Data are expressed as means ± SEM of triplicate determinations on each of *n* animals per treatment group. For groups with $n = 2$, mean values from triplicate determinations on tissues from each animal appear in parentheses. Details of treatments and analytical technique under Materials and Methods.

Lysosomal peptidase enzyme activity. In young rats the activity of the lysosomal proteolytic enzymes cathepsin D (a carboxyl endopeptidase), and cathepsin B (a thiol endopeptidase) was nearly doubled when assayed 24 hr after discontinuing administration of gasoline (Table 3). Conversely, the activity of acid phosphatase, which acts on phosphoric monoesters such as those in nucleic acids, was unaffected by gasoline. The basal levels of both acid phosphatase and cathepsin B were reduced in saline-treated 26-month-old rats compared to those of similarly treated 3.5-month-old rats (Table 3). In gasoline-treated senescent male rats the activities of renal cortical acid phosphatase and cathepsin B were not different from those of age-matched controls (Table 3). However, cathepsin D activity in gasoline-treated old rats was increased to 232% of the value observed in saline-treated age-matched controls.

DISCUSSION

Two previously unreported observations relevant to chemically induced hyaline drop-

TABLE 3

EFFECT OF GASOLINE ADMINISTRATION ON RENAL LYSOSOMAL HYDROLASE ACTIVITIES IN YOUNG AND AGED MALE FISCHER 344 RATS

Treatment	n	Lysosomal enzyme activities		
		Acid phosphatase (nmol/min/mg protein)	Cathepsin B (nmol/min/mg protein)	Cathepsin D (μmol/min/mg)
Young adult males (3.5 month)				
Saline	4	5.5 ± 0.2	206.6 ± 20.5	27.7 ± 0.9
Gasoline	6	5.4 ± 0.1	336.3 ± 6.4*	45.3 ± 5.4*
Senescent males (26 month)				
Saline	4	4.0 ± 0.3**	61.3 ± 4.6**	34.5 ± 12.5
Gasoline	6	4.0 ± 0.2	64.5 ± 5.7	80.0 ± 6.6*

Note. Data are expressed as means ± SEM of triplicate determinations on each of *n* animals per treatment group. Details of treatments and analytical technique under Materials and Methods.

* Different from age-matched saline-treated group, $p < 0.05$ (one-way analysis of variance and Dunnett's test).

** Different from value for saline-treated 3.5-month-old rats, $p < 0.05$.

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Cathepsin D (μmol/min/mg)
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34.5 ± 12.5 80.0 ± 6.6*

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let nephropathy of male rats were made in this study: the spontaneous age-related decrease in PCT epithelial cell hyaline droplet number, and the failure of gasoline to cause hyaline droplet nephropathy in aged male rats. The apparent decrease and eventual loss of spontaneously occurring hyaline droplets which react with methylene blue–basic fuchsin stain (Fig. 1) was unexpected and not entirely consistent with observations made on tissue prepared with toluidine blue staining (Fig. 2). The methylene blue–basic fushcin method stains proteins nonspecifically; therefore, the nature of the age-dependent alterations in protein composition of hyaline droplets cannot be determined by this method alone. However, electrophoretic (Fig. 4) and RIA data (Table 2) from the kidneys of saline-treated old rats indicate that by 26 months of age α_{2u}-globulin is absent. This information is consistent with that reported previously by Roy and co-workers (Roy *et al.*, 1983) and suggests that decreased production of α_{2u}-globulin contributes directly to the decrease in hyaline droplet number and age-dependent alteration of the staining properties of the remaining droplets. The precise stoichiometry between the declining rates of α_{2u}-globulin synthesis and the decrease in hyaline droplet number remains to be defined.

After puberty male rats excrete large amounts of protein in the urine (Gray and Purmalis, 1965; Neuhaus and Flory, 1978). Thus, the renal proximal tubules of these animals are continually stressed by the requirement to reabsorb and process large quantities of proteins. However, no comprehensive biochemical characterization of proteins in constitutive or hydrocarbon-induced hyaline droplets in rats of various ages has been published. Nor have detailed publications on age-related alterations in renal histology of male rats mentioned changes in the number or characteristics of hyaline droplets (phagolysosomes) found in PCT epithelial cells of rats older than 12 months (Andrew and Pruett, 1957; Gray *et al.*, 1974; Coleman *et al.*, 1977; Haley and Bulger, 1983). On the other hand, reports of increased glomerular permeability and altered urinary protein composition with increasing age (Gray and Purmalis, 1965; Neuhaus and Flory, 1978) point to the possibility that hyaline droplet number and composition may change as a function of the proteins present in the urine. A gradual age-dependent decline in the rate of synthesis of α_{2u}-globulin by the liver can by noted as early as 150 days of age and beyond 750 days hepatic synthesis of α_{2u}-globulin is almost undetectable (Roy *et al.*, 1983). Furthermore, at 5–7 months of age, the amount of albumin and other large proteins passing the renal glomerular filtration barrier of rats begins to increase such that, as early as 9 months of age, the relative amount of α_{2u}-globulin in the urinary filtrate has been greatly reduced (Neuhaus and Flory, 1978). Since renal proximal tubules resorb individual proteins on the basis of both ionic properties and mass contribution to total urinary protein, it seems likely that the majority of resorbed protein in rats at 1 year of age or more is not α_{2u}-globulin. Considering this point and the age-dependent changes reported here, we conclude that the chemical composition of renal hyaline droplets is altered markedly in the kidneys of aged rats.

α_{2u}-Globulin is the primary urinary protein of young adult male rats (Roy and Neuhaus, 1966) and the toxic effects of many hydrocarbons are unique to male rats of ages at which α_{2u}-globulin excretion is high (Table 1). α_{2u}-Globulin has also been shown to be a principal component of hyaline droplets accumulated during gasoline intoxication in rats about 3.5 months of age (Garg *et al.*, 1987; Olson *et al.*, 1987, 1988). Conversely, the failure of gasoline to alter hyaline droplet number in aged male rats (Figs. 2, 3) and the absence of α_{2u}-globulin in kidneys of gasoline-treated old rats (Fig. 4, Table 2) suggest strongly that the presence of this unique protein contributes directly to the susceptibility of young postpubescent rats to hyaline droplet nephropathy. Furthermore, the age-related differential induction of renal lysosomal peptidase enzymes after administration of gasoline (Table 3) suggests that the contents

of phagolysosomes in gasoline-treated young rats differ from those in old rats. In young rats which are competent to produce α_{2u}-globulin, the accumulation of this protein by renal phagolysosomes in response to gasoline (Fig. 4, Table 2) probably causes a compensatory increase in the activity of cathepsins B and D (Table 3). Substrate-mediated induction of lysosomal proteolytic enzymes is known to occur in many tissues (Helminen *et al.*, 1968; Salminen, 1985). We have shown previously that specific inhibition of cathepsin B by administration of leupeptin *in vivo* results in rapid accumulation of α_{2u}-globulin in the kidney of young male rats (Olson *et al.*, 1988). Thus, cathepsin B activity appears to be intimately linked to the renal metabolism of α_{2u}-globulin. In contrast, aged rats, which lack α_{2u}-globulin, when treated with gasoline show an increase in only cathepsin D activity. Whether this is due to accumulation of proteins other than α_{2u}-globulin is unknown. Therefore, the primary response of the rat kidney to gasoline, i.e., α_{2u}-globulin accumulation, as well as a secondary or compensatory response to protein overloading caused by gasoline differs between young and aged male rats.

Chronic exposure to several of the hyaline droplet-inducing nephrotoxic hydrocarbons is associated with an increased incidence of renal carcinoma in male rats (National Cancer Institute, 1977; National Toxicology Program, 1983; MacFarland *et al.*, 1984; National Toxicology Program, 1988). In these animals, necrosis of individual cells in the PCT epithelium, perhaps due to phagolysosomal protein overloading, has been observed following short-term hydrocarbon exposure (Short *et al.*, 1986, 1987). Repeated rounds of cell death and regeneration are suggested to contribute to renal carcinogenesis by promoting clonal expansion of a small number of spontaneously occurring premalignant cells, increasing the number of cells becoming premalignant or both (Short *et al.*, 1987; Gibson and Bus, 1988). Such an indirect mechanism of tumorigenesis is consistent with both the failure to demonstrate genetic toxicity of hyaline droplet-inducing nephrocarcinogens and the relatively low incidence with which renal tumors appear in gasoline-exposed male rats (MacFarland *et al.*, 1984).

With respect to the mechanism of renal carcinogenesis just detailed, the failure to induce hyaline droplet nephropathy in gasoline-treated old animals is especially significant since this suggests that α_{2u}-globulin is an essential component of the hydrocarbon-induced nephropathic response. Alden and coworkers (1984) have shown previously that prepubertal male rats, which have neither α_{2u}-globulin nor spontaneously occurring hyaline droplets, are resistant to nephropathy caused by Decalin. The findings with senescent rats, however, extend the prepubertal rat paradigm significantly because we show that even in rats which have a spontaneously occurring population of hyaline droplets and are presumably albuminuric, the absence of α_{2u}-globulin is sufficient to cause the animals to become refractory to the toxicity of gasoline. Thus, α_{2u}-globulin is essential and necessary for the occurrence of the acute consequences of gasoline exposure. Furthermore, if the mechanism proposed to explain gasoline-induced renal carcinogenesis in male rats is operative, these results imply that α_{2u}-globulin is essential for the occurrence of nephrocarcinogenesis during chronic gasoline exposure. The final extension of this reasoning then suggests that species which lack α_{2u}-globulin, or at least high levels of structurally very similar proteins, will be refractory to both the acute and the chronic effects of gasoline demonstrated in male rats.

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