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Renal function in a rat model of analgesic nephropathy: effect of chloroquine

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GFR = glomerular filtration rate

L-NAME = N^G-nitro-L-arginine methyl ester

PGE_2 = Prostaglandin E_2

PGI_2 = Prostaglandin I_2

SNK test = Student Newman Keuls test

HETE = hydroxyeicosatetraenoic acid

EET = epoxyeicosatrienoic acid

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Abstract

The antimalaria drug chloroquine is often taken against a background of analgesic nephropathy caused by NSAIDs such as paracetamol (acetaminophen). Chloroquine has marked effects on the normal kidney and stimulates an increase in plasma vasopressin via nitric oxide. The aim of this study was to determine the renal action of chloroquine in a model of analgesic nephropathy. Sprague Dawley rats (n = 6-8 per group) were treated with paracetamol (500 mg kg^{-1} day^{-1}) for 30 days in drinking water to induce analgesic nephropathy; control rats received normal tap water. Under Intraval anaesthesia (100 mg kg^{-1}) rats were infused with 2.5% dextrose for 3 hours to equilibrate and after a control hour they received either vehicle, chloroquine (0.04 mg h^{-1}), L-NAME (N^G-nitro-L-arginine methyl ester, nitric oxide synthase inhibitor , 60 μg kg^{-1} h^{-1}) or combined chloroquine and L-NAME over the next hour. Plasma was collected from a parallel group of animals for vasopressin radioimmunoassay. Long term paracetamol treatment resulted in a decrease in glomerular filtration rate (p<0.05), sodium excretion (p<0.001) and urine osmolality (p<0.001), but no change in urine flow rate compared with untreated animals. Chloroquine administration in paracetamol treated rats induced a significant reduction (p<0.05) in urine flow rate and a significant increase in plasma vasopressin (p<0.001). These effects were blocked by co-administration of L-NAME and thus appear to be mediated by a pathway involving nitric oxide. However, these responses contrast with the chloroquine-induced diuresis previously observed in untreated rats, possibly reflecting paracetamol inhibition of renal prostaglandin synthesis and consequent moderation of vasopressin's action.

Paracetamol (known as acetaminophen in the USA) is one of the most commonly used non-steroidal anti-inflammatory drugs (NSAID) (Hardman et al., 2001). It is a rapid, reversible, non-competitive inhibitor of cyclo-oxygenase activity and thus products of the arachidonic acid cascade. In addition to its analgesic properties, paracetamol also has direct actions on the kidney. Colletti et al. (1999) demonstrated that administration of paracetamol to dogs fed either a normal or low sodium diet (renal prostaglandin-dependent state) resulted in a decrease in renal blood flow, glomerular filtration rate (GFR) and prostaglandin E_2 (PGE_2) excretion. In the isolated perfused rat kidney, administration of paracetamol resulted in a decrease in GFR and PGE_2 (Trumper et al., 1998). Similarly, in normal human volunteers treated with paracetamol for 3 days, a reduction in urinary PGE_2 and sodium excretion were observed. In addition, paracetamol induced a delay in the onset of diuresis following an acute water load (Prescott et al., 1989).

Paracetamol also exerts acute and chronic nephrotoxic effects. Acute ingestion of large doses (10-15g) is characterised by necrosis and damage to the proximal tubule. However it is recognised from both clinical and experimental studies that much lower doses (500-1000mg) can produce renal damage, especially in patients with hepatic disease or those taking enzyme inducer drugs (carabamazepine and phenytoin) or in the malnourished (Blantz, 1996). Chronic ingestion of paracetamol results in analgesic nephropathy. This is defined as habitual ingestion of an analgesic, which after an insidious onset, leads to renal papillary necrosis and chronic interstitial nephritis with progressive renal failure (Henrich, 1998). Epidemiological studies show that long term regular consumption of paracetamol increases the relative risk of chronic renal disease to 3.2 (Sandler et al., 1989), while the odds ratio for end stage renal disease was 2.1 for the heaviest

annual intake of paracetamol and 2.4 for cumulative lifetime intake of more than 5000 tablets containing paracetamol (Perneger et al., 1994). Burrell et al. (1990) found that paracetamol (380 mg kg^{-1} body wt day^{-1}) and aspirin (230 mg kg^{-1} body wt day^{-1}) given for 21 weeks to female Fischer 344 rats resulted in papillary necrosis and impaired ability to concentrate urine, though a lower dose of paracetamol alone (120 mg kg^{-1} body wt day^{-1}) did not induce significant renal damage.

Paracetamol and other NSAID are often prescribed as antipyretic agents to reduce fever associated with malaria. Hence, people living in regions where malaria is prevalent are likely to ingest paracetamol on a regular basis over a long period of time. Indeed, there is evidence of widespread chronic paracetamol ingestion in many developing countries (Chada, 1998; Eddleston, 2000). The consequences of clinical and sub-clinical analgesic nephropathy may be exacerbated in these populations by anti-malaria drugs which can also affect renal function.

We have shown (Ahmed et al., 2003) that one such drug, chloroquine, causes a marked increase in GFR, urine flow rate and urinary sodium excretion in the rat. These effects were reversed by L-NAME, suggesting that nitric oxide mediates, at least in part, these renal effects of chloroquine. As chronic paracetamol ingestion impairs urinary concentrating ability in the rat (Burrell et al., 1990), chloroquine administration against a background of analgesic nephropathy might be expected to cause a pronounced diuresis which may be of clinical significance in patients suffering from dehydration or electrolyte imbalance. Accordingly, the aims of this study were to develop a sub-clinical model of paracetamol-induced analgesic nephropathy in the rat without overt renal dysfunction or gross kidney morphological changes and then to determine the

effects of chloroquine on renal function in this model. The rational for choosing a sub-clinical level was to model the more common level of renal impairment seen with long term NSAID use in both Western and tropical countries. This would allow the subsequent study of the confounding effects of chloroquine against a background of a moderately damaged kidney in which capacity to adapt fluid and ion balance may be impaired. As we have previously shown that chloroquine's actions on renal function and vasopressin secretion are mediated, at least in part, by nitric oxide (Ahmed et al., 2003), we have also studied the effect of nitric oxide inhibition by L-NAME on the responses to chloroquine administration in paracetamol treated rats.

Methods

All experiments were performed under the authority of a UK Home Office Project Licence and received local ethical approval.

Induction of analgesic nephropathy

Male Sprague Dawley rats (345-400g) were purchased from Charles River UK Limited (Margate, Kent, UK) and were held in the School of Biological Sciences where they had free access to food (Beekay Rat and Mouse Standard Diet, Bantin and Kingman Ltd., Hull, UK) and water, with a 12 hours light and 12 hours dark cycle prior to experimentation.

Paracetamol (4-acetamidophenol, Sigma, Poole, Dorset, UK) was dissolved in drinking water (500 mg kg^{-1} body wt day^{-1}) and the pH was adjusted to 6.7 by addition of NaOH (Burrell et al., 1991b). Rats received paracetamol for 30 days before renal function study during which time daily water intake did not differ significantly from untreated animals receiving tap water alone.

Surgical preparation

Animals were anaesthetised with Intraval (100 mg kg^{-1} body weight, thiopentone sodium BP, Rhône-Poulenc Rorer Limited, Nenagh, Co Tipperary, Ireland) and transferred to a hot plate which maintained body temperature, monitored by a rectal probe, at 37°C throughout the

experiment. Cannulae were inserted into an external jugular vein, carotid artery and the bladder and a tracheotomy was performed, as previously described (Ahmed et al., 2003).

Servo-controlled fluid replacement

Euvolaemic fluid replacement of spontaneous urine output was achieved using a servo-controlled fluid replacement system, as previously described (Ahmed et al., 2003). Briefly, urine flow rate, determined gravimetrically, is transmitted to an adjustable pump via a computer. A programme developed at the University of Manchester (Burgess et al., 1993) allows the infusion rate of the pump to be automatically adjusted to precisely replace intravenously the volume of fluid lost as urine.

The rat was positioned so that all urine produced flowed directly from the bladder catheter into a pre-weighed plastic vial placed on an electronic balance (model L2200 P, Sartorious, GMBH, Gottingen, Germany), which reset automatically at the end of each loop (10 minutes). The balance was connected to a computer (PC model 1460, Amstrad plc, Brentford, Essex, UK) which detected changes in urine flow rate and activated an adjustable pump (Perfusor Secura, B. Braun Medical Limited, Melsungen, Germany) with a syringe containing 2.5% dextrose. In addition, a constant slow infusion was maintained at a rate of 1ml h^{-1} via a second infusion pump (Precidor type 5003, Infors HT, Bottmingen, Switzerland) which allowed the delivery of clearance marker (^{3}H inulin in 2.5% dextrose, 6μCi h^{-1}, Amersham International plc, Little Chalfont, Bucks, UK) for the determination of glomerular filtration rate. The infusates were mixed via a metal three-way connector. The flow rate of the adjustable pump was set by the

computer to precisely replace 2.5% dextrose at a rate matching the urine flow rate of the previous 10 minute cycle, taking into account fluid delivery from the constant infusion pump.

Experimental protocol

Following surgery, a bolus dose of ^{3}H inulin (6μCi) was injected via the venous cannula and servo-controlled fluid replacement initiated. All animals were allowed a 3 hour equilibration period, following which paracetamol treated animals were assigned to paracetamol (n = 6), paracetamol / chloroquine (n = 6), paracetamol / L-NAME (n = 6) and paracetamol / chloroquine / L-NAME (n = 6) groups. All rats, including an additional group of untreated controls (vehicle, n = 8) then received 2.5% dextrose replacement for a 1 hour control period, following which the control and paracetamol only groups continued to receive 2.5% dextrose for the remaining 2 hours of the experiment. In the chloroquine-treated group, infusion of chloroquine (0.04 mg h^{-1} chloroquine diphosphate, Sigma, UK, previously shown in our hands to affect renal function in the anaesthetised rat at this dose (Ahmed et al., 2003)) was started via the constant infusion pump for 1 hour, following which the infusate was switched to 2.5% dextrose for the final hour of the experiment. In the L-NAME treated group, L-NAME (60μg kg^{-1} h^{-1}, N^{G}-nitro-L-arginine methyl ester, Sigma, UK, previously shown to be effective in our hands at this dose in inhibiting nitric oxide synthase in the anaesthetised rat with no alteration in blood pressure (Ahmed et al., 2003)) was infused for 2 hours after the control period. In the final group, combined L-NAME and chloroquine infusion began after the 1 hour control period. Chloroquine infusion ceased after 1 hour and rats continued to receive L-NAME for the final hour. Urine samples were collected every 10 minutes after the equilibration period and blood samples were collected at 0.5,

1.5 and 2.5 hours post-equilibration. The blood samples (0.6 ml) were collected from the carotid artery and a similar volume of dextrose solution was replaced. Plasma was separated by centrifugation and stored at 4°C prior to analysis.

Parallel groups of animals (*n* = 6 per group) equivalent to those used for renal studies were prepared specifically to collect blood for vasopressin radioimmunoassay. Blood samples were taken from animals undergoing servo-controlled fluid replacement midway through the drug (chloroquine, L-NAME or in combination) treatment hour. Animals were decapitated and trunk blood (5-7 ml) was collected into tubes held on ice containing 100µl 0.125 mol EDTA (Sigma, UK) and 250µl ammonium heparin (BDH, UK). Plasma was separated following centrifugation for 10 minutes and stored at -20°C prior to measurement of plasma vasopressin concentration by radioimmunoassay as previously described (Warne et al., 1994).

Analysis

Urine and plasma sodium concentrations were measured by flame photometry (Corning 480, Corning Ltd, Halstead, Essex, UK) and osmolality by freezing point depression (Roebling osmometer, LH Roebling, Berlin, Germany). ^{3}H inulin was determined using a 1900CA Tri-Carb Liquid Scintillation Analyser (Canberra Industries, Meriden, CT) β-counter.

Statistical analysis

Data are presented as the mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using SPSS for Windows (Standard version 10.1.0, SPSS UK Ltd., Woking, Surrey, UK). Comparisons between paracetamol treated and untreated rats were by Student's unpaired *t*-test. In paracetamol treated rats receiving chloroquine ± L-NAME comparisons over time were by repeated measures ANOVA and comparisons within groups between control, treatment and recovery periods were by ANOVA followed by Student Newman Keuls (SNK) test. Significance was ascribed at the 5% level.

Results

Baseline renal function in paracetamol treated rats

Data presented in table 1 represent renal excretion rates over the control hour immediately following the equilibration period and prior to subsequent drug administration (chloroquine or L-NAME). Paracetamol treatment resulted in a significant reduction in GFR ($p<0.05$) compared with untreated animals. Urine flow rate tended to be higher in paracetamol treated rats which was associated with a significant reduction in urine osmolality ($p<0.001$) and sodium excretion rate ($p<0.001$). Despite these marked effects on renal concentrating ability, paracetamol treatment at the dose employed in this study had no significant effect on the gross histological morphology of the kidney by comparison with untreated rats (data not shown). Mean arterial blood pressure did not differ between paracetamol treated rats and untreated rats (untreated, n = 8, 121 ± 3 vs paracetamol treated, n = 24, 126 ± 4 mmHg). Mean arterial blood pressure remained stable in all four paracetamol treated groups over the course of the whole experiment and did not differ between groups (paracetamol 126 ± 4, paracetamol / chloroquine 126 ± 2, paracetamol / L-NAME 127 ± 4, paracetamol / chloroquine / L-NAME 123 ± 7 mmHg).

Chloroquine administration in paracetamol treated rats

Urine flow rate

The urine flow rates of paracetamol treated rats infused with chloroquine ± L-NAME are shown in figure 1. Repeated measures ANOVA revealed significant differences both over time ($F_{4,80}$ = 25.6, p<0.001) and between drug treatments ($F_{3,20}$ = 3.1, p<0.05). Urine flow was stable and similar in all groups of animals immediately prior to chloroquine or L-NAME infusion. During the hour of chloroquine infusion in paracetamol treated rats there was a significant reduction in urine flow rate (*post hoc* SNK test vehicle vs chloroquine, p<0.05), followed by a significant diuresis in the recovery hour (p<0.05) (Fig 1a). Co-infusion of L-NAME with chloroquine completely abolished the antidiuresis seen with chloroquine infusion alone (Fig 1b). The urine flow rate in paracetamol / chloroquine / L-NAME rats started to increase immediately upon the infusion of chloroquine and L-NAME (p<0.05) and continued to rise even after chloroquine administration ceased (p<0.05, Fig 1b). The same pattern was also observed in the paracetamol treated rats receiving L-NAME alone.

There were no differences in GFR (Fig 2, ANOVA 1st hour, $F_{3,23}$ = 0.86, p = 0.48) and urine osmolality (Fig 4, $F_{3,23}$ = 0.52, p = 0.67) during the initial post-equilibration control hour between the four groups, though Na^+ excretion was somewhat lower in the group due to receive chloroquine and L-NAME (Fig 3, $F_{3,23}$ = 4.9, p = 0.01). Thus, for ease of comparison, in subsequent graphs the mean values are presented for the control, post equilibration hour (1st hour), the hour of drug treatment (2nd hour) and the recovery hour (3rd hour).

Glomerular filtration rate:

The GFR, as determined from the clearance of inulin, is shown in figure 2. During the hour of chloroquine administration no significant differences were seen between the groups. In the subsequent hour, rats that had been infused with chloroquine showed a significant increase in GFR (ANOVA 3rd hour, $F_{3,23} = 6.86$, $p = 0.002$; *post hoc* SNK test vehicle vs chloroquine $p<0.01$) by comparison with vehicle infused paracetamol treated rats. L-NAME alone induced a significant increase in GFR during the 3rd hour ($p<0.01$) by comparison with vehicle infused paracetamol rats. However, despite elevating GFR when infused individually, a combined infusion of chloroquine and L-NAME had no significant effect on GFR which remained at baseline levels.

Sodium excretion

The mean sodium excretion rate is shown in figure 3. Within the first hour of chloroquine or L-NAME administration there was a significant increase in sodium excretion by comparison with vehicle infused paracetamol treated rats (ANOVA 2nd hour, $F_{3,23} = 9.72$, $p < 0.001$; *post hoc* SNK test vehicle vs chloroquine $p<0.001$, vehicle vs L-NAME $p<0.001$). However, when infusion of chloroquine was combined with L-NAME there was no effect on sodium excretion. Sodium excretion continued to increase following cessation of chloroquine infusion in the chloroquine only group (ANOVA 3rd hour, $F_{3,23} = 86.6$, $p < 0.001$; *post hoc* SNK test vehicle vs chloroquine $p<0.001$). Animals receiving L-NAME alone also showed elevated sodium

excretion in the 2nd and 3rd hours (vehicle vs L-NAME $p<0.001$). Sodium excretion remained at baseline levels over the 3rd hour in rats receiving the combined chloroquine and L-NAME infusion, which was significantly lower than that in rats receiving chloroquine alone ($p<0.001$).

Urine osmolality

Urine osmolality is shown in figure 4 as a measure of urine concentrating ability. During chloroquine infusion there was a significant increase in urine osmolality (ANOVA 2nd hour, $F_{3,23} = 7.39$, $p < 0.01$; *post hoc* SNK test vehicle vs chloroquine $p<0.01$) compared with vehicle infused, paracetamol treated rats, which is consistent with the associated fall in urine flow rate (Fig. 1a). The combination of chloroquine and L-NAME returned urine osmolality to baseline values. Over the following hour, following the cessation of chloroquine infusion, osmolality remained elevated in the chloroquine infused group (ANOVA 3rd hour, $F_{3,23} = 3.79$, $p < 0.05$; *post hoc* SNK test vehicle vs chloroquine $p<0.05$), but this was lower than during the preceding hour. L-NAME, with or without chloroquine, had no effect on urine osmolality by comparison with vehicle infused, paracetamol treated rats.

Plasma vasopressin

The sensitivity of the vasopressin assay was 1.2 fmol ml^{-1}; coefficients of variation were determined using a pool of plasma with a measured vasopressin concentration of 4 pg ml^{-1}, inter-assay variation was $8.2 \pm 0.8\%$ (n = 5) and intra-assay variation was $11.4 \pm 1.5\%$ (n = 10).

Samples for the measurement of plasma vasopressin were taken from a parallel group of animals mid-way through the hour of chloroquine infusion (Fig. 5). This corresponded with the maximum reduction in urine flow rate (Fig 1a) and increase in urine osmolality (Fig. 4) when chloroquine was infused in paracetamol treated rats. Chloroquine infusion was associated with a significant increase in plasma vasopressin (ANOVA, $F_{3,23} = 20.95$, $p<0.001$; *post hoc* SNK test vehicle vs chloroquine $p<0.001$) by comparison with vehicle infused, paracetamol treated rats. The administration of L-NAME with or without chloroquine induced a significant reduction in plasma vasopressin by comparison with vehicle infused paracetamol treated rats ($p<0.01$) and rats receiving chloroquine ($p<0.001$).

Figure 6 depicts a model which could explain the differing actions of chloroquine on water reabsorption by collecting duct cells in (A) untreated and (B) paracetamol treated rats.

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Discussion

Establishing a model of analgesic nephropathy

The paracetamol treatment regime used in this study induced changes in the renal excretion of water and ions, suggesting that a sub-clinical level of analgesic nephropathy had been achieved. The administration of paracetamol for 30 days had no effect on gross kidney morphology, nor on the growth or general health of the animals. The dose of 500 mg kg^{-1} day^{-1} is considerably higher than the normal adult dose of 14.3 mg kg^{-1} day^{-1} in a human, and is also higher than the (UK) maximum recommended dose of 4000 mg (57 mg kg^{-1}) (British Medical Association, 2002). This reflects the relative resistance of the rat to the induction of analgesic nephropathy. Previous studies have shown that a dose of 500 mg kg^{-1} day^{-1} for 20 weeks was required to induce analgesic nephropathy in the rat with typical histopathological changes (papillary necrosis and interstitial nephritis) (Nanra et al., 1973). At lower doses of 140-210 mg kg^{-1} day^{-1} renal morphological changes were not induced even when the period of administration was extended up to 117 weeks (Johansson, 1981; Burrell et al., 1991a). Only by combining paracetamol (380 mg kg^{-1} day^{-1}) with aspirin (230 mg kg^{-1} day^{-1}) for 21 weeks were Burrell et al. (1990) able to produce renal papillary necrosis. In accordance with these earlier approaches, we did not observe any renal histological changes following paracetamol treatment at 500 mg kg^{-1} day^{-1} for 4 weeks, suggesting that analgesic nephropathy at the clinical level had not been induced. However, a number of marked functional changes were induced. Most notably a reduction in concentrating ability was observed which contrasts with the effects of acute paracetamol infusion in which urine osmolality increased (Ahmed et al. 2002). These observations suggest that the

altered renal function displayed by the paracetamol treated rats reflects sub-clinical nephropathy rather than an acute action of paracetamol remaining in the circulation.

Paracetamol treatment was associated with a 30% reduction in GFR. GFR is regulated, in part, by angiotensin II which stimulates both afferent and efferent arteriolar constriction and by prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2) which, in conjunction with nitric oxide, stimulate vasodilatation (Schlondorff, 1986). Paracetamol inhibits prostaglandin synthesis (Hardman et al., 2001) which is likely to shift this balance in favour of vasoconstriction and a reduction in GFR. PGI_2 and PGE_2 are also found within the mesangium (Klahr et al., 1988) where they act to relax mesangial cells, thereby increasing GFR. Inhibition of these prostaglandins may lead to contraction of the mesangium and a reduction in GFR (Navar, 1998). Tubuloglomerular feedback is also dependant on prostaglandins and may be impaired during inhibition of prostaglandin synthesis, leading to an inappropriate GFR (Zenser et al., 1981).

An inhibitory effect on prostaglandin synthesis may also account for the effects of paracetamol on sodium excretion. Paracetamol inhibits PGE_2 synthesis, which is the primary prostaglandin affecting medullary haemodynamics and sodium and water handling (Dunn, 1998). Prostaglandins increase renal blood flow, reducing proximal reabsorption of sodium (Ichikawa and Brenner, 1980), while directly inhibiting sodium reabsorption by the thick ascending limb (Kaojarern et al., 1983). Thus inhibition of PGE_2 synthesis is likely to cause a reduction in sodium excretion, as was observed in paracetamol treated rats.

Paracetamol treatment also resulted in a reduction in urine osmolality, which may reflect an additional failure in the ability of the paracetamol treated rat kidney to concentrate urine. Humans with analgesic nephropathy have been reported to have lower urine osmolality than control subjects, even after administration of desamino-D-arginine vasopressin (dDAVP) (Wambach et al., 1989). This did not appear to be mediated by a reduction in AVP-induced cAMP, as urinary excretion rates were comparable between the two groups. During the early stages of analgesic treatment in rats (Burrell et al., 1990), the changes in urinary concentrating ability were reversible, but after prolonged analgesic treatment, maximum urinary concentrating ability failed to recover, suggesting that papillary damage was permanent (Burrell et al., 1991b).

Chloroquine administration in paracetamol treated rats

Administration of chloroquine in rats previously treated for 30 days with paracetamol led to a reduction in urine flow rate of over 50% by comparison with vehicle infused rats. The urine flow rate decreased steadily and reached its lowest level 30 minutes after the onset of chloroquine infusion, before rising again to a maximum after 80 minutes. This contrasts with our previous observation in non-paracetamol treated rats in which chloroquine had no antidiuretic effect. Indeed, chloroquine induced a significant diuresis despite concurrently stimulating an increase in plasma vasopressin (Ahmed et al., 2003).

One explanation for this lies in the observation that NSAID treatment enhances the sensitivity of the kidney to the action of vasopressin. Stimulation of arginine vasopressin V_1 receptors leads to increased prostaglandin synthesis, which inhibits cAMP production in the collecting duct and

diminishes the antidiuretic effect mediated by the V_2 receptor. Paracetamol, by inhibiting prostaglandin synthesis, may potentiate the antidiuretic effect of arginine vasopressin in the collecting duct (Fejes-Toth et al., 1977; Walker et al., 1994). Against this background, the chloroquine-induced increase in vasopressin may be sufficient to overcome the diuretic influences of chloroquine and thus result in antidiuresis. The subsequent reversal in urine flow rate upon cessation of chloroquine infusion may reflect a fall in plasma vasopressin concentration, however, we did not measure plasma vasopressin at this time.

Nitric oxide appears to play an important role in mediating these actions of chloroquine, though its effects seem to be moderated by the actions of paracetamol. In our previous study (Ahmed et al., 2003), chloroquine induced a diuresis and an increase in plasma vasopressin concentration in naive rats which could be prevented by co-infusion of L-NAME. In the current study, L-NAME blocked the antidiuretic action of chloroquine and inhibited the increase in plasma vasopressin concentration in paracetamol treated rats. Figure 6 depicts a model which could explain the apparently contradictory actions of chloroquine on urine flow rate and the role of nitric oxide in mediating its actions.

In the non-paracetamol treated rat (Fig 6a), chloroquine stimulates an increase in vasopressin secretion via a nitric oxide-dependent pathway, leading to an increase in cAMP generation in collecting duct cells via V_2 receptors. However, nitric oxide also increases cGMP generation and prostaglandin synthesis, both of which inhibit cAMP generation. If this inhibitory influence on cAMP is large enough, water permeability will not increase and a diuresis will ensue. In the paracetamol treated rat (Fig 6b) the inhibitory effect of prostaglandins is lost as paracetamol

inhibits prostaglandin synthesis, thus sufficient cAMP is generated to cause an increase in aquaporin 2 insertion into the apical membrane and water permeability increases, resulting in an antidiuresis. L-NAME prevents these actions of chloroquine by lowering the plasma vasopressin concentration and blocking nitric oxide-mediated cGMP generation and prostaglandin synthesis.

Chloroquine administration in paracetamol treated rats also induced a profound increase in sodium excretion by comparison with vehicle infused controls. This was reversible by L-NAME in the 3rd hour post equilibration, but not in the 2nd hour during chloroquine administration. Nitric oxide has been shown to inhibit proximal tubular fluid reabsorption by inhibiting sodium reabsorption (Eitle et al., 1998), suggesting that this may be one site of action for chloroquine. However, this does not explain why the chloroquine-induced natriuresis in the 3rd hour was not blocked by L-NAME, nor why L-NAME administration alone in paracetamol treated rats also resulted in a natriuresis.

A paracetamol-induced shift in the arachidonic acid cascade towards the cytochrome P-450 monocyclo-oxygenase pathway could offer an explanation. The P-450 (CYP) pathway leads to the production of hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs) (Murray and Brater, 1993). 20-HETE is the primary product of CYP and has been reported to be an essential component of tubuloglomerular feedback and renal autoregulation, modulating sodium transport in the medullary thick ascending limb and proximal tubules (Oyekan et al., 1999). Increased production of 20-HETE by the proximal tubules and medullary thick ascending limb reduced sodium reabsorption in these regions (Nowicki et al., 1997). Nitric oxide inhibits renal CYP activity, thus L-NAME administration could lead to an increase in the synthesis of 20-

HETE which in turn results in an increase in sodium excretion (McGiff and Quilley, 1999). Clearly, further work is required to establish the complex relationship between prostaglandins and nitric oxide in rats treated with paracetamol.

In summary, we have developed a sub-clinical model of analgesic nephropathy based on the administration of a high dose of paracetamol over a short period of time. This approach does not lead to gross changes in renal morphology, but clearly results in perturbations in renal function. Using this model, we have also shown that long term paracetamol ingestion alters the renal response to chloroquine compared with naive rats in our earlier study (Ahmed et al., 2003). Most notably, the chloroquine-induced increase in vasopressin was associated with an antidiuresis in paracetamol treated rats compared with a diuresis in non-treated rats, despite a similar increase in plasma vasopressin concentration. These effects were reversed by L-NAME, suggesting a role for nitric oxide. The reason for these differing effects may lie in the inhibitory effect of paracetamol on renal prostaglandin synthesis and their role in moderating AVP-induced cAMP generation.

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Tables

Table 1. Effect of 30 days paracetamol ingestion (500 mg kg^{-1} body wt day^{-1} via drinking water) on renal function in anaesthetised rats receiving euvolaemic fluid replacement. Control, untreated rats received normal tap water. Values represent the mean ± SEM urinary excretion rates, standardised to 100g body weight, over one hour. Statistical analysis was by Student's unpaired *t*-test; * $p<0.05$, *** $p<0.001$ untreated vs paracetamol treated rats.

	Untreated (n = 8)	Paracetamol treated (n = 24)
Glomerular filtration rate (ml min^{-1})	0.74 ± 0.07	0.50 ± 0.06 *
Urine flow rate (ml h^{-1})	0.23 ± 0.03	0.51 ± 0.17
Sodium excretion rate (µmol h^{-1})	9.9 ± 1.2	4.1 ± 0.4 ***
Urine osmolality (mOsm kg^{-1} H_2O)	453 ± 46	174 ± 10 ***

Figure Legends

Figure 1. Urine flow rate in paracetamol treated rats infused with (A) vehicle (n = 6) or chloroquine (n = 6) or (B) L-NAME (n = 6) or chloroquine and L-NAME (n = 6). Data are presented as the mean ± SEM. The 1st hour represents the control period during which all animals received 2.5% dextrose. In the 2nd hour chloroquine and / or L-NAME infusion commenced, while the 3rd hour represents the post-chloroquine recovery phase. Repeated measures ANOVA was significant over time ($F_{4,80}$ = 25.6, p<0.001) and between drug treatments ($F_{3,20}$ = 3.1, p<0.05).

Figure 2. GFR in paracetamol treated rats infused with vehicle (n = 6), chloroquine (n = 6), L-NAME (n = 6) or chloroquine and L-NAME (n = 6). Data are presented as the mean ± SEM. Statistical analysis across all groups and time points was by one-way ANOVA ($F_{11,71}$ = 4.12, p<0.001) and SNK test. Statistical differences from the vehicle infused group are shown as ** p<0.01 within each hour of the experiment.

Figure 3. Urinary sodium excretion in paracetamol treated rats infused with vehicle (n = 6), chloroquine (n = 6), L-NAME (n = 6) or chloroquine and L-NAME (n = 6). Data are presented as the mean ± SEM. Statistical analysis across all groups and time points was by one-way ANOVA ($F_{11,71}$ = 43.1, p<0.001) and SNK test. Statistical differences from the vehicle infused group are shown as ** p<0.01, *** p<0.001 and between chloroquine and chloroquine/L-NAME treated groups as +++ p<0.001 within each hour of the experiment.

Figure 4. Urine osmolality in paracetamol treated rats infused with vehicle (n = 6), chloroquine (n = 6), L-NAME (n = 6) or chloroquine and L-NAME (n = 6). Data are presented as the mean ± SEM. Statistical analysis across all groups and time points was by one-way ANOVA ($F_{11,71}$ = 8.25, p<0.001) and SNK test. Statistical differences from the vehicle infused group are shown as * p<0.05, ** p<0.01 and between chloroquine and chloroquine/L-NAME treated groups as ++ p<0.01 within each hour of the experiment.

Figure 5. Plasma vasopressin concentration midway through the hour of drug administration (2nd hour) in rats treated with chloroquine, L-NAME , chloroquine/L-NAME and vehicle infused rats (n = 6 per group). Data are presented as the mean ± SEM. Statistical analysis across all groups was by one-way ANOVA ($F_{3,23}$ = 20.95, P < 0.001) and SNK test. Statistical differences from the vehicle infused group are shown by ** p<0.01, *** p<0.001 and between chloroquine and chloroquine/L-NAME treated groups by +++ p<0.001.

Figure 6. Proposed model of chloroquine's actions on water reabsorption by collecting duct cells in (A) untreated and (B) paracetamol treated rats. NO = nitric oxide, AVP = vasopressin, V_1 = type 1 vasopressin receptor, V_2 = type 2 vasopressin receptor, PG = prostaglandins, cAMP = adenosine 3':5'-cyclic monophoshate, cGMP = guanosine 3':5'-cyclic monophoshate, AQP = aquaporin 2

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Figure 1

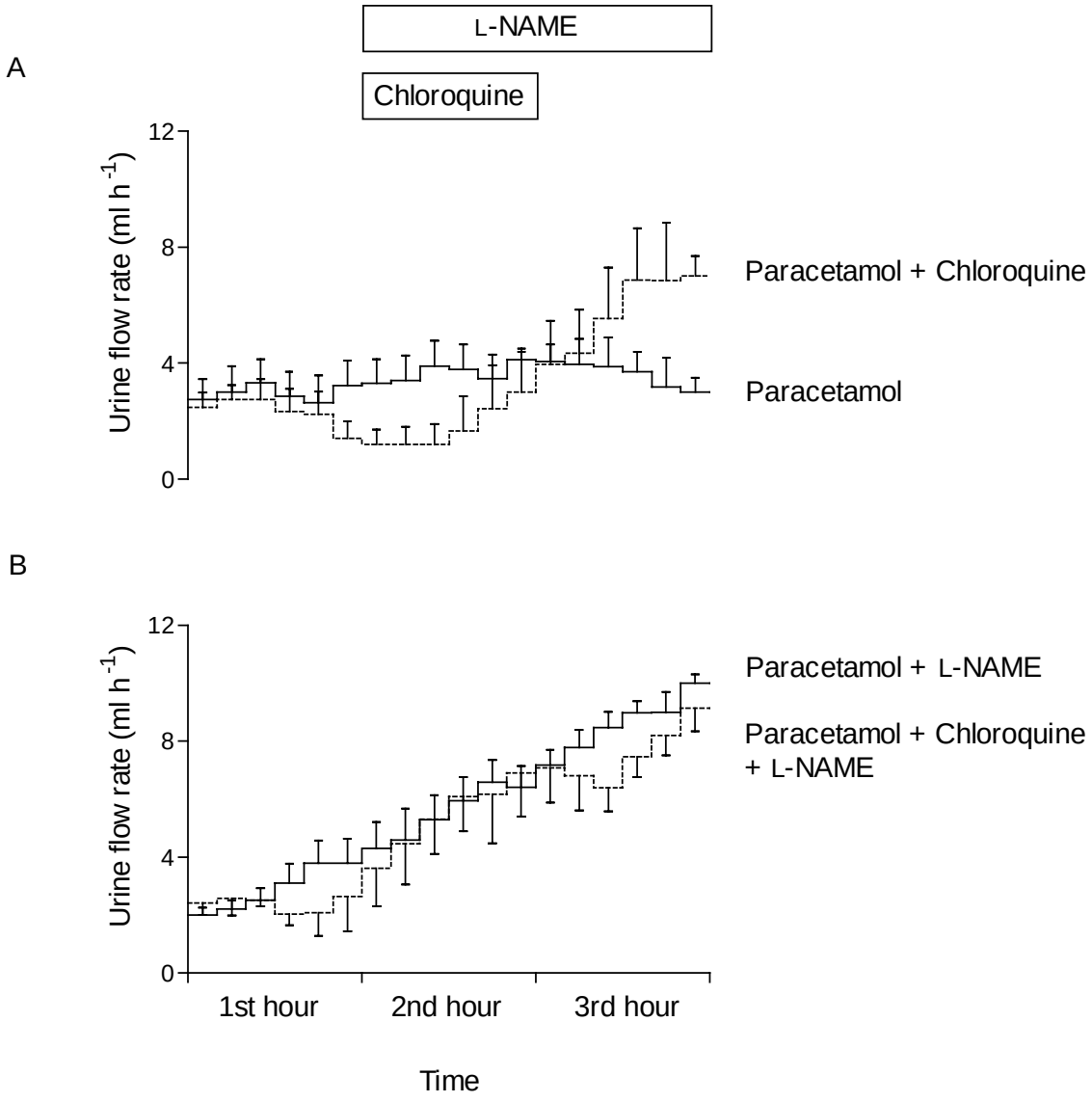

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Figure 2

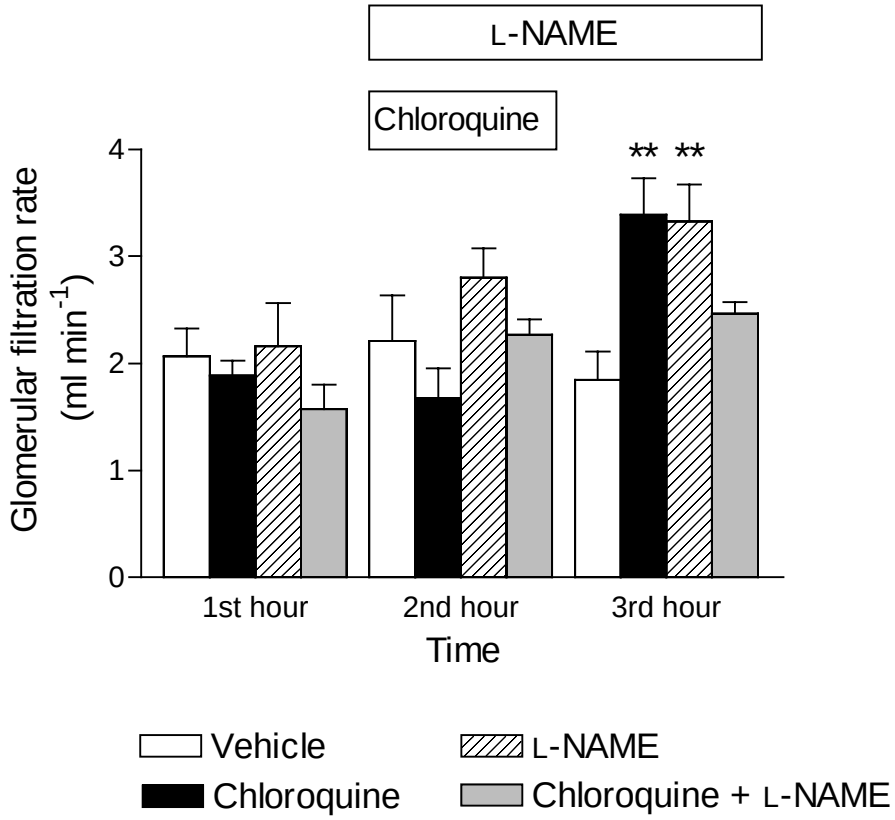

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Figure 3

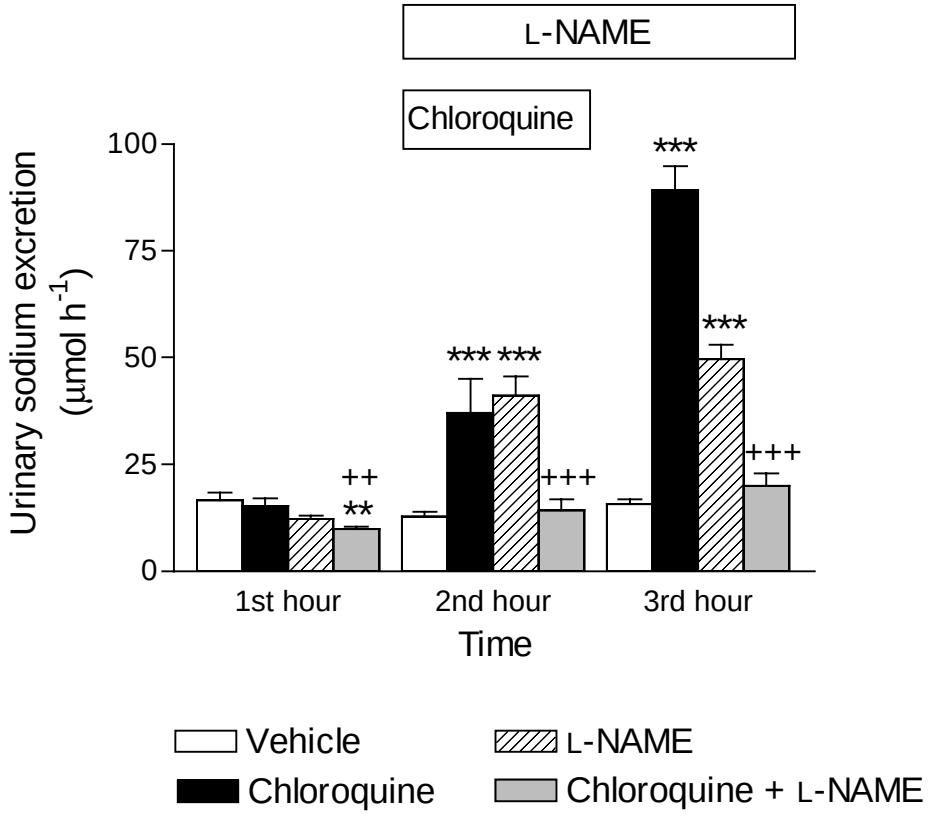

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Figure 4

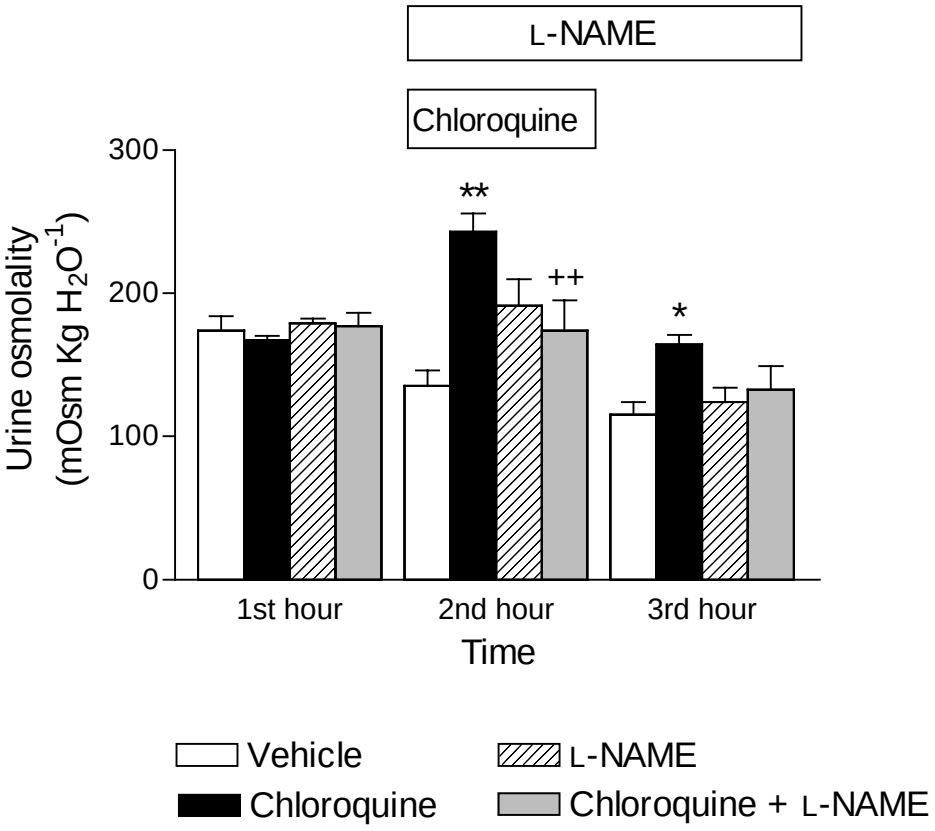

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Figure 5

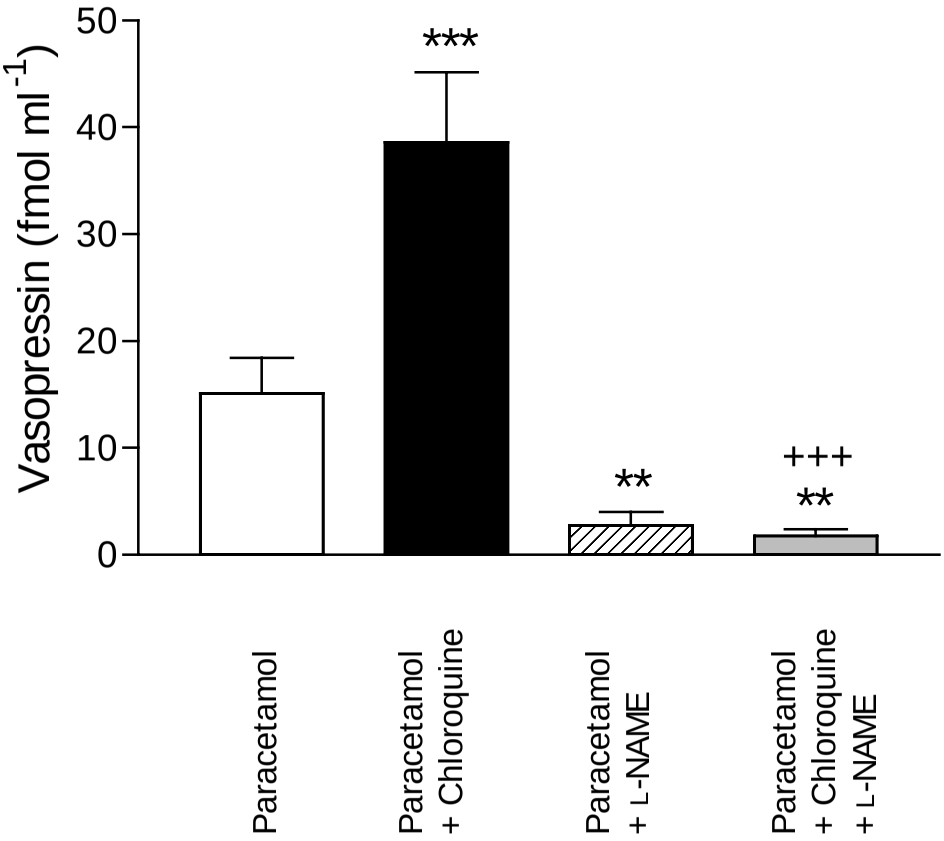

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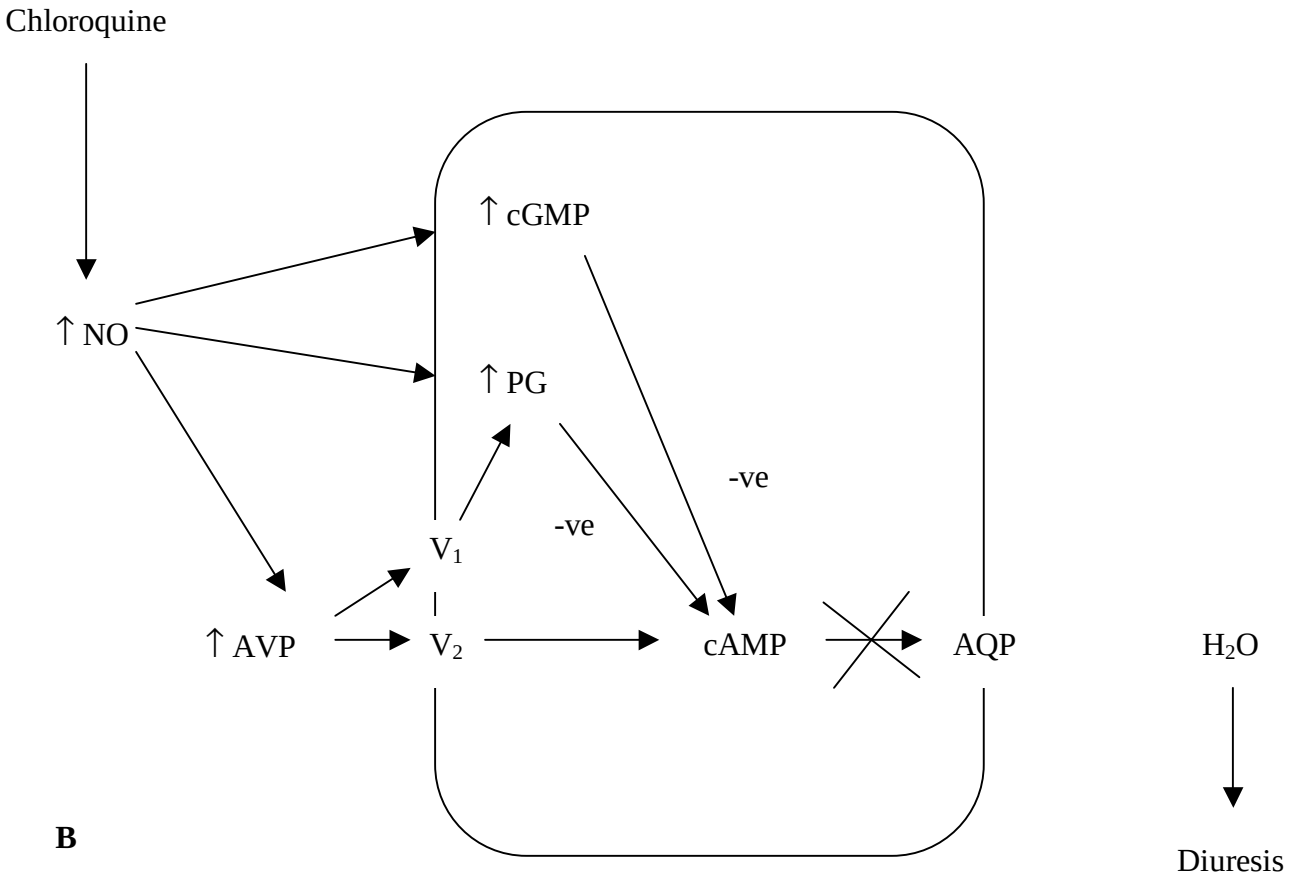
Figure 6

A



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