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RAPID COMMUNICATION

THE ROLE OF α2u GLOBULIN SYNTHESIS IN THE PRODUCTION OF RENAL HYALINE DROPLETS BY ISO-OCTANE.

Linda Carruthers, Kate Reeves, Moses Paul, Andrew Searle, William Templeton[1] & Alan J Paine*

DHSS Dept of Toxicology & [1]Dept of Biochemistry, St Bartholomew's Hospital Medical College, West Smithfield, London ECIA 7BE, UK.

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INTRODUCTION: Exposure of mature male rats to iso-octane (2,2,4-trimethylpentane), a constituent of unleaded gasoline, results in the formation of renal "hyaline droplets" and an increased incidence of renal tumours (1-2). The "hyaline droplets" produced in the renal proximal tubules, following administration of iso-octane, contain the protein α2u globulin (2) which is the major urinary protein of the mature male rat (3). Since the proximal tubules of the kidney are involved in protein reabsorption and catabolism (4) this may suggest that iso-octane is affecting the renal handling of α2u globulin. However, it is well established that the administration of large amounts of natural proteins can also result in the formation of renal "hyaline droplets" (5,6). Therefore another mechanism to account for the formation of iso-octane-induced renal hyaline droplets is that this xenobiotic increases the synthesis of α2u globulin and that this overloads the renal clearance mechanism. The major site of α2u globulin synthesis is the liver and there is no synthesis of this protein in the kidney (7). Accordingly a simple way to determine the involvement of the hepatic synthesis of α2uglobulin in the pathogenesis of renal hyaline droplets produced by iso-octane is to co-administer an inhibitor of protein synthesis such as cycloheximide because cycloheximide is known to block hepatic protein synthesis by more than 90% (8,9).

The present work, employing a novel ELISA assay of α2u globulin, shows that the co-administration of cycloheximide does indeed prevent the iso-octane-mediated increase in α2u globulin as well as the histological appearance of renal hyaline droplets. These findings suggest that the induction of α2u globulin synthesis by iso-octane is related to the formation of renal hyaline droplets. However the administration to rats of purified α2u globulin, in amounts comparable to that produced by iso-octane administration, did not result in its renal accumulation or the formation of renal hyaline droplets. Collectively these findings demonstrate that the ability of iso-octane to produce renal hyaline droplets is in part due to it inducing α2u globulin synthesis but must also be related to its effects on the renal handling of this protein.

MATERIALS AND METHODS

Treatment of animals: Male CD rats weighing 180-250g, purchased from Charles River Ltd., Margate, Kent, U.K., were fasted overnight prior to dosing. Drinking water was provided ad libitum. Rats were given 2,2,4-trimethylpentane (Analar grade, B.D.H. Chemicals, Poole, Dorset, U.K.) as a single dose, of 12 mmoles/kg body weight, by gavage in corn oil (2ml/kg body weight). Cycloheximide (Sigma Chemical Co., Poole, Dorset, U.K) in sterile, non-pyrogenic, 0.9%(w/v) saline was given intravenously at a dose of 1.5mg/kg body weight. Control animals were dosed with an equivalent volume of corn-oil by gavage and saline intravenously. Urine samples were collected for 24hr following dosing and then the rats were killed with CO_2 and blood collected from the inferior vena cava.

Tissue Preparation: A 10% (w/v) homogenate of the left kidney or liver was prepared in 3% (w/v) sodium deoxycholate in 10mM Tris-HCI pH8.2 containing 2mM phenylmethylsulphonyl

Footnote *address correspondence to A J Paine

fluoride by using an Ultra-Turrax TP18/10 blender in 3 x 10 sec bursts. After 30min at room temperature to solubilize the protein, the homogenate was centrifuged at 10^5g for 1hr and the supernatant was used for assay of α2u globulin. For this assay the kidney preparation was routinely diluted to 1mg tissue/ml and a final deoxycholate concentration of 0.5%(w/v).

Antibody preparation: α2u globulin was purified from male CD rat urine by an ammonium sulphate precipitation, ion-exchange chromatography on CM-cellulose and by Sephadex G50-SF chromatography essentially as described previously (2). The purified protein was found, in agreement with other workers (10), to have a microheterogeneity and consequently an M_r of 18,000-20,000 on sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). 1mg of this preparation emulsified in 800µl of Freund's complete adjuvant was injected into a half-lop rabbit at 2 intramuscular, 1 subcutaneous and 1 intraperitoneal sites. Three weeks later a booster of 1mg α2u globulin emulsified in Freund's incomplete adjuvant was administered and serum was prepared from the rabbit one week later.

ELISA: α2u globulin levels were determined in samples of kidney and urine using the horseradish peroxidase conjugate method of ELISA. In summary, 50µl of serial dilutions of tissue preparations or urine were added to glutaraldehyde pre-treated flat-bottomed microtitre plates and incubated overnight at 37°C. The samples were then aspirated and the wells filled with 3% (w/v) bovine serum albumin in phosphate buffered saline (PBS) and the plates left at room temperature for 1hr after which the solution was aspirated and the wells washed three times with PBS followed by a wash with 0.2% (w/v) Tween 20 in PBS. Then 50µl of a 2000-fold dilution of rabbit anti-rat α2u globulin serum in PBS was added to each well and the plates left at room temperature for 2hr. The antiserum was removed and the wells washed as described above. Then 50µl of 1000-fold dilution of horseradish peroxidase conjugated to goat anti-rabbit IgG (H + L chains), purchased from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, was added to each well. After 30min at room temperature this antiserum was removed and the plates washed again as described previously. Then 100µl of a 10mM o-phenylene diamine in PBS containing 5mMH_2O_2 was added to each well. After 30 min of incubation, in the dark, at room temperature the absorbance was determined at 450nm in a Titertek microtitre plate reader. The concentration of α2u globulin was calculated from "standard curves" produced from purified α2u globulin.

Liver cell cultures were prepared from male CD rats as previously described (11) and after 4 hr of incubation the culture medium changed to Williams medium E, without methionine, containing 5% dialysed foetal calf serum and 50µCi ^{35}S methionine at a specific radioactivity of 1.38Ci/umole (Amersham International, Bucks, U.K.) was added per 3ml culture medium. 20hr later the medium was aspirated and the hepatocyte monolayer was scraped into 3ml of 1% Nonidet NP-40 in 0.15M NaCl containing 10mM Tris-HCl pH 7.4, 1mM EDTA, 1mM (designated "lysis buffer") and 1mg bovine serum albumin/ ml. A cell homogenate was prepared with an Ultra-Turrax TP18/10 blender for 10sec, centrifuged at 10^5g for 1hr and the α2u globulin in the supernatant and culture medium precipitated by the addition of 5µl of rabbit anti-rat α2u globulin serum for 30min followed by 50µl of a 10% (w/v) suspension of S. aureus cells. After 1hr at 4°C the immunoprecipitate was recovered by centrifugation for 2min in an Eppendorf microfuge and the pellet washed with 3xlml of lysis buffer. The pellet was resuspended in 50µl SDS-PAGE sample buffer boiled for 1min and 25µl used for the determination of radioactivity. The remainder was loaded onto a 15% (w/v) SDS-PAGE gel. Subsequent electrophoresis and autoradiography showed that radioactivity was exclusively localised to protein(s) of M_r 18,000-20,000 which is identical to that found for purified α2u globulin. Preimmune serum from rabbits precipitated less than 0.5% of the radioactivity of the samples studied but this was not found by SDS-PAGE autoradiography to be associated with any protein.

2,4,4-trimethylpentanoic acid was synthesized from 2,4,4-Trimethylpentanol (obtained from

Aldrich Chemical Co. Gillingham, Dorset) 52gm (0.4mole), dissolved in 250ml acetone previously refluxed with $KMnO_4$ for 2hr and distilled twice through a Vigreux column. To this ice-cold solution 60g, (0.6mole) of CrO_3 in 225ml of water containing 50ml conc. H_2SO_4 was added dropwise over 2hr and the temperature kept below 15°C. After stirring for a further hour, the reaction mixture was kept overnight at room temperature and then treated with excess solid $Na_2S_2O_5$ (to reduce unreacted CrO_3) followed by 100ml ether. The organic layer was separated and washed twice with water and the combined aqueous layer and washings were extracted twice with ether. The ether extracts were added to the original organic layer, which was washed twice with water, dried over anhydrous Na_2SO_4 and stripped of solvent by rotary evaporation under reduced pressure. After further drying over Linde 4A molecular sieve, the residue was distilled under reduced pressure to give TMP acid as a colourless oil (51.5g, 89% yield): b.p. 73.5-74.5°C at 2.5mm Hg: i.r. (thin film), 3000(br), 1708(s), 1465(m), 1415(w), 1290(w), 1230(m), 950(w) cm^{-1}. Anal. Calc. for $C_8H_{16}O_2$: C, 66.63;H, 11.18. Found: C, 66.41; H, 11.07.

Results: The results in Table 1 show that untreated, mature male rats excrete 2-4mg of ∝2u globulin in their urine per day and that approximately 2mg of this protein is present in their kidneys. In contrast ∝2u globulin was not detected by the sensitive ELISA assay employed (limit of detection 50ng/ml) in samples of rat liver or plasma, even after TMP treatment (data not shown for brevity). Accordingly there are only two major compartments of ∝2u globulin in the mature male rat, namely the kidney and urine. However, treatment of mature male rats with 12mmoles TMP/kg body weight only significantly increased the amount of ∝2u globulin found in the kidney and was without effect on the urinary excretion of this protein (Table 1). As mentioned previously, in the introduction, this effect of TMP administration could be due to the induction of the hepatic synthesis of ∝2u globulin overloading the renal clearance of this protein. Accordingly the effect of administering cycloheximide, in doses that are known to inhibit hepatic protein synthesis by more than 90% (8,9), on the renal accumulation of ∝2u globulin produced by TMP administration were determined. The results, presented in Table 1, show that the co-administration of cycloheximide is able to completely prevent the TMP-mediated increase of ∝2u globulin in the kidney. Concomittantly treatment with cycloheximide also prevented the histological appearance of renal hyaline droplets (histological data not shown for brevity). Unequivocal evidence that TMP is an

Table 1

Effect of TMP ± cycloheximide treatment, and the intravenous administration of ∝2u globulin, on the renal concentration and urinary excretion of ∝2u globulin.

Treatment	Cycloheximide pre-treatment	∝ 2u globulin in:- Urine (mg/24hr)	Kidney (mg/gm kidney)
Control (n=8)	-	2.1 ± 1.6	1.5 ± 0.5
	+	1.8 ± 1.3	1.1 ± 0.8
TMP (n=8)	-	4.1 ± 2.2	24.0 ± 4.4
	+	1.1 ± 0.9	2.2 ± 1.3*
20mg 2u i.v. (n=3)	-	20.5 ± 1.2	1.4 ± 0.6

The results are expressed as the mean ± S.D. and n=the number of individual animals.

* Denotes significantly different ($p = 0.01$) from group not treated with cycloheximide.

inducer of hepatic α2u globulin synthesis could not be obtained in liver cell culture because of the low solubility/miscibility of TMP with aqueous media. However a metabolite of TMP in the rat is known to be 2,4,4-trimethylpentanoic acid (TMP acid) (12) and in view of its much greater water solubility the effects of TMP acid on the synthesis of α2u globulin in rat hepatocyte culture was determined. The results in Table 2 show that TMP acid is an inducer of the synthesis of α2u globulin but not of other secretory proteins i.e. rat serum albumin.

Table 2

Induction of α2u globulin synthesis by TMP acid in rat hepatocyte culture

Treatment	Total radioactivity (dpm x10^{-4}) incorporated by cultured rat hepatocytes into immunoprecipitates of:-	
	α2u globulin	albumin
None	45.4 ± 5.5	55.6 ± 4.3
1mM TMP acid	88.9 ± 4.0*	44.9 ± 12.5

For experimental details see section on Methods.

* Denotes significantly different ($p = 0.01$) from untreated value

Discussion: The results collectively demonstrate that TMP or its metabolites are inducers of hepatic α2u globulin synthesis and that this property is indeed related to the formation of renal hyaline droplets. However, the finding that the intravenous administration to mature male rats of purified α2u globulin, in amounts comparable to that induced by TMP administration, did not result in its renal accumulation (Table 1) or the formation of renal hyaline droplets suggests that this phenomenon must also be related to the effects of the xenobiotic on the renal handling of α2u globulin. The administration of TMP to rats has not been found to produce overt nephrotoxicity (4) and we have found, in preliminary experiments, that it also does not produce a generalised impairment of glomerular filtration rate. Accordingly the ability of TMP to cause the renal accumulation of α2u globulin is in part due to an induction of hepatic α2u globulin synthesis but must also be related to the effect of this xenobiotic on the renal handling/degradation of the protein.

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