THE EFFECT OF LYSINE ON HAEMOGLOBIN INDUCED RENAL DAMAGE

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ABSTRACT

Large amounts of free haemoglobin in the circulation following intravascular haemolysis may cause many adverse effects, especially to the kidneys. Some attribute the deleterious renal effects to the stroma of red blood cells. However, there is evidence showing that intratubular haemoglobin plays an active role in the pathogenesis of renal damage. Intracellular uptake of haemoglobin is thought to be an important step leading to impairment of renal function, though the mechanism of the renal damage is as yet unclear. It is also thought that haemoglobin shares with other low molecular weight proteins the same uptake sites which are nonspecific and can be influenced by certain amino acids and positively charged substances. In this study, attempts were made to mitigate the renal damage caused by haemolysis by reducing the uptake of haemoglobin of the proximal tubular cells. The amino acid lysine which had been shown to increase the excretion of some proteins which are normally reabsorbed by the proximal tubule was employed to achieve this end.

In vivo haemolysis was induced in rats by intra-arterial bolus injection of 4 ml distilled water over 5 seconds. This resulted in haemoglobinaemia, haemoglobinuria, and a reduction in inulin clearance. However, renal function was preserved when the experiment was repeated with concomitant infusion of 30 mM lysine. This concentration of lysine was
demonstrated not to affect inulin clearance in normal rats within the experimental period. In addition to the inulin clearance measurement, the excretion of the enzyme N-acetyl-\(\beta\)-D-glucosaminidase (NAG) was also used as an index of proximal tubular cell damage. When in vivo haemolysis was produced in rats, an increase in the excretion of NAG was observed. When 30 mM lysine was simultaneously infused, the degree of increase of NAG excretion was less. These findings lend support to the hypothesis that haemolysis induced renal damage is in some way related to renal haemoglobin uptake. Lysine inhibits this process, and therefore minimizes renal damage.
The oxygen transporter, haemoglobin, is normally contained in erythrocytes. However, there are many situations in which haemoglobin is liberated into the circulation. These conditions include malarial infections; the use of valvular prosthesis in some patients; prolonged use of extracorporeal circulation in open heart surgery; mismatched blood transfusion; administration of chemical agents such as glycerol and benzene; genetic defects such as glucose-6-phosphate dehydrogenase deficiency and paroxysmal nocturnal cold haemoglobinuria; injection of venoms of such snakes as rattlesnake and coral snake, and the inadvertent introduction of large quantities of distilled water into the bloodstream as for example during irrigation of the bladder in transurethral prostatectomy (1). Moreover, the potential use of haemoglobin solution as a blood substitute will also lead to increased free haemoglobin in blood, i.e. haemoglobinemia.

Disposition of free haemoglobin

Free haemoglobin in plasma binds to plasma haptoglobin which is an $\alpha_2$-globulin (2). The haemoglobin-haptoglobin complex does not undergo renal glomerular filtration. It is disposed of by the macrophages of the reticuloendothelial system in the spleen, liver, bone marrow and other organs (3). Haemoglobin is a tetramer consisting of two pairs of unlike chains. The molecule dissociates reversibly into two symmetrical dimers, monomers, globin, and free
Introduction

haem (2). The ferrous ion chelated by the haem molecule is readily oxidized into ferric ion by plasma oxidases (2). Oxidized haem forms complexes with serum haemopexin and serum albumin. Dimeric and monomeric free haemoglobin in excess of the binding capacities of the circulating proteins undergoes renal glomerular filtration.

After filtration, the haemoglobin is reabsorbed in the proximal tubule (4), and the iron released is stored in the kidney, possibly as ferritin. The absorbed globin is probably catabolized in situ under the influence of cytoplasmic lysosomes. The porphyrin is catabolized rapidly in the proximal tubular cells (5,6).

When the amount of haemoglobin is so large that the binding capacity of plasma haptoglobin as well as the absorptive capacity of the proximal tubular cells are exceeded, haemoglobin appears in the urine.

It has been known for a long time that there is some correlation between haemoglobinaemia and impairment in renal function. Intra renal obstruction was observed when investigation on the pathology of blackwater fever was conducted by injecting rabbits with solutions of haemoglobin (7). Renal function impairment in patients suffering from incompatible transfusions was attributed to the effect of haemoglobin (7). When haemoglobinuria was produced in rabbits by intravascular haemolysis or by the injection of fresh autologous and heterologous haemolysates, epithelial necrosis with regeneration was found to involve the terminal parts of the proximal tubules in all animals (8). Besides, when haemoglobin solution was infused to dogs suffering from haemorrhagic shock, there was a subsequent transient depression of the urea clearance suggesting the possibility that renal damage may be caused by the haemoglobin solution (9). Some investigators proposed
Introduction

that the acute renal effects of haemolysis or haemoglobin are secondary to intratubular obstruction and concomitant toxic tubular damage (10).

Despite the apparent deleterious renal effects of haemoglobin solution, active research on haemoglobin has never ceased. One aspect of the interest in studying the effects of haemoglobin stemmed from the possible application of haemoglobin solution as a blood substitute.

Blood substitutes

Since the first successful blood transfusion in 1914, this therapeutic procedure has become relatively safe and efficient. However, despite a continuing campaign to recruit donors, all blood banks experience periodic shortfalls in the supply of whole blood. Moreover, the shelf-life of blood preserved in the liquid state is limited, normally being twenty one to thirty five days only. When blood transfusion is required, blood typing and cross matching have to be done. This is relatively time-consuming as every minute is critical in real emergency. At times, the blood type needed may not be available in the blood bank. If there are natural calamities when many people are injured and need blood simultaneously, the blood bank may not be able to meet the demands.

People in the past have already recognized such drawbacks and looked for blood substitutes. An ideal blood substitute should be able to carry oxygen and at the same time exert enough oncotic pressure to maintain blood volume. Plasma expanders such as dextran and albumin expand the plasma well but they are unable to carry
Introduction

oxygen. Therefore, attempts have been made to use special organic liquids, such as perfluorinated compounds which have high physical solubility for oxygen.

The fundamental disadvantage of the purely physical transport of oxygen in the presently available aqueous emulsions of perfluorinated compounds is the shallow slope of the oxygen saturation curve. Under physiological partial pressures of 5.3 to 13.3 kPa, oxygen transport by these emulsions remains insufficient to effect adequate oxygenation of the tissues (11). Moreover, several potential problems might be encountered. (a) Many fluorocarbons have a very high vapour pressure, vapourizing rapidly at body temperature and causing embolism. (b) Fluorocarbons are not miscible with water; hence, the preparations must be stable emulsions. (c) Fluorocarbon compounds of lower vapour pressures are not eliminated and are permanently taken up by the reticuloendothelial system (12). Some of the new types of fluorocarbons reduce the severity of these shortcomings (13). However, the concern about in vivo tissue accumulation of these compounds have impeded their acceptance for clinical use.

Haemoglobin, a component of normal blood, can be prepared from outdated human erythrocytes, and does not require typing or crossmatching prior to use. In addition to its oncotic activity, haemoglobin can transport and exchange oxygen (14). Moreover, it has lower viscosity than blood, does not cause microaggregates, and may not be allergenic. Furthermore, haemoglobin is highly soluble in physiologic solutions and can be stored for extended periods of time.

Although there are many theoretical advantages of haemoglobin solution, reports of renal damage occurring after the administration of haemoglobin solution have deterred developmental work on this
Introduction

potential blood substitute (7,8,9).

In 1967, Rabiner and co-workers, by making use of millipore filter, were able to prepare haemoglobin solution which was free of fragments of the lysed erythrocytes (i.e. stroma). They proposed that renal damage, following administration of haemoglobin solution could be due to coagulant activity of red cell stromal contaminants. The coagulant activity of stroma was considered responsible for the disseminated intravascular coagulation which might play an important role in the pathogenesis of renal damage. These investigators postulated that stroma-free haemoglobin solution had no acute or chronic renal effects. No change in urinary output was observed and the clearance of urea, creatinine or para-aminohippuric acid did not change. Moreover, histological sections failed to demonstrate any lesions attributable to haemoglobin toxicity (14). This study rekindled the interest in employing haemoglobin solution as a blood substitute.

Later experiments lent further support to Rabiner's theory. When stroma-free haemoglobin solution was infused into cynomolgus monkeys even with prior stress in the form of dehydration, phlebotomy, or both, no alterations in clearance of inulin, endogenous creatinine, para-aminohippuric acid or renal architecture were observed (15). Relihan and co-workers failed to observe any change in renal function when stroma-free haemoglobin solution was infused into dogs (16). Moreover, histologic examinations of renal tissues taken before the infusion and various times after renal artery occlusion and infusion showed no essential differences between the kidneys of control dogs infused with saline and those infused with the haemoglobin solution (16).
Nephrotoxicity of stroma-free haemoglobin solution

With more studies on stroma-free haemoglobin, people became not so sure of whether haemoglobin molecule was non-nephrotoxic. In 1977, Savitsky et al conducted a clinical safety trial of stroma-free haemoglobin in which stroma-free haemoglobin was administered by slow intravenous infusion to 8 healthy men (17). During the infusion of the stroma-free haemoglobin solution and in the immediate hours after infusion, there was a significant decrease in urine output and endogenous creatinine clearance. There was a significant interference with normal renal functions during the period of haemoglobinemia and haemoglobinuria. However, this interference was transient and lasted for only a few hours (the period of haemoglobinemia). The changes were reversible and all subsequent measurements were normal. Savitsky attributed the temporary interference with renal function to haemoglobin rather than to the stroma because removal of 99% of stroma did not prevent these changes (17).

Haupt et al used rats to study the ultrastructural changes of proximal tubular cells after a haemoglobin load with or without temporary renal ischaemia. Electron microscopic examination was performed on kidneys removed 48 hours after treatment. The authors found that the size of lysosomes were altered. The lysosomes were four times larger than mitochondria in rats infused with haemoglobin, while lysosomes were rarely larger than mitochondria in groups given no haemoglobin. Moreover, the worst lesions were found in kidneys from rats that underwent renal ischaemia and haemoglobin load, the authors concluded that their results support the view that absorption
Introduction

of haemoglobin by the tubular cells made these cells more vulnerable to damage by ischaemia (18).

In 1988, Tam and Wong infused stroma-free haemoglobin solution to one group of rats and infused dextran-haemoglobin solution to another group (19). The dextran used has a molecular weight of 20000. Haemoglobin coupled to the dextran will not undergo renal glomerular filtration due to the large molecular size. The authors found that there was a decrease in glomerular filtration rate (GFR) in the former group. In contrast, there was no change in GFR in the latter group. Moreover, the excretion of the enzyme N-acetyl-β-D-glucosaminidase (NAG), a lysosomal enzyme located predominantly in the proximal tubules, showed a ten fold increase in the former group while there was only a slight increase in the latter group. Hence, this study showed that stroma-free haemoglobin had adverse effects on kidney. Moreover, the fact that there was no renal function impairment when the haemoglobin was made non-filterable by coupling it to dextran implied an etiologic role for intratubular haemoglobin in causing the derangement in renal function. Furthermore, this provides a possible means of preventing haemoglobin induced renal injury. (19)

Adopting a similar line of thought, Yoshioka et al investigated the possibility of using haptoglobin to prevent renal failure following thermal injury. In burns patients, there is a destruction of red blood cells due to direct thermal action which results in a massive release of free haemoglobin into the blood stream. Moreover, there is continued haemolysis secondary to intravascular coagulation and other humoral factors. Ten burns patients were divided into two groups, five patients received haptoglobin and five patients did not. The authors found that none of the five patients treated with
haptoglobin developed acute renal failure, whereas one patient in the control group died of acute renal failure. However, it should be noted that the better prognosis seen in the haptoglobin treated group cannot be attributed to the haptoglobin treatment because severity of the injury was not uniform in all patients and the number of patients in the series was too small. Nonetheless, administration of haptoglobin incurred prophylactic as well as therapeutic effects on renal injury secondary to haemolysis. (20)

It seems that if haemoglobin is rendered non-filterable by either coupling it with dextran or binding it with haptoglobin, there was no impairment in renal function. It is also of interest to find out whether there is an aggravation of the impairment of renal function in the presence of a factor which enhances reabsorption, such as tubular obstruction.

It is now thought that tubular obstruction promotes proximal tubular cell haemoglobin endocytic uptake as urinary stasis allows greater time for endocytosis to occur. The resulting increase in proximal tubular cell haemoglobin burden facilitates the expression of its nephrotoxic effects. Several lines of evidence are in support of this notion. First, in the presence of ureteral obstruction an otherwise subtoxic haemoglobin dose induced giant haem-stained proximal tubular cell endolysosomes and proximal tubular cell necrosis. Second, tubular obstruction when induced by ischaemic acute renal failure, also led to proximal tubular cell giant endolysosomes despite the administration of a low-dose of haemoglobin. Third, giant endolysosomes and proximal tubular cell necrosis were only seen in conjunction with extensive cast formation. Fourth, those tubular segments with the greatest degree of proximal tubular cell
Introduction
degenerative changes were those with the most striking endolysosome formation, strongly suggesting a link between endolysosome formation and proximal tubular cell necrosis (21).

The cellular mechanism whereby haemoglobin exerts deleterious effect on kidney remains to be defined. One attractive hypothesis involves the stimulation of hydroxyl radical formation by the haem iron which leads to oxidant tissue injury. Iron catalyzes the Haber-Weiss reaction whereby superoxide radical and hydrogen peroxide yield hydroxyl radical (OH.)

\[
\begin{align*}
O_2^- + Fe^{3+} &\rightarrow Fe^{2+} + O_2 \\
2O_2^- + 2H^+ &\rightarrow H_2O_2 + O_2 \\
H_2O_2 + Fe^{2+} &\rightarrow OH^- + OH^- + Fe^{3+}
\end{align*}
\]

Normally, iron is bound to ferritin and does not participate in this reaction. However, it was found that denatured haemoglobin was able to promote OH. formation. Paller injected glycerol intramuscularly to a group of rats to produce myoglobinaemia, haemoglobinaemia, myoglobinuria, and haemoglobinuria and infused haemoglobin in amounts exceeding plasma binding capacity to attain haemoglobinaemia and haemoglobinuria in another group of rats. In each group, some rats concomitantly received deferoxamine while others did not. He found that deferoxamine had a protective role in both models. The glomerular filtration rate did not show any marked decrease in GFR in those rats treated with deferoxamine in contrast to those without. Moreover, deferoxamine also decreased lipid peroxidation, showing that iron was involved in this process. Hence, it was postulated that iron was released from myoglobin and
Introduction

haemoglobin and catalyzed OH. formation by the Haber-Weiss reaction. This OH. then initiated lipid peroxidation and other free radical-mediated reactions which cause injurious effects to the cells (22). The aforementioned events seem to follow entry of haemoglobin into kidney cells. One would logically suppose that if the entry of haemoglobin into tubular cells can be blocked, then the harmful effect of haemoglobin can be reduced. It is therefore logical to ask and investigate whether there would be any advantageous effects on kidney if the entry of haemoglobin can be blocked. Success in blocking the entry of haemoglobin into kidney cells lies in the knowledge of how haemoglobin is handled by the kidney or rather how protein is handled by the kidney.

Renal handling of low molecular weight proteins

Low molecular weight proteins are reabsorbed by segregation into endocytotic vesicles at the apical border of tubular cells. The vesicles then migrate to the interior of the cell and fuse with lysosomes. The proteins are digested in the lysosomes. The reabsorbed protein is catabolized within the secondary lysosomes, which contain the hydrolytic enzymes: acid phosphatase, β-N-acetylhexosaminidase, arylsulfatase, and cathepsin A, B, and D. This process of protein uptake and degradation applies to large proteins such as albumin, haemoglobin and small proteins such as insulin (23).

The first step in endocytosis in the proximal tubule involves binding of protein to the luminal plasma membrane through the attachement of a positively-charged moiety of the protein to the luminal membranes, which are thought to be negatively charged. Thus,
Introduction

The infusion of positively charged amino acids such as ornithine, lysine or arginine into human subjects increases the excretion of proteins (24). Moreover, basic amino acids have been shown to reduce renal lysozyme accumulation in rat kidney, tubular lysozyme reabsorption in microperfusion experiments, and protein binding to isolated renal brush border membranes (25). On the contrary, when neutral or anionic amino acids were infused, the excretion of plasma proteins was not increased (26).

The plasma proteins filtered at the glomerulus appear to be reabsorbed by transport mechanisms that are shared with other proteins and have certain specific characteristics. This view was mainly based on the observation made by Hardwicke and Squire in the fifties that the intravenous infusion of albumin in nephrotic patients produces an increase in the clearance of globulins proportional to the albumin clearance (27). It has been reported that $\beta_2$-microglobulin and albumin can inhibit each other's tubular reabsorption in rat. The proteins filtered through the glomeruli are taken up by common tubular endocytotic sites irrespective of their physico-chemical features (23). Haemoglobin possibly enters renal tubular cells by the same mechanism via endocytotic sites just like other proteins. Moreover, it is probable that haemoglobin shares with other low molecular weight proteins the same receptors rather than using separate, specialized endocytotic sites. It is because in normal circumstances, haemoglobin does not exist in the circulation and therefore there is no haemoglobin filtered through the glomerulus and reabsorbed by the proximal tubular cells.
Introduction

Aim of study

If uptake of haemoglobin causes renal injury, then it would be worthwhile to investigate whether the blockade of its uptake prevents the damage. Moreover, it is useful to find out whether haemoglobin uptake utilizes the same uptake mechanism as other low molecular weight proteins. As lysine has been shown to increase the excretion of proteins such as $\beta_2$-microglobulin, kappa chains, lambda chains, albumin, transferrin, and IgG (24); it is therefore of interest to find out whether lysine would also inhibit haemoglobin reabsorption and, furthermore, whether lysine would confer any protective effect in renal injury secondary to haemolysis by the possible inhibition of haemoglobin reabsorption.
In order to elucidate the mechanism of haemolysis induced renal damage, the effect of concomitant infusion of lysine was studied. Rats were employed as the experimental animal. The investigation consisted of several sets of experiments. In each set, the rats were divided into 4 groups. In the first group, the normal control group, the rats received only isotonic saline infusion. In the second group, the lysine control group, the rats received only lysine (in isotonic saline) infusion. In the third and fourth groups, the "haemolysis" groups, haemolysis was induced 90 minutes after the start of experiment. In the third group, isotonic saline was infused while lysine (in isotonic saline) was infused in the fourth group.

One of the reasons for dividing the study into several sets of experiments was that radioactive inulin was used in the measurement of inulin. These samples were immediately discarded and suitably disposed of once the measurement was taken and were not used in the measurement of other parameters to avoid radioactive contamination. Besides, the amount of urine was often not sufficient to be used in several measurements.

The first set of experiments involved the investigation of the effects of haemolysis on kidney function assessed by the measurement of tritiated inulin clearance. In the second set of experiments, the effect of haemolysis on the excretion of N-acetyl-β-D-glucosaminidase was studied. In the third set of experiments, retinol binding protein was measured to examine how lysine and haemolysis affect the excretion
Methodology of this protein.

In addition to the above sets of experiments, experiments investigating the effects of lysine on kidney function and experiments checking the purity of inulin were also done.

All rats used were male overnight fasted Sprague-Dawley rats weighing 350 to 400 g. The animals were anaesthetized by the use of 4.5% sodium pentobarbital (60 mg/kg i.p.). A cannula was placed in the jugular vein for infusion purposes. The carotid artery was cannulated to facilitate blood sampling, blood pressure monitoring, and the injection of distilled water. A tracheostomy was done so that an airway could be placed in it. A low median incision was made in the lower abdomen and the urinary bladder was carefully dissected out. A small opening was incised in the bladder and a catheter was placed and fixed in it with ligature to allow accurate collection of urine. Moreover, the penis was ligated to prevent extravasation of urine. The temperature of the rats was maintained either by the use of a thermostatically controlled heat plate or a lamp shining directly on the rat. The blood pressure was monitored throughout the experiment.

When the surgical procedures were completed, experiments were started by first giving a priming dose (0.7 ml) of either inulin (6.7 kBq) or isotonic saline to the rats through the jugular vein. If inulin clearance was to be determined, the former was used. Otherwise, the latter was used. Immediately after the priming dose, all rats started receiving infusions of either isotonic saline or lysine in isotonic saline throughout the experiment. Whether tritiated inulin was added to the infusion fluid in any experiment was dictated by the need to measure inulin clearance in that particular experiment. The sustaining solution contained 3.7 kBq/ml of inulin if inulin clearance
Methodology

was to be measured. The infusion rate was set at 3.3 to 3.4 ml/h.
In vivo haemolysis was induced by the bolus injection of 4 ml of
 distilled water through the carotid artery over five seconds. Blood
 sampling (about 0.5 ml) and urine collections were made every 45
 minute starting from either 90 minute or 45 minute as in the case of
 the NAG and the RBP studies. If haemolysis was induced, a blood
 sample would be collected 5 minutes after induction of haemolysis.

The experimental procedure is schematically shown below:

The glomerular filtration rate at 90, 135 180, 225 minutes after
the start of experiment was assessed by the measurement of tritiated
inulin clearance (19). Urine N-acetyl-β-D-glucosaminidase was
measured by the MCP-NAG method (28,29). Urine retinol binding protein
was measured by enzyme linked immunosorbent assay (30).

Plasma and urine haemoglobin were measured by the method of
Drabkin and Austin (31). In this method, haemoglobin was oxidized with
potassium hexacyanoferrate (III) to methaemoglobin which then reacted
with potassium cyanide to form cyanmethaemoglobin. The optical density
of which was then compared with the standard. 0.04 ml of the plasma
or urine were added to 2.5 ml Drabkin's solution in a cuvette. The
Methodology

optical densities of the samples and the standard were measured with a spectrophotometer at 540 nm. The concentration of the haemoglobin in the samples were then determined by the use of Beer's Law.

For the determination of inulin clearance, 50 μl of plasma or urine specimens collected at various time points were pipetted into scintillation vials. Ten millilitres of the scintillation cocktail was then added to the vial. The cocktail contained the solvent, toluene; the primary solute, 2,5-diphenyloxazole (PPO); the secondary solute, 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP); and the emulsifier, octoxynol, also known as Triton. The vials were then counted in a Beckman LS 7000 liquid scintillation system. Each sample was counted for a maximum of 10 minutes.

Many of the samples contained haemoglobin which may cause colour quenching. Quenching results in a reduction in the counting efficiency because haemoglobin may absorb strongly in the wavelength region where the fluors luminesce, causing a severe shift in the observed spectrum even at very low concentrations. This problem was circumvented by using external standardization employing the H number method for correction. Tritium quenched standards containing the same amount of activity but different quantities of quenching agent were counted. The H numbers were measured for each standard and a quench curve generated which related the counting efficiency of each standard to its corresponding H number. One typical set of data is shown in Fig. 2.1.

The analysis of data involved the use of multivariate analysis of variance, multivariate analysis of covariance, and the Dunnett's test. When the aim was to find out whether a set of repeatedly measured variables would be different among several groups, the analysis of
Methodology

variance would be employed. When there was a need to control for the baseline level, the analysis of covariance was used instead. If the purpose was to find out whether there was a change in the repeatedly measured variable compared with the control value, the Dunnett's test was employed. The calculations were done by the use of the statistical package, SPSS, in an IBM personal computer.
Fig 2.1. The quench curve relating the counting efficiency and the H number.
Determination of the purity of inulin

CHAPTER THREE

DETERMINATION
OF THE PURITY
OF INULIN

In the study, the glomerular filtration rate was assessed by the inulin clearance method. Inulin can be determined by a modification of the method of Fjeldbo and Stamey (32) in which inulin is hydrolyzed to fructose with concentrated HCl and FeCl₃ and then reacted with resorcinol, producing a red condensation product that is measured spectrophotometrically at 480 nm. However, it is more convenient to use the radioisotope, tritiated inulin.

The $^3$H Inulin ($C_6H_{11}O_6$)($C_6H_{10}O_5$)$_n$($C_6H_{11}O_5$) is a polysaccharide with a molecular weight of approximately 5200. It was purchased from the Amersham International Plc. $^3$H Inulin on storage might yield decomposition products such as D-fructose or D-glucose. Such decomposition products can be metabolized by the body in contrast to the parent molecule. One of the metabolites might include tritiated water. These products will cause a distortion in the measurement of inulin clearance. Therefore, it is clear that every endeavour has to be made to ensure this would not happen. Hence, the purity of the inulin was checked in the first place by employing gel-filtration chromatography.
Determination of the purity of inulin

Method

Sephadex G-25 was added to distilled water and the suspension was then placed in a water bath at 90°C for 1 hour to accelerate swelling. This also served to remove any air bubbles present in the suspension. The gel was packed in a Bio-rad column 10 cm long and an internal diameter of 1.0 cm. Phosphate buffer saline (pH=7.4) was used as the eluent. For the preparation of about 100 ml of phosphate buffer, 19.0 ml of 0.2 M sodium di-hydrogen phosphate was added to 81.0 ml 0.2 M di-sodium hydrogen phosphate and the pH was adjusted to 7.4 with the use of a pH meter and the addition of further amount of either the conjugate acid or the conjugate base. Two column volumes of eluent were passed through the column in order to stabilize and equilibrate it with the bed. The red haemoglobin molecule was used to determine the void volume. The haemoglobin solution was layered on top of the bed. The column outlet was then opened and the sample was allowed to drain into the bed, taking care not to let the bed run dry. A small amount of eluent was used to wash any sample which remained on the bed surface and on the wall into the bed. The column was then connected to a flask, the reservoir, containing the eluent and the column was operated at a flow rate of 0.30 ml/min. After the determination of void volume, methylene blue was used to find out the elution profile of a small molecule. Finally, the procedure was repeated with 35 μl of tritiated inulin solution (22.2 kBq/ml) which was the sample of interest. The effluent was collected in 0.5 ml portions up to a total volume of 2.5 ml. Thereafter, 1 ml portions were collected to an elution volume of 6 ml. After mixing 0.2 ml of each collected fraction with scintillation fluid, the effluent was counted using the
Determination of the purity of inulin

Beckman LS 7000 liquid scintillation system.

During the packing of the column, the following points were observed. 1) No air bubbles were present in the suspension as the gel had been swollen on a boiling water bath. 2) No air bubbles were present in the dead space under the net. 3) The well-mixed gel suspension was poured down the column in one smooth maneuver to avoid any heterogeneities or stratification of the column. 4) The column was vertical.

Results

The void volume was 2.9 ml. Methylene blue was eluted at an elution volume from 10 ml to 22 ml. The elution profile of tritiated inulin is shown in Figure 3.1 in which counts per minute (CPM) was plotted against elution volume in millilitre.

Discussion

The advantages of inulin clearance include the following: 1) Inulin is freely filtered and not secreted nor reabsorbed. 2) Inulin is inert and nontoxic. 3) Inulin does not bind with plasma proteins. 4) Inulin is not metabolized.

Sephadex which was a bead-formed gel prepared by cross-linking dextran with epichlorohydrin was used as the gel filtration media. As the molecular mass of the tritiated inulin is 5200, Sephadex G-25
Determination of the purity of inulin was employed since the fractionation range of it in terms of molecular mass was 1000-5000. In addition, it has the advantages of good rigidity, easy handling, and good flow characteristics. Inulin would elute at the void volume.

During sample application on the bed surface, every effort had been made to ensure that the bed would not run dry and there was no disturbance on the bed surface.

Haemoglobin solution exhibits a red colour and haemoglobin has a molecular mass of approximately 60,000. Therefore, haemoglobin is a suitable molecule in determining the void volume which is the elution volume of molecules which are only distributed in the mobile phase because they are larger than the largest pores in the gel. Moreover, its red colour enabled us to check the homogeneity of the bed by running through a sample solution of red haemoglobin solution.

To gain an idea of the volume at which small molecules would be eluted, methylene blue was a suitable candidate as it had a blue colour and it was a small molecule with a molecular mass of 373.90. Monosaccharide such as fructose or glucose would elute at a volume similar to that of methylene blue.

From Figure 3.1, the large molecule haemoglobin eluted at a volume from 2.9 to 6 ml. A single peak in the value of CPM was observed in similar fractions, signifying the presence of the large molecule tritiated inulin. Methylene blue eluted at 10 ml. No peak was noticed in these fractions. If any degradation products such as tritiated fructose and glucose or the metabolite, tritiated water, were present, a peak or even several peaks would have occurred in the same fractions in which methylene blue was eluted. Absence of such peaks suggests that the inulin we used contained little, if any,
Determination of the purity of inulin degradation products. The results would be reliable and the tritiated inulin clearance would be a good measure of glomerular filtration rate.
Determination of the purity of inulin

Figure 3.1. Elution profile of inulin. Haemoglobin was used to determine the void volume. Methylene blue was used as an index to demonstrate when small molecules started to be present in the effluent.
Determination of the optimal lysine concentration

CHAPTER FOUR

DETERMINATION
OF THE
OPTIMAL LYSINE
CONCENTRATION

Lysine, 2,6-diaminohexanoic acid, is an amino acid which is positively charged at physiological pH. It is essential in human nutrition, as it is not synthesized by the human body. It is also essential with respect to its growth effect in rats.

In 1977, Mogensen and Solling were able to demonstrate that substances with a positively charged group located terminally in the molecule (ornithine, lysine, arginine, epsilon-aminocaproic acid, and Cyclocaprone) inhibited instantaneously tubular protein reabsorption and lysine was the most effective molecule tested (24). It is therefore logical to make use of this property of lysine to inhibit tubular haemoglobin reabsorption, and explore whether lysine infusion would have a beneficial effect in renal damage secondary to haemolysis.

Before infusing lysine into rats, however, one problem remained to be solved. What is the concentration of lysine that should be infused into the rats? Despite the fact that amino acids are present in living organisms, high doses of amino acids are toxic. Nausea, vomiting, hypotension, tachycardia, azotemia, kidney tubular cell damage, kaliuresis, bicarbonaturia, and even acute renal failure have been reported when too high a dose of amino acid was administered (33,34,35,36,37,38,39).
Determination of the optimal lysine concentration

As our aim in using lysine was to ameliorate the haemolysis induced renal damage, it was therefore essential to determine a dose which would cause the least harmful renal effects in the rats.

Method

In this set of experiments, 500 mM lysine in isotonic saline infused at a rate of 3.3-3.4 ml/h was tested first. As described in Chapter 2, infusion was immediately carried out upon the completion of the surgical procedure. Inulin clearance was measured 90, 135, 180, 225 minutes after the start of infusion. Should the inulin clearance not be stable over time with the use of lysine at this concentration, that is, 500 mM; the concentration of lysine was halved and the experiment repeated again. This process would be repeated until a concentration of lysine which had no adverse effects on the inulin clearance was found. In addition, the tritiated inulin clearance was also measured in rats receiving only isotonic saline infusion for control purposes.

To confirm that lysine would not depress inulin clearance at the optimal concentration of lysine found in the above experiments, isotonic saline was infused into rats for 90 minutes and the inulin clearance measured, then lysine at the concentration determined in the above experiments was substituted for isotonic saline and infused for two 45 minute periods and the inulin clearance was measured in each period.
Determination of the optimal lysine concentration

Statistical analysis

Dunnett's test was used to compare the inulin clearances at 135, 180, 225 minute with the baseline inulin clearance at 90 minute within a group. Multivariate analysis of covariance was used to explore between-groups patterns of differences on the set of inulin clearance in toto. All values are expressed in mean ± SEM.

Results

When 500 mM lysine was tested, the glomerular filtration rate as measured by the inulin clearance showed a large decrease, dropping from an initial value of 2.81±0.26 ml/min to the final value of 0.59±0.11 ml/min (p<0.01; Fig. 4.1). A decline in inulin clearance was still observed when the concentration of lysine was halved to 250 mM, the inulin clearance dropped from the baseline level of 2.52±0.29 ml/min to a minimum of 1.68±0.13 ml/min (p<0.05) at 180 minute (Fig 4.2). Deterioration in inulin clearance also occurred when the concentration of lysine was halved again to 125 mM, as manifested by a rapid drop from 2.97±0.13 ml/min to 2.33±0.18 ml/min (p<0.01) at 135 minute and further down to the minimum of 2.09±0.14 ml/min (p<0.01) at 180 minute (Fig 4.3). The inulin clearance seemed to be rather stable when 60 mM lysine was tested (Fig 4.4), however, the set of inulin clearance considered in toto with the inulin clearance at 90 minute as covariate exhibited a difference between this group and the control group (p<0.01). When 30 mM of lysine was infused, no difference
Determination of the optimal lysine concentration

between the control group and the 30 mM lysine group was observed (MANCOVA) (Fig. 4.5, Fig. 4.6).
Determination of the optimal lysine concentration

Figure 4.1. Temporal profile of the inulin clearance when 500 mM lysine was infused. Data are the mean±SEM. Dunnett's test was used for data analysis. (** p < 0.01 vs inulin clearance at 90'; n=6)
Determination of the optimal lysine concentration

Figure 4.2. Temporal profile of the inulin clearance when 250 mM lysine was infused. Data are the mean±sem. Dunnett's test was used for data analysis. (* p < 0.05 vs inulin clearance at 90'; n=7)
Determination of the optimal lysine concentration

Figure 4.3. Temporal profile of the inulin clearance when 125 mM lysine was infused. Data are the mean±sem. Dunnett's test was used for data analysis. (** p < 0.01 vs inulin clearance at 90'; n=8)
Determination of the optimal lysine concentration

60 mM LYSINE INFUSION

Figure 4.4. Temporal profile of the inulin clearance (ClIN) when 60 mM lysine was infused. Data are the mean ± sem. Dunnett's test was used for data analysis. No significant difference in ClIN was found at 135', 180', and 225' when compared with that at 90'. (n=8)
Determination of the optimal lysine concentration

30 mM LYSINE INFUSION

Figure 4.5. Temporal profile of the inulin clearance ($Cl_{IN}$) when 30 mM lysine was infused. Data are the mean±SEM. Dunnett's test was used for data analysis. There was no significant difference between the $Cl_{IN}$ at 90', and that at 135', or 180' or 225'. (n=7)
Figure 4.6. Temporal profile of the inulin clearance ($C_{\text{IN}}$) when isotonic saline was infused. Data are the mean±sem. When Dunnett's test was used to compare the $C_{\text{IN}}$ at various time points with that at 90', no significant difference was found. (n=7)
Figure 4.7. The baseline inulin clearance (Cl\textsubscript{IN}) was obtained during isotonic saline infusion. After 90 minutes of saline infusion, the infusion fluid was shifted to 30 mM lysine. The Cl\textsubscript{IN} were obtained 45 and 90 minutes post lysine infusion and they were found to be not statistical significant different from the baseline value. Data are the mean±sem. (n=6)
Determination of the optimal lysine concentration

In the group of rats infused with saline for 90 minutes and then changed to lysine infusion for another 90 minutes, there was essentially no change between the baseline inulin clearance obtained at 90 minute, 3.28±0.20 ml/min, and the inulin clearance obtained 45 and 90 minutes after changing to 30 mM lysine infusion which were 3.12±0.21 ml/min and 3.02±0.23 ml/min respectively. (Fig. 4.7)

Discussion

This study indicates that high doses of the amino acid lysine produce a decrease in renal function, clearly demonstrating its nephrotoxic potential. Obvious changes in inulin clearance were found when lysine was infused at a rate of 19.4 μmol/kg/min (125 mM at 3.4 ml/h to 350 g rat) or greater. This finding is in agreement with other studies (35,36,37,38). However, our data also showed that renal function was unaffected when a low concentration of lysine was used.

It is well known that high doses of amino acids are toxic. In dogs, an oral dose of about 7 mmol/kg of glycine, L-serine, L-alanine, or L-threonine was well tolerated. However, the same dose of L-lysine, L-arginine, and several other amino acids caused the dogs to vomit. Different amino acids exert different toxic effects which might be generalized or specific to particular target organs. For example, methionine induces nausea, vomiting, hypotension, periodic disorientation, tachycardia, and azotemia (33), while ethionine and D-serine seem to be particularly nephrotoxic (34). 5-hydroxy-L-tryptophan was reported to cause renal damage in rats and the LD50 dose of this amino acid was 1.7 mmol/kg in Sprague-Dawley rats.
Determination of the optimal lysine concentration

Intravenous infusion of up to 240 μmol/kg/min basic amino acids L-lysine, L-arginine, or L-ornithine into dogs caused a marked kaliuresis (39).

Infusion of L-lysine at 110 μmol/kg/min produced massive bicarbonaturia in dogs and marked accumulation of this amino acid was found in the kidney (38). Moreover, in the same study, it was shown that the glomerular filtration rate in dogs dropped from the control value of 63.4±7.3 ml/min to 39.5±7.4 ml/min 180 minutes after the start of experiments. Walker et al proposed that lysine could enter the cell in its unionized form (39), and as a result, reduce hydrogen ion secretion from proximal cell through trapping of intracellular protons by the accumulated unionized lysine. An alternative hypothesis is that lysine, existing mostly in cationic form in the tubular fluid, simply behaves as a poorly reabsorbable cation which forces the excretion of bicarbonate and chloride to maintain electroneutrality (38).

When Zager and co-workers infused lysine to rats at a rate of 125 μmol/kg/min for 80 minutes, they found that there was a 63 % decrease in glomerular filtration rate (37). If lysine had been infused for a longer time in Zager's studies, there might have been a larger drop in the glomerular filtration rate as indicated by our results.

When L-lysine was infused at 55 μmol/kg/min (13 mmol/kg total) to rats, true nephrotoxicity was observed as manifested by a decrease in inulin and creatinine clearances and an increase in 125I-albumin clearance (36). The inulin clearance dropped to a value of about 0.29 ml/min after lysine infusion for four hours. This data is comparable to results found here. When lysine was infused at 80.9 μmol/kg/min for 3.75 hours, the inulin clearance dropped to a value of 0.59
Determination of the optimal lysine concentration ml/min (Fig. 4.1).

To further establish that the renal failure observed in the above 4-hour studies was not just a transient phenomenon, Malis et al. performed 20-hour studies in which lysine 1.9 g/kg was administered intraperitoneally to rats (36). Blood and urine samples were collected after twenty hours. It was found that the creatinine clearance in the lysine treated group had a value of 0.8±0.2 ml/min while the control group had a value of 1.8±0.2 ml/min (36).

Racusen and co-workers reported that when lysine was given to rats in a dose of about 15 mmol/kg (61 μmol/kg/min) over 4 hours, increases in serum urea nitrogen and creatinine concentrations were found. Moreover, urea nitrogen and creatinine clearances fell markedly. In addition, intratubular proteinaceous debris, desquamation of individual tubular epithelial cells, and considerable tubular regeneration with mitoses, hyaline casts were found in kidney sections. On the contrary, these authors observed no changes in serum BUN, serum creatinine, urea and creatinine clearances in rats infused with lysine at lower concentrations, the largest of which tested was a total of 2.74 mmole at a rate of 40.7 μmol/kg/min. In addition to the observation that there was no functional change in the rats infused with a lower concentration of lysine, there was no structural change either (35,37).

The mechanisms of lysine induced depression in renal function is as yet not clear. Several possibilities have been proposed: 1) increased renal vascular resistance and decrease in renal blood flow; both of these phenomena follow the initial elevations in tubular pressure and increase in tubular diameter, tubular obstruction may cause compression of peritubular capillaries by distended tubules and
Determination of the optimal lysine concentration

also a delayed afferent arteriolar constriction due perhaps to a feedback mechanism. 2) increased delivery of lysine to distal, more sensitive areas of the nephron, possibly with direct toxic effects at high doses when the $T_{\text{max}}$ for lysine reabsorption is exceeded. 3) effects on tubular structure and formation of casts, resulting in obstruction and perhaps non-functioning nephrons; lysine blocks tubular protein reabsorption and it seems possible that the high protein concentration in tubular fluid might increase the tendency of cast formation in tubule (35).

Perhaps one may query the use of lysine in our future sets of experiments in the light of the potential nephrotoxicity of lysine. As a matter of fact, nearly all amino acids are toxic but only when used in high doses. The positively charged amino acid arginine as well as other amino acids such as glutamic acid, aspartic acid, alanine, and glycine have also been shown to cause a reduction in glomerular filtration rate (37). Our study as well as the study of Racusen et al (35) showed that a stable glomerular filtration rate could be obtained with a low dose of lysine. Moreover, it was well documented that lysine was very effective in inhibiting protein reabsorption in the proximal tubule (24). It is this property that makes it a potential agent to prevent deterioration in renal function induced by haemolysis.
After determining the dose of lysine which is safe, the potential protective effect of lysine on haemoglobinuric renal damage was investigated. It is well known that lysine inhibits small molecular weight protein uptake and, if haemoglobin uptake is the central step leading to renal damage, this property of lysine could be used to decrease the uptake of haemoglobin in the proximal tubules and thus lessen any damage resulting from it. Therefore, this study was conducted to examine if there was any beneficial effect of lysine on renal function in haemolysis in vivo.

**Method**

In this set of experiments, the tritiated inulin clearance of four groups of rats were compared. The first group was the control group which received 0.9% saline infusion only. The second group, the lysine only group, was the one that received 30 mM lysine in 0.9% saline infusion. The third group, the haemolysis-only group, received 0.9% saline infusion throughout the experiment and haemolysis was induced 90 minutes after the start of experiment. The fourth group, the haemolysis-plus-lysine group, received 30 mM lysine in isotonic saline throughout the experiment and haemolysis was induced at 90 minute. Haemolysis was produced by bolus injection of 4 ml distilled
The protective role of lysine water through the carotid artery over 5 seconds. Tritiated inulin clearance was determined 90, 135, 180, 225 minutes after the start of experiment as mentioned in Chapter 2. Plasma and urine haemoglobin concentration were determined 5, 45, 90, and 180 minutes after haemolysis. This was done by mixing the samples with Drabkin's reagents and the optical density of the mixture was then measured with a spectrophotometer at 540 nm. The haemoglobin concentration of the samples was then calculated by the Beer's equation. The plasma haemoglobin concentration five minutes after haemolysis was used as a selection criterion to include those rats which had similar degree of haemolysis in the analysis.

Statistical Analysis

All data are expressed in mean±SEM. Dunnett's test was used for intra-group comparison. Multivariate analysis of covariance was used for inter-group comparison of inulin clearance. Multivariate analysis of variance was used for inter-group comparison of plasma haemoglobin concentration and the amount of haemoglobin excreted.

Results

For the haemolysis-only-group, the inulin clearance showed a rapid deterioration (Fig. 5.1). The baseline inulin clearance was 2.75±0.06 ml/min. Forty five minutes after haemolysis treatment, there was already a rather marked decrease of the inulin clearance to a level of 1.94±0.25 ml/min (p<0.01). The inulin clearance finally
The protective role of lysine reached a value of 1.82±0.21 which was significantly different from the baseline clearance value (p<0.01). In contrast, in the haemolysis-plus-lysine group, no fall in the inulin clearance was noted after haemolysis treatment (Fig. 5.2). The inulin clearance remained rather stable throughout the experiment for this group as well as in the control group (Fig. 5.3) and lysine-only group (Fig. 5.4). Multivariate analysis of covariance using the inulin clearance at 90 minute (the inulin clearance just before haemolysis) as a covariate showed that there was no statistical difference between the lysine-only, haemolysis-plus-lysine and the control group. A statistical significant difference existed between the control group and the haemolysis-only group (p<0.002). Moreover, the differences in inulin clearance between the haemolysis-only group and the haemolysis-plus-lysine group were of statistical significance (p<0.05; Fig. 5.5).

The plasma haemoglobin concentration five minutes after haemolysis was similar for the haemolysis-only and haemolysis-plus-lysine groups. The haemoglobin concentration were 6.28±1.13 and 6.00±0.66 g/l respectively, implying that there was no difference in the degree of haemolysis. On the other hand, as shown in Figure 5.6, the haemolysis-only group seemed to have a higher level of haemoglobin 45, 90, 135 minutes after haemolysis than the haemolysis-plus-lysine group. However, the difference was not statistically significant. In addition, the haemolysis-plus-lysine group showed a greater degree of haemoglobin excretion at 135 and 180 minutes (Fig. 5.7), but, again, there is no statistical significant difference between the haemolysis-only and the haemolysis-plus-lysine group in this aspect.
The protective role of lysine

Figure 5.1. Temporal profile of the inulin clearance ($Cl_{IN}$) in the group receiving isotonic saline infusion. Haemolysis was induced at 90 minute. After haemolysis, the $Cl_{IN}$ dropped significantly. (* $p < 0.01$ vs $Cl_{IN}$ at 90 minute; n=9)
The protective role of lysine

Figure 5.2. Temporal profile of the inulin clearance ($C_{\text{IN}}$) in the group receiving 30 mM lysine in saline infusion. Haemolysis was induced at 90 minute. The $C_{\text{IN}}$ at various time points are not significantly different from $C_{\text{IN}}$ at 90 minute. (n=9)
Figure 5.3. Temporal profile of the inulin clearance in the group receiving isotonic saline infusion. This group received no haemolysis treatment. No significant differences were found between the Cl\textsubscript{IN} at 90 minute and those at other time points thereafter. (n=5)
The protective role of lysine

Figure 5.4. Temporal profile of the inulin clearance in the group receiving 30 mM lysine infusion. Haemolysis was not induced in this group. There was no significant difference between the $C_{\text{IN}}$ at 90 minute and those at other time points thereafter. (n=7)
The protective role of lysine

Figure 5.5. The inulin clearance of the haemolysis-only group (n=9) and the haemolysys+lysine group (n=9) are compared in this figure. The differences between these groups are statistically significant. (p < 0.05)
The protective role of lysine

Figure 5.6. The level of plasma haemoglobin in the haemolysis-only group (n=9) and the haemolysis+lysine group (n=9). Haemolysis was induced at 90 minute. No significant differences were observed between the two groups.
The protective role of lysine

Figure 5.7. The excretion of haemoglobin in the haemolysis-only group (n=9) and the haemolysis+lysine group (n=9) collected at 135, 180, and 225 minutes after the start of experiment. Haemolysis was induced at 90 minute. No significant differences were observed between the two groups.
The protective role of lysine

Discussion

In this set of experiments, there was a rapid decline of inulin clearance when haemolysis was induced in the haemolysis-only group. The results confirm that in vivo haemolysis does induce a depression of renal function. In the haemolysis-plus-lysine group, there was no alteration in the inulin clearance. The only difference between these two groups was the additional infusion of lysine in the latter group. Therefore, it is logical to infer that lysine indeed offers some protection against haemolysis associated renal function impairment. We postulate that the mechanism of the protective role of lysine may well be related to its well documented ability to inhibit the reabsorption of protein in the proximal tubules. The positively charged molecule lysine may occupy the binding sites that trigger endocytosis so that there is less haemoglobin uptake and hence a lesser degree of injury. Although the haemoglobin data (Fig. 5.7) could not prove that the excretion of haemoglobin did increase significantly in the haemolysis-plus-lysine group, it is interesting to note that this group appeared to have a greater degree of haemoglobin excretion and a lower plasma haemoglobin concentration in comparison with the haemolysis-only group. The trend in the excretion of haemoglobin was reversed in this group upon the termination of experiment at 225 minute. This could be due to the lower plasma concentration. A low haemoglobin concentration will lead to a low haemoglobin excretion. It should be pointed out that despite a tendency for higher haemoglobin excretion, the urinary excretion of haemoglobin may not explicitly reflect the change. If only a small
The protective role of lysine

amount of haemoglobin uptake is blocked by lysine to achieve a protective effect, the change in the quantity of excreted haemoglobin may not be clearly evident.
In Chapter 5, we have seen that lysine seems to offer protection against in vivo haemolysis associated renal injury. The renal function was preserved in the haemolysis-plus-lysine group but not in the haemolysis-only group. An alternative way to demonstrate the protective effect of lysine would be to make use of the lysosomal enzyme, N-acetyl-\(\beta\)-D-glucosaminidase (NAG). This is a widely distributed lysosomal enzyme, located predominantly in the renal proximal tubules. It has been established that the NAG concentration in the urine increases with proximal tubular damage \((40,41)\), and it is considered to be a sensitive index of renal disease and drug nephrotoxicity. NAG was quantitated in this study by the method of Noto et al. \((28)\)

**Method**

As in the previous study, the rats were divided into four groups, the first group received only isotonic saline infusion, the second group received only 30 mM lysine in isotonic saline infusion, the third group received isotonic saline infusion and haemolysis treatment, while the last group received 30 mM lysine in saline infusion and haemolysis treatment. Plasma and urine samples were collected 45, 90, 135, 180, and 225 minutes after the start of experiment.
An enzyme study

The plasma and urine haemoglobin 45, 90, and 135 minutes after haemolysis were determined using the method of Drabkin and Austin (31). In addition, the plasma haemoglobin concentration 5 minutes after haemolysis was measured to ensure that the degree of haemolysis was similar.

NAG was assayed at 37°C in a centrifugal analyzer (Cobas Bio) by its action on the substrate sodium m-cresolsulfonphthaleinyl N-acetyl-β-D-glucosaminide. NAG hydrolyzes this compound to N-acetyl-glucosamine and m-cresolsulfonphthalein (MCP). The concentration of MCP can then be measured spectrophotometrically. In this assay, the substrate solution contained borax and m-cresolsulfonphthaleinyl N-acetyl-β-D-glucosaminide in citrate buffer. After mixing 5 µl of the urine sample with 100 µl of the substrate solution, the mixture was incubated for 999 seconds at 37°C. The absorbance of the mixture at 580 nm was measured 990 seconds after the start of incubation. The reaction was terminated by the addition of 30 µl of 300 mM sodium carbonate solution and the absorbance of the mixture at 580 nm was again measured (28,29). The amount of MCP formed could be derived from the difference between the two absorbance readings. One unit of enzyme activity is defined as the amount of enzyme catalyzing the formation 1 µmol of MCP per hour under the conditions described above.

As the haemoglobin has its peak absorbance at 540 and 580 nm, the presence of haemoglobin in the samples may interfere with the measurement of NAG. To solve this problem, various dilutions of a standard haemoglobin solution were prepared in isotonic saline, 0.2 ml of aliquots of urine sample was added to 0.2 ml of these solutions so that the resulting mixture had different haemoglobin concentration but identical NAG concentration. The NAG concentration of the '0'
An enzyme study

haemoglobin solution in which there was no haemoglobin (0.2 ml aliquot of urine + 0.2 ml isotonic saline) was measured and used as a reference value. The NAG values were measured in these mixtures. The ratio of the NAG concentration in the presence of haemoglobin to that without ('O' haemoglobin solution) was calculated and plotted against haemoglobin concentration. The NAG concentration of the rat urine samples were corrected for the presence of haemoglobin using this graph.

The interference of haemoglobin on NAG determination was also studied by employing known concentrations of haemoglobin solutions which contained no NAG. These solutions were then treated the same way as the samples which contained NAG, that is, they were incubated with the substrate solution and then the stop reagent as mentioned above. This was performed to find out whether NAG activity could be measured in these solutions and whether there was any relationship between the measured activity and the haemoglobin concentration.

Statistical analysis

Data are the mean ± SEM. Multivariate analysis of covariance was used for inter-group comparison of N-acetyl-β-D-glucosaminidase data with the average of the values obtained at 45 and 90 minutes as the control value. Dunnett's test was used for intra-group comparison with the control value. Multivariate analysis of variance was used for inter-group comparison of haemoglobin data.
An enzyme study

Results

The ratio of measured NAG concentration of the mixtures to the expected NAG concentration was plotted against haemoglobin concentration, and a regression line was drawn through the points. The correlation coefficient was 0.93 (Fig. 6.1), the slope was 0.083 and the constant term was 1.03. In this regression line, the abscissa was the haemoglobin concentration while the ordinate was the correction factor. The NAG concentration of the samples were corrected for the presence of haemoglobin by first determining the concentration of haemoglobin and then the correction factor corresponding to this haemoglobin concentration would be read off from this regression line. The concentration of the NAG was then divided by this correction factor to obtain the true value of NAG concentration.
Figure 6.1. The regression line obtained by plotting the 'NAG correction ratio' against haemoglobin concentration. The 'NAG correction ratio' was obtained by dividing the NAG of the sample with that of the '0' haemoglobin solution. The sample and the '0' haemoglobin solution contained the same amount of NAG.
An enzyme study

The average rate of N-acetyl-β-D-glucosaminidase excretion in the first two groups, that is, the normal control and the lysine control group, did not show significant fluctuation (Fig. 6.2, Fig 6.3), implying the absence of insults to proximal tubular cells. In contrast, the third and fourth groups, namely, the haemolysis-only and the haemolysis-plus-lysine groups, showed a marked increase in NAG excretion rate after in vivo haemolysis. In the haemolysis-only group, the average baseline value of NAG excretion rate was 24.9±1.6 mU/min., and it increased to 116.2±16.2 mU/min. immediately after haemolysis (Fig. 6.4). The peak value of NAG excretion, 160.9±14.6 mU/min. occurred within the 135-180 minute period. For the fourth group, the haemolysis-plus-lysine group, there was also an elevation of NAG excretion after haemolysis, but the degree of elevation was less (Fig. 6.5). The average baseline excretion of NAG in this group was 24.4±3.7 mU/min., and a three fold increase to 73.5±5.6 mU/min was observed immediately after in vivo haemolysis. The peak excretion rate, 115.0±19.1 mU/min., occurred in 135-180 minute period, similar to that in the third group. At all time intervals, the NAG excretion was less in the haemolysis-plus-lysine group than the haemolysis-only group. (Fig. 6.6) The differences in these two groups were statistically significant (p<0.01) as revealed by the use of multivariate analysis of covariance.
Figure 6.2. The average rate of NAG excretion in the normal control group which received only isotonic saline infusion. The excretion rates in various time periods exhibited no statistical difference when compared with the average baseline value in the first ninety minutes. (n=7)
Figure 6.3. The average rate of NAG excretion in the lysine control group which received 30 mM lysine in saline throughout the experiment. No statistical differences were found between the baseline value and that in the time period thereafter. (n=6)
Figure 6.4. The average rate of NAG excretion in the haemolysis-only group. This group received isotonic saline infusion throughout the experiment. Haemolysis was induced at 90 minute. A sharp rise in the excretion rate was observed after haemolysis. (n=11)
Figure 6.5. The average NAG excretion rate in the haemolysis-plus-lysine group. This group was infused with 30 mM lysine in saline throughout the experiment. Haemolysis was induced at 90 minute. A rise in the excretion rate was noted after haemolysis. (n=12)
An enzyme study

Figure 6.6. A summary of the NAG excretion rate of the four groups. NAG excretion rose sharply after haemolysis. The haemolysis-only group (n=11) showed a greater degree of rise than the haemolysis-plus-lysine group (n=12). (p < 0.01) The NAG excretion rate in the isotonic saline infusion group (n=7) and the 30 mM lysine infusion group (n=6) were stable throughout the experimental period.
An enzyme study

The plasma haemoglobin concentration five minutes after haemolysis in the haemolysis-only group and the haemolysis-plus-lysine group were very similar. The values were 6.33±1.1 g/l and 6.60±0.47 g/l respectively. The plasma haemoglobin concentration was lower in the latter group at all times but the differences were not statistically significant (Fig 6.7). The quantity of haemoglobin excreted also showed no statistically significant difference (Fig. 6.8).
An enzyme study

Figure 6.7. The plasma haemoglobin concentration in the haemolysis-only group (n=11) and the haemolysis-plus-lysine group (n=12). No significant difference was observed between the two groups.
An enzyme study

Figure 6.8. The amount of urinary haemoglobin excreted in the haemolysis-only group (n=11) and the haemolysis-plus-lysine group (n=12). No significant difference was observed between the two groups.
An enzyme study

When haemoglobin solutions of various concentrations containing no NAG were assayed for NAG activity with the MCP-NAG method using a centrifugal analyzer, NAG activity could be measured despite the absence of the enzyme, NAG, in these solutions. When the NAG activity of these solutions were plotted against the haemoglobin concentration, a regression line (slope=8.06, intercept=1.72) with a correlation coefficient of 0.993 was obtained (Fig.6.9). From this graph, it can be observed that the measured NAG activity was proportional to the haemoglobin concentration.
Figure 6.9. The NAG activity measured in the various concentrations of haemoglobin solutions. No NAG was present in these solutions.
The interference of NAG measurement by haemoglobin is established in this experiment. This effect is well accounted for by a change in the absorbance of the haemoglobin in conditions of different pH. The absorbance of haemoglobin in a neutral or an alkaline environment is higher than that in an acidic one. In NAG determination, a substrate solution with a pH of 4.9 was initially added to the urine samples. After incubation, the basic sodium carbonate (300 mM) was added to the mixture to terminate the reaction. Consequently, there was a change in the pH of the reaction mixture and this change in pH would lead to an increase in the absorbance of the haemoglobin. This change in absorbance would be falsely attributed to the formation of MCP if the role of haemoglobin was not recognized.

The results showed that the degree of haemolysis in the two groups that received haemolysis treatment was similar as revealed by the plasma haemoglobin concentration at five minutes after haemolysis. With respect to the plasma haemoglobin concentration and haemoglobin excretion, the lack of statistical significance between the two groups prevents us from drawing any conclusion from the data. However, it should be pointed out that the results do not contradict the ability of lysine to increase haemoglobin excretion. It is possible that such measures are simply not sensitive enough to detect subtle changes in haemoglobin excretion.

It was shown in the last chapter that haemolysis alone causes a depression of renal function but the addition of lysine preserves it, thus suggesting that lysine may have a beneficial effect. The present study was designed to substantiate this theory from a different
An enzyme study perspective. In this study, the lysosomal enzyme, NAG, was utilized as an index of proximal tubular cell damage. Measurement of N-acetyl-β-D-glucosaminidase in urine is helpful in detecting damage to proximal tubular cells in acute and chronic renal disease (40,41). For example, it has been used as an index of early rejection of kidney graft after renal transplantation (42) and a sign of drug nephrotoxicity (43,44). This study showed that NAG excretion increased after haemolysis and, in conformity with the results in Chapter 5, the increase was significantly lower in the haemolysis-plus-lysine group. The results provided the evidence that less damage seemed to have occurred in association with in vivo haemolysis in the presence of lysine. Thus, these results lend further support to the theory that lysine plays a protective role in haemolysis induced renal damage.

It is obvious that the present data and those in the previous chapter showed that lysine mitigates renal damage secondary to haemolysis as manifested by the maintenance of glomerular filtration rate and a lower NAG excretion rate when lysine was infused in addition to haemolysis treatment. One interesting issue that follows is how changes in the tubules affect renal function. Why is it that a drop of glomerular filtration rate occurs in parallel with an increase in NAG excretion which signifies the presence of proximal tubular cell damage. The drop in glomerular filtration rate might be attributed to both vascular and tubular factors. The vascular effects may reflect in vivo activation of tubuloglomerular feedback. The tubuloglomerular feedback is a regulatory mechanism which lowers glomerular filtration rate whenever the solute delivery or solute concentration at the macula densa is raised (45,46). Tubular damage produced by
An enzyme study

haemoglobin might impair sodium reabsorption and the increased macula densa flow rate leads to an increased chloride ion transport. The macula densa senses changes in delivery and subsequent reabsorption of chloride ions. The importance of chloride is probably related to the chloride dependence of the Na-K-2Cl carrier in the luminal membrane that promotes the entry of these ions into the cell (47). Afferent arteriolar constriction and decreased capillary permeability due to contraction of mesangium may ensue. Consequently, a depression of glomerular filtration rate follows (48,49).

The renin-angiotensin system has been proposed to modulate the tubuloglomerular feedback (45,48), but its role remains controversial. Studies have shown that angiotensin antagonists and inhibitors of the converting enzyme can blunt, but not eliminate, tubuloglomerular feedback (50,51). One reason for it may be attributed to the difficulty in blocking effectively the intrarenal renin-angiotensin mechanism operating locally at the level of the juxtaglomerular apparatus, its glomerulus and glomerular vessels (52). Other substances such as adenosine, prostaglandins, and intracellular calcium have also been proposed to participate in feedback regulation.

In addition to the tubuloglomerular feedback, the tubules may contribute to the depression of renal function by increased reabsorption of luminal fluid through damaged epithelium (53,54,55). Moreover, tubular obstruction may be yet another contributing factor to the decline of glomerular filtration rate (1). It is possible that it is the interplay of all these factors that is responsible for the depression of renal function secondary to in vivo haemolysis.
A plasma protein study

CHAPTER SEVEN

A PLASMA PROTEIN STUDY

As described in the previous two chapters, it seems that lysine plays a protective role in haemolysis induced renal damage. It is believed that this effect of lysine may be due to the inhibition of tubular protein reabsorption. Thus far, the data are suggestive, rather than conclusive of this point. Therefore, attention was focused on a small molecular weight protein which is filtered and reabsorbed. One such plasma protein is retinol binding protein which is the specific transport protein for vitamin A in the rat. This protein has a molecular weight of 21000 and occurs in serum complexed to prealbumin and retinol. After retinol is delivered to target cells, retinol binding protein rapidly dissociates from prealbumin, is filtered through the glomerulus, and is reabsorbed by the tubule. If the beneficial effect of lysine is mediated via inhibition of haemoglobin reabsorption, it will also do the same to retinol binding protein and renal excretion of retinol binding protein is expected to be increased after lysine infusion or haemolysis. This point was examined in this chapter.

Method

Double antibody sandwich enzyme linked immunosorbent assay (ELISA) was employed to quantitate the urine concentration of retinol
A plasma protein study

binding protein (30). Rabbit antibody to human retinol binding protein and to retinol binding protein conjugated to horseradish peroxidase (Dakopatt Glostrup, Denmark) were kindly provided by Professor R. Swaminathan. The wells of the microtiter plate were each coated with 100 µl of retinol binding protein antibody. The antibody was prepared by diluting 8 µl of stock antibody (A040) with 10.4 ml of buffer A (Phosphate buffer saline, pH 7.2). After coating, the plate was left overnight at 4°C. The plate was washed with buffer B (prepared by the addition of 1.0 ml of Tween 20 and 20.75 g of NaCl to 1 litre of Buffer A) and then filled with non-fat milk to saturate the non-specific binding sites. The antibody coated plate was then washed three times with buffer B. One hundred microlitres of the test samples, the standards, and the control were each added to the wells in duplicate. The plate was covered and incubated at room temperature for two hours, and then it was washed three times with buffer B. One hundred microlitres of diluted peroxidase conjugated antibody was added to each well, and incubated at room temperature for one hour. The peroxidase conjugated antibody was diluted by adding 25 µl of the antibody (PC-Ab P304) to 10 ml of buffer B. The plate was again washed three times with buffer B. One hundred microlitres of the colour substrate was added to each well. The colour substrate was prepared by mixing 12 ml of buffer C (Citric Acid-Phosphate Buffer, pH 5.0), 5 µl of 30% hydrogen peroxide, with 8 mg of 1,2-phenylenediamine dihydrochloride. The plate was covered and left in a dark place to develop the color. After incubating for 10 to 15 minutes, 100 µl of 1 M sulphuric acid was added to each well to stop the reaction. The absorbance of the wells were then determined by a plate reader at 492 nm.
A plasma protein study

The urinary retinol binding protein levels was measured in the usual four groups, namely, the normal control, the lysine control, the haemolysis-only, and the haemolysis-plus-lysine groups. The urine specimens were collected at 45, 90, 135, 180, and 225 minutes after the start of experiment. Haemolysis was induced after the second collection of urine specimen at 90 minute in the usual way by bolus injection of 4 ml distilled water.

Since the antibody employed in this study was intended for use in human specimens, problems such as the possible lack of cross reactivity between rat retinol binding protein and the antibody as well as the presence of potential inhibitors in urine interfering with the assay might arise. Therefore, it was necessary to test the validity of the antibodies in this study first. To achieve this end, aliquots of rat urine (100 μl) were added to 100 μl of the various standards and the retinol binding protein of the mixture were then determined. The same procedure was repeated using human urine and the buffer B.

Results

A typical standard curve in the ELISA of retinol binding protein is shown in Figure 7.1. When aliquots of rat urine were added to the different standards, the absorbance of these mixtures did not show any significant increase above the baseline level of the absorbance of the mixtures consisting of standards and buffer B. On the contrary, an increase in the absorbance was noted when aliquots of human urine were added to the standards. These results are summarized in Figure 7.2.
A plasma protein study

When retinol binding protein was measured in the urine specimens in the normal control, lysine control, haemolysis-only, and the haemolysis-plus-lysine groups, the absorbance obtained were some values around zero. These data are shown in Table 7.1.

Table 7.1. The absorbance of rat urine in retinol binding protein measurement in normal saline control (N/S), lysine control (Lysine), hemolysis-only (Hem), and hemolysis-plus-lysine (Hem+Lys) groups. The samples were obtained from eight rats and collected 45, 90, 135, 180, and 225 minutes after the start of experiments.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Rat1</th>
<th>Rat2</th>
<th>Rat3</th>
<th>Rat4</th>
<th>Rat5</th>
<th>Rat6</th>
<th>Rat7</th>
<th>Rat8</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/S Lysine Hem Hem+Lys N/S Lys Hem Hem+Lys</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>-0.065</td>
<td>-0.06</td>
<td>-0.052</td>
<td>-0.017</td>
<td>0.007</td>
<td>-0.039</td>
<td>-0.051</td>
<td>-0.082</td>
</tr>
<tr>
<td>90</td>
<td>-0.071</td>
<td>-0.045</td>
<td>-0.036</td>
<td>-0.037</td>
<td>-0.050</td>
<td>-0.015</td>
<td>-0.045</td>
<td>-0.075</td>
</tr>
<tr>
<td>135</td>
<td>-0.022</td>
<td>-0.009</td>
<td>-0.021</td>
<td>-0.02</td>
<td>-0.081</td>
<td>-0.028</td>
<td>-0.015</td>
<td>-0.001</td>
</tr>
<tr>
<td>180</td>
<td>-0.015</td>
<td>-0.02</td>
<td>-0.041</td>
<td>-0.006</td>
<td>-0.090</td>
<td>-0.066</td>
<td>0.021</td>
<td>-0.068</td>
</tr>
<tr>
<td>225</td>
<td>-0.042</td>
<td>-0.029</td>
<td>-0.037</td>
<td>-0.023</td>
<td>0.021</td>
<td>-0.029</td>
<td>0.021</td>
<td>-0.058</td>
</tr>
</tbody>
</table>
Figure 7.1. The retinol binding protein standard curve.
Figure 7.2. The absorbance of the mixtures of standard (Std) plus buffer, standard plus aliquots of rat urine, and standard plus aliquots of human urine. A parallel upward shift is observed in Std+Human urine when compared with Std+Buffer.
Discussion

Nephrotoxic agents such as gentamicin damage the renal proximal tubular parenchyma, thus resulting in increased urinary excretion of low molecular weight proteins. Tubular function can be assessed by measuring specific marker proteins in the urine. One such protein is retinol binding protein. Rat retinol binding protein has a molecular weight of 21000 and circulates in the plasma in the form of a protein-protein complex, with an apparent molecular mass of about 60000 to 70000 (56). It is a rather stable protein to use as a monitor of renal tubular function. In principle, the level of retinol binding protein in urine would be higher in the lysine control group and the haemolysis-only group when compared with the normal control group. It is because the amino acid lysine and the protein haemoglobin will block the uptake sites of small molecular weight proteins. As a result, the excretion of retinol binding protein will be increased. By the same token, the urinary retinol binding protein will be even higher in the haemolysis-plus-lysine group because lysine and haemoglobin will both be present and a larger degree of blockade of the uptake sites may consequently ensue.

The absorbance data of rat urine we obtained are values that are close to zero. This signifies that either there is no retinol binding protein in urine or there is something wrong with the antibody. However, the human urine data revealed that the antibody worked and retinol binding protein could be detected in human urine. Therefore, the data might reveal that there was no cross reactivity of the antibody with rat retinol binding protein. This confirmed some
previous immunodiffusion findings that there was minimal immunological cross-reactivity between human and rat retinol binding protein (56). These results indicate that human and rat retinol binding protein are immunologically distinct and different from each other. Certain important structural differences may exist between the two proteins, particularly with respect to those aspects of structure involved in antigenic determination. It is therefore inappropriate for us to apply the retinol binding protein antibody intended for human use in determining rat retinol binding protein. Some choice other than retinol binding protein has to be selected to illustrate that lysine increases protein excretion.
In this master's project, the purity of tritiated inulin was first confirmed by performing gel filtration chromatography. An optimal lysine concentration which did not affect glomerular filtration rate in rats was determined. Then the potential protective effect of lysine in haemolysis induced renal damage was explored by performing a functional study in which tritiated inulin clearance was used as an index and an enzyme study by the use of N-acetyl-β-D-glucosaminidase. Finally, attempts were made to further clarify the mechanism by doing a plasma protein study.

The study stemmed from the fact that in vivo haemolysis produced renal function impairment and lysine increases the excretion of small molecular weight proteins; and the belief that this particular property of lysine may offer protection in haemolysis induced renal damage.

It is well established that in vivo haemolysis produces renal function impairment but it seems that there is still a controversy on whether haemoglobin molecule per se is nephrotoxic. Rabiner attributed the renal damage done in haemolysis to stroma, the fragments of lysed erythrocytes (14). However, Tam and Wong established the etiological role of intratubular haemoglobin in causing renal function impairment (19).
Epilogue

In our experimental model, haemolysis was induced in vivo in rats by bolus injection of distilled water. This created a condition in which haemoglobin as well as stroma would be present in the plasma. Our results showed that the inulin clearance dropped immediately after haemolysis. But it is interesting to delineate the differential role of each factor. Our data showed that when lysine was added to the infusion fluid, glomerular filtration rate was preserved. If this effect is mediated via inhibition of tubular haemoglobin reabsorption, it implies that haemoglobin plays a major role in causing haemolysis associated renal damage. This is in accord with the Yoshioka's report that haptoglobin has prophylactic and therapeutic effects in renal injury secondary to haemolysis (20). In such instances, haptoglobin's role is to bind haemoglobin. This further attests the postulate that haemoglobin plays a more important role in haemolysis associated renal damage.

According to our data, the addition of lysine preserves renal function and mitigates proximal tubular cell damage in haemolysis. We postulate that this effect be due to the property of lysine to inhibit tubular reabsorption of haemoglobin. As a matter of fact, our data did suggest such a trend. Of course, it would be imprudent to draw any conclusion from such data in the absence of statistical evidence. As the increase in the number of haemoglobin molecules excreted due to the action of lysine might be small, only a small percentage increase in haemoglobin excretion might ensue. Therefore, a sensitive method of detection was needed. However, our results seemed to show that our way of determining haemoglobin excretion was not sensitive enough to detect the possible subtle changes in haemoglobin excretion.
Epilogue

In order to provide a more complete picture, it would be ideal if we could have some data that showed lysine did increase protein excretion instead of taking this property for granted as reported by other authors. We tried to do this by using retinol binding protein as our marker. However, this was a futile attempt because of the lack of cross reactivity of the antibody that are available to us. Yet our results in the retinol binding protein study confirmed the results reported by other authors.

In this study, we have illustrated several points. Firstly, a high concentration of lysine impairs renal function. Secondly, in vivo haemolysis causes a depression of renal function. Thirdly, 30 mM lysine preserves renal function and minimizes proximal tubular cell disruption in haemolysis.

Based on the present findings, several future follow-up extended studies might be deemed suitable. To substantiate the hypothesis that the beneficial role of lysine derives from its positively charged property, similar studies using other positively charged molecules such as arginine and metallothionein should be carried out. Moreover, the experimental model should be extended to include myoglobinaemia. Furthermore, $\beta_2$-microglobulin and cell culture technique should be used to elucidate the mechanism of the effect of lysine in the prevention of haemoglobin uptake of renal tubular cells. If the results are favourable, lysine might find a wide application in many clinical disturbances in which haemoglobinaemia occurs. To name a few, they include mismatched blood transfusion and burns. This, of course, has to await clinical studies to validate the use of lysine in such conditions.
In conclusion, despite the setback that we cannot perfect our study by showing that lysine increases the excretion of the low molecular weight protein, the retinol binding protein, we have successfully showed in this master's project that lysine plays a protective role in haemolysis associated renal damage.
References


