Synergistic Effects of Nitric Oxide and Prostaglandins on Renal Escape from Vasopressin-induced Antidiuresis

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Abbreviated title: NO and PG in AVP escape

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ABSTRACT

Recent results from our laboratories indicate that renal escape from vasopressin (AVP)-induced antidiuresis is accompanied by marked down-regulation of kidney aquaporin-2 (AQP2) and AVP V2 receptors. The present studies evaluated the effect of nitric oxide (NO) and prostaglandins (PG) synthesis blockade on escape from antidiuresis. DDAVP-infused rats were water loaded (WL) for 5 days. L-NAME, an NO synthesis inhibitor, or diclofenac, a cyclooxygenase inhibitor, was infused subcutaneously beginning one day before WL. As early as 2 days after WL, urine volume increased and urine osmolality decreased, indicating the onset of escape. Endogenous NO synthesis, measured as urinary NO$_2$ + NO$_3$ excretion, was significantly increased in the WL group compared to the non-WL controls during all 5 days of WL. L-NAME (20 mg/kg/day) markedly decreased urine volume on days 4 and 5 of WL, indicating inhibition of the escape phenomenon. Kidney AQP2 protein was significantly increased by this dose of L-NAME as well. A lower dose of L-NAME (10 mg/kg/day) or diclofenac (2.5 mg/kg/day) did not significantly affect the escape phenomenon by themselves, but the combination of L-NAME and diclofenac showed a marked inhibitory effect on the escape phenomenon, which also was accompanied by a significant increase in kidney AQP2 expression. These results therefore suggest that renal NO and PG both play important roles in escape from AVP-induced antidiuresis by acting synergistically to down-regulate kidney AQP2 expression.

KEYWORDS: vasopressin, nitric oxide, prostaglandins, aquaporin-2, escape
INTRODUCTION

In animal models of the syndrome of inappropriate antidiuretic hormone secretion (SIADH), sustained administration of vasopressin and water results in water retention and a secondary natriuresis, leading to progressive hyponatremia. However, after several days this is then followed by increased free-water excretion despite sustained administration of vasopressin (1-6). This phenomenon is known as "vasopressin escape" from antidiuresis. With the onset of vasopressin escape, water excretion increases despite continued administration of vasopressin, allowing water balance to be re-established and the serum sodium to be stabilized at a steady, albeit decreased, level.

Studies over the years have provided convincing evidence that vasopressin escape is secondary to body fluid expansion. Cowley et al. reported that vasopressin escape did not occur in dogs in which total body weight was precisely servo-controlled by adjusting fluid intakes, so that an expansion of body fluid volume was prevented (1). Similarly, Gross et al. observed that when a positive water balance was prevented by matching the rate of hypotonic fluid infusion to the urine output in rats, vasopressin escape did not occur (2). Hall et al. showed that vasopressin escape did not occur in dogs in which renal perfusion pressure was servo-controlled, suggesting that vasopressin escape is mediated by increased renal perfusion pressure that causes both diuresis and natriuresis (3). In previous studies from our laboratories, we have shown that escape from vasopressin-induced antidiuresis is associated with a marked vasopressin-independent decrease in kidney aquaporin-2 mRNA and protein expression (4), as well as vasopressin V2 receptor
binding (5), and that this down-regulation of aquaporin-2 does not appear to be closely related to either plasma or tissue osmolality (6). These combined results indicate that ECF volume expansion is critical for the onset of vasopressin escape. However, the mechanisms by which the water retention induces escape remain unclear.

It has been reported that urinary prostaglandin E2 excretion is increased concomitant with the onset of escape and that prevention of this increase in urinary prostaglandin E2 excretion with indomethacin delays the onset of escape (2). Moreover, kidney nitric oxide (NO) and prostaglandins (PG) have been reported to antagonize the antidiuretic effect of vasopressin (7; 8). Both NO and PG have also been considered to be involved in pressure diuresis and volume expansion-induced natriuresis (9-11). These findings suggest the possibility that kidney NO and PG may somehow be involved in the mechanisms of vasopressin escape as well. In the present study, we evaluated the effects of N^G^-nitro-L-arginine methyl ester (L-NAME), an NO synthetase inhibitor, and diclofenac, a PG synthetase inhibitor, on renal aquaporin-2 expression and the onset and maintenance of escape during vasopressin-induced antidiuresis.

MATERIALS AND METHODS

Animals and escape model.

Male Sprague-Dawley rats (300-350 g; Taconic Farms, Germantown, NY) were maintained under the controlled conditions (24°C, lights on 0600-1800 h). Under light
methoxyflurane (Metofane) anesthesia, osmotic mini-pumps were implanted subcutaneously (model 2002; Alzet, Palo Alto, CA) to deliver 5 ng/h of dDAVP (Rhone-Poulenc Rorer, Collegeville, PA). After 4 days of dDAVP administration, during which time all rats received ad libitum pelleted chow and water, the experimental groups were water loaded by substituting daily feedings of a liquid formula (AIN-76; Bio Serve, Frenchtown, NJ, in a volume of 70 ml) while non-water-loaded control group received AIN-76 powder. This amount of liquid diet provides sufficient calories for weight maintenance in adult rats. Thus, to maintain their caloric intake, the rats were forced to consume substantial quantities of water as well. According to the manufacturer’s information, the sodium, potassium and chloride contents of the AIN-76 liquid diet are 0.0113, 0.0054 and 0.0110 mEq/l, respectively. The rats were maintained in metabolic cages, allowing quantitative urine collections. All procedures were carried out in accordance with the National Institute of Health guidelines on the care and use of animals and an animal study protocol approved by the Georgetown University Animal Use and Care Committee.

Effect of L-NAME and diclofenac on escape from vasopressin-induced antidiuresis.

Separate water-loaded groups were infused with L-NAME (10 or 20 mg/kg/day) or diclofenac (2.5 mg/kg/day) via osmotic mini-pumps (model 2001; Alzet, Palo Alto, CA), which were implanted one day before the start of water loading. This dose of diclofenac has been reported to effectively decrease kidney prostaglandin E2 production in rats (12). Since diclofenac has a potential problem to cause gastrointestinal bleeding, ranitidine (10 mg/kg/day, Sigma Chemical, St. Louis, MO) was administered
subcutaneously via osmotic mini-pumps to all groups in the experiments using diclofenac. Rats were confirmed that they did not have gastrointestinal bleeding at the time of sacrifice. L-NAME (Sigma) was dissolved in saline, and diclofenac (Sigma) was dissolved in 50% DMSO. Urine volume and osmolality were measured daily. On day 5 after water loading, rats were euthanized by decapitation and both kidneys were rapidly removed and frozen in dry ice. The kidneys were stored at -80°C until processing for immunoblotting. Plasma samples were collected from trunk blood at the time of decapitation for measurement of plasma sodium concentration and osmolality.

**Urinary NOx excretion measurement.**

Since urinary NOx excretion has been reported to be affected by dietary NOx intake in the food (13), the same amount of food was given to the water-loaded group as to the liquid diet group, and to the non-water-loaded group as to the powder diet group by pair feeding. Urine was collected into a container with penicillin G (2,000 IU, Bristol-Myers Squibb, Princeton, NJ) and streptomycin (2,000 IU, Sigma) to inhibit bacterial growth. Thereafter, the urine was centrifuged, and a aliquot was stored at –70°C until measurement of NOx. Urinary NOx was measured by chemiluminescence (Sievers Instruments, Boulder, CO). NO₃ was catalytically converted to NO₂, from which NO was evolved under acid hydrolysis and reacted with O₃, yielding photons that were measured using a photomultiplier tube (14).
**Immunoblotting**

Left whole kidneys were homogenized in chilled membrane-isolation solution containing 250 mM sucrose, 10 mM triethanolamine, 1 µg/ml leupeptin (Bachem California, Torrence, CA), and 0.1 mg/ml phenylmethylsulfonyl fluoride (United States Biochemical Corp., Cleveland, OH) adjusted to pH 7.6. Protein concentration was measured by BCA protein assay reagent kit (Pierce Chemical CO., Rockford, IL). All samples were then diluted with isolation solution to a protein concentration of 2 µg/µl then diluted to 1 µg/µl with NOVEX sample buffer (NOVEX, San Diego, CA). SDS-PAGE was carried out on 12% Tris-Glycine polyacrylamide minigels using an Xcell II Mini-Cell electrophoresis apparatus (NOVEX). The proteins were transferred from the gels electrophoretically to nitrocellulose membranes using a Bio-Rad mini-Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA). The nitrocellulose membranes were probed with the affinity-purified rabbit polyclonal antibodies directed against rat aquaporin-2 (L127) (15). The secondary antibody was donkey anti-rabbit IgG conjugated to horseradish peroxidase (No. 31458; Pierce Chemical Co.) used at a concentration of 0.16 µg/ml. To visualize sites of antibody-antigen reaction, blots were exposed to a luminol-based enhanced chemiluminescence reagent (LumiGLO; Kirkegarrd and Perry Laboratories, Gaithersburg, MD) before exposure to x-ray film (FUJI FILM, Kanagawa, Japan). Relative quantitation of the band densities from immunoblots was carried out by densitometry using NIH image software.

**Statistics**
Data are presented as mean values ± S.E. Urine data were statistically analyzed by two-way repeated measures ANOVA followed by Student Newman-Keuls multiple comparison. Results from immunoblot densitometry were analyzed by one-way ANOVA followed by Student Newman-Keuls multiple comparison. Differences were considered statistically significant at p<0.05.

RESULTS

Time course of urine volume and urine osmolality. Fig. 1A shows the time course of changes in daily urine volume in an initial group of rats. Urine volume began to increase by day 2 of water loading despite sustained administration of dDAVP. Fig. 1B shows the time course of changes in urine osmolality in the same experiment. Urine osmolality was significantly decreased by day 2 of water loading. These results indicate that escape from vasopressin-induced antidiuresis began by 2 days after the onset of water loading.

Effects of water loading on urinary NO\textsubscript{2} and NO\textsubscript{3} excretion. Figure 2 shows daily urinary NO\textsubscript{2} and NO\textsubscript{3} excretion after water loading. Endogenous NO synthesis, measured as urinary NO\textsubscript{2}+NO\textsubscript{3} excretion, was significantly increased in the water-loaded group compared to the non-water-loaded controls during all 5 days of water loading, and peaked on day 2 (WL = 6.8±0.9, control = 4.7±0.4 µmol/day, p<0.05). L-NAME (20
mg/kg/day) significantly decreased urinary NO$_2$+NO$_3$ excretion on all days to levels that were the same as, or less than, those of the non-water-loaded controls.

*Effects of L-NAME on urine volume and osmolality.* Figure 3 shows the effects of L-NAME (20 mg/kg/day) on urine volume and urine osmolality. Rats ate 70 ml of liquid diet on the first day, but drank only approximately 10 ml on days 2 and 3 of water loading, likely because of anorexia caused by the induced hyponatremia, and the food intake began to recover thereafter (Table 1). There were no differences in the food intake between the L-NAME treated group and the water-loaded control group. As in the initial time course studies, urine volume increased and urine osmolality decreased by 2 days after the onset of water loading, indicating ongoing renal escape from vasopressin-induced antidiuresis. Infusion of a high dose of L-NAME (20 mg/kg/day) did not affect urine volume and osmolality on day 1 and day 2; however, urine volume markedly decreased and urine osmolality slightly increased on day 3 of water loading and thereafter, indicating inhibition of the progress of the escape process. Plasma sodium concentration was also slightly increased in the L-NAME treated group compared to the water-loaded control group (Table 2). Urinary sodium excretion was markedly increased on day 2 in water-loaded rats causing negative balances (Fig. 4A). In the L-NAME treated group, urinary sodium excretion tended to decrease on day 3 of water loading and thereafter compared to the water-loaded controls. Negative potassium balances were also seen on all days (Fig. 4B). Consequently, the total sodium and potassium balances between day 1 and 5 were negative in all groups, but the electrolyte loss was significantly decreased in the L-NAME treated group compared to the water-loaded control group (Table 3).
Effects of L-NAME on kidney aquaporin-2 protein expression after water loading.

Fig. 5A shows immunoblots of rat whole kidney homogenates on day 5 of water loading probed with anti-aquaporin-2. The aquaporin-2 protein bands, which are decreased in the water-loaded groups compared with the non-water-loaded controls, appeared to be decreased to a lesser degree by treatment with L-NAME (20 mg/kg/day). Densitometry confirmed that L-NAME significantly increased aquaporin-2 protein expression compared with the water-loaded controls (175±7% of water-loaded controls, p<0.05; Fig. 5B).

Effects of L-NAME on urine volume and osmolality in non-water-loaded rats.

L-NAME (20 mg/kg/day) slightly increased urine volume and decreased urine osmolality in non-water-loaded rats, but these changes were not significant (data not shown). Aquaporin-2 immunoblots showed that this dose of L-NAME (20 mg/kg/day) did not affect kidney aquaporin-2 expression in non-water-loaded rats (data not shown).

Effects of combination of L-NAME and diclofenac on urine volume and osmolality after water loading.

There were no significant differences in the food intake among the treatment groups (Table 4). Figure 6 shows the effects of a lower dose of L-NAME (10 mg/kg/day) and diclofenac (2.5 mg/kg/day) on urine volume and osmolality after water loading. L-NAME (10 mg/kg/day) or diclofenac (2.5 mg/kg/day) did not significantly affect the urine volume and osmolality by themselves. However, the combination of L-NAME and diclofenac markedly decreased urine volume and increased urine osmolality.
on day 3 of water loading and thereafter, indicating inhibition of the progress of the escape process. Despite this inhibition of escape, there were no significant differences in plasma sodium among the groups (Table 5). In the L-NAME and diclofenac treated group, urinary sodium excretion tended to decrease on day 3 of water loading and thereafter compared to the water-loaded controls (Fig. 7A). The total sodium and potassium balances between day 1 and 5 were negative in all groups, but the negative balance tended to be less in the L-NAME + diclofenac treated group (Table 6).

**Effects of L-NAME and diclofenac on kidney aquaporin-2 protein expression after water loading.** Figure 8A shows immunoblots of rat whole kidney homogenates on day 5 of water loading probed with anti-aquaporin-2. Neither L-NAME (10 mg/kg/day) nor diclofenac (2.5 mg/kg/day) significantly affected kidney aquaporin-2 protein expression by themselves. However, the combination of L-NAME and diclofenac appeared to increase the aquaporin-2 bands compared to the water-loaded controls. Densitometry confirmed that the combination of L-NAME and diclofenac significantly increased kidney aquaporin-2 expression (166±5% of water-loaded controls, p<0.05; Figure 8B).

**DISCUSSION**

To study the potential roles of NO and PG in the escape from vasopressin-induced antidiuresis, we first evaluated NO synthesis during water loading. Urinary prostaglandin E2 excretion has been reported to increase concomitantly with the onset of escape (2; 16).
However, there have been no reports about changes in NO synthesis after water loading. Our results showed that endogenous NO synthesis, measured as urinary NO$_2$+NO$_3$ excretion, was significantly increased after water loading. This up-regulation of NO synthesis was seen as early as one day after initiation of water loading and peaked on day 2, which coincided with the onset of escape. After 3 days, NO synthesis decreased somewhat, which could be because of the decrease in body water retention as a result of increased urine flow due to the ongoing escape. Thus, endogenous NO synthesis after water loading may be related to the ECF volume status. Although it is still unclear what the sources of NO or PG actually are, it seems reasonable to hypothesize that these vasoactive substances are synthesized in the vasculature. In this regard, it is notable that Hall et al. showed that vasopressin escape did not occur in dogs in which renal perfusion pressure was servo-controlled, suggesting that vasopressin escape is mediated by increased renal perfusion (3). Increased renal perfusion pressure due to ECF volume expansion would be expected to cause stretching of renal vascular endothelial cells, as well as glomerular mesangial cells (17; 18). It has been reported that shear stress stimulates the production of NO and PG through stretch-activated ion channels in vascular endothelial cells (19-23).

In the present study, we showed that L-NAME has inhibitory effects on vasopressin escape, though predominantly during the later stages of escape (i.e., days 3-5). This may be explained as follows. This dose of L-NAME has been reported to significantly increase blood pressure (24). It is well known that elevation of renal blood pressure increases urine flow and sodium excretion, which has been termed pressure
natriuresis (25). Such increases in blood pressure would be enhanced by increased ECF volume; therefore, it is possible that the antidiuretic effect of dDAVP was overcome by a pressure diuresis produced by the hypertensive effect of L-NAME. However, any L-NAME-induced increases in blood pressure would be expected to be maximal on day 1 or day 2 after water loading when water retention was maximal, whereas the effects of L-NAME were seen mainly during the later stages of escape. Interestingly, L-NAME did not significantly decrease urine flow in non-water-loaded rats treated with DDAVP infusion. This suggests that inhibitory effects of L-NAME on vasopressin escape are probably not caused by non-selective effects such as renal toxicity, and further supports the idea that increased NO synthesis after water loading may be an important contributing factor to the induction and/or maintenance of vasopressin escape.

The results of present study also showed that the inhibitory effect of L-NAME on vasopressin escape was accompanied by a significant blunting of the water-loading-induced decreases in kidney aquaporin-2 protein expression. There are two possibilities for this inhibitory mechanism of L-NAME. One is that L-NAME acts directly on collecting duct cells to reverse an inhibitory effect of NO on the antidiuretic effect of vasopressin. Vasopressin regulates kidney collecting duct water permeability via aquaporin-2 by stimulating its intracellular shuttling (27) or by increasing its abundance (28). Both of these mechanisms have been reported to be induced by increases in cellular cAMP levels (26; 27). It has been reported that NO inhibits vasopressin-stimulated cAMP synthesis by increasing cGMP in kidney collecting ducts (28). Therefore, it is possible that NO might down-regulate kidney aquaporin-2 expression by inhibiting
cellular cAMP production, in which case L-NAME would reverse this down-regulation of aquaporin-2 expression by inhibiting NO synthesis. Recently, Bouley et al. reported that NO stimulates insertion of aquaporin-2 in renal epithelial cells by increasing intracellular cGMP, indicating that NO has an antidiuretic effect via a vasopressin-independent mechanism (29). However, this study did not report the effects of NO on vasopressin-induced aquaporin-2 translocation. A second potential mechanism is that L-NAME might increase aquaporin-2 expression indirectly by modulating renal hemodynamics. L-NAME is known to vasoconstrict the renal artery and reduce renal blood flow by decreasing local synthesis of NO, a potent vasodilator (24). As mentioned above, because an increase in renal perfusion appears to be critical for vasopressin escape, it is possible that L-NAME might reverse down-regulation of aquaporin-2, in part, by decreasing renal blood flow. Together with our previous reports showing marked down-regulation of kidney aquaporin-2 expression during vasopressin escape (4; 6), the results from the present studies suggest that down-regulation of aquaporin-2 plays a pivotal role in the escape phenomenon. However, we cannot exclude the possibility of other mechanisms. For example, factors such as wash-out of the medullary osmotic concentration gradient, local changes in renal blood flow, changes in systemic blood pressure, and other factors that are known to influence renal concentrating ability, may also play an important role in the escape phenomenon. Further studies will be required to assess the relative contributions of these factors to vasopressin escape.

It is interesting that the plasma sodium concentration did not decrease further after L-NAME (20 mg/kg/day) or L-NAME + diclofenac treatment despite marked decreases
in urine volume. Since decrease in urine volume would result in more water retention in the body, it would be expected that the plasma sodium concentration would decrease in these rats as a result of plasma dilution. To address this point, we calculated the sodium and potassium balances during the experiments. The urinary sodium excretion was markedly increased on day 2 resulting in negative sodium balance, which is characteristic of SIADH. The urinary sodium excretion of L-NAME (20mg/kg/day) or L-NAME + diclofenac group tended to decrease on days 3, 4 and 5 compared to the water-loaded control group. The total sodium and potassium balance between day 1 and 5 was negative in all groups, but it was higher in the L-NAME (20mg/kg/day) group compared to the control group. It was also higher, although not statistically significant, in the L-NAME + diclofenac group. These results indicate that rats in the L-NAME (20mg/kg/day) or L-NAME + diclofenac group have more sodium in their bodies at the end of experiments compared to the control group, and this may explain why the plasma sodium concentrations did not decrease further in the L-NAME (20mg/kg/day) and the L-NAME + diclofenac groups despite additional water retention caused by a decrease in urine volume.

Perhaps most importantly, the results of present study suggest that there is a potentially important interaction between NO and PG in maintaining renal escape from vasopressin-induced antidiuresis. Neither a lower dose of L-NAME (10 mg/kg/day), nor diclofenac, significantly affected vasopressin escape when they were administered separately. However, it is striking that the combination of L-NAME and diclofenac produced a marked inhibitory effect on the escape phenomenon, which also was
accompanied by significant increase in kidney aquaporin-2 expression. It has been reported that diclofenac causes a decrease in GFR (30; 31), and we cannot deny the possibility that the decrease in urine flow may be caused by the decrease in GFR. However, aquaporin-2 expression was increased by diclofenac when it was administered with L-NAME. Therefore, it seems reasonable to think that a decrease in GFR is not the only contributor to the observed decrease in urine volume. Although the present study was not designed to elucidate the mechanism of this interaction between NO and PG, it seems possible that since both NO and PG are potent vasodilators, an inhibition of either NO or PG alone might be overcome by a compensatory increase in the activity of the other. In this case, inhibition of NO or PG individually would not effectively inhibit vasopressin escape. However, when infused at higher dose, L-NAME alone did inhibit vasopressin escape to some degree. This may be because the vasoconstricting activity of L-NAME is more potent than the vasodilating effect of PG, and consequently renal blood flow was significantly decreased by the higher dose of L-NAME alone. Another possibility is that L-NAME activates mechanisms other than modulating renal hemodynamics, such as direct effects on renal collecting duct cells. In this case, compensation by PG might not occur, or may be less potent with higher doses of L-NAME.

In conclusion, results from the present study indicate that renal NO and PG both play important roles in escape from vasopressin-induced antidiuresis, and suggest that they might act synergistically to down-regulate kidney aquaporin-2 expression during escape from vasopressin-induced antidiuresis.
ACKNOWLEDGEMENTS

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REFERENCES


LEGENDS TO FIGURES

Fig. 1. Time course of changes in urine volume (A) and urine osmolality (B) after water loading. All rats were implanted with dDAVP osmotic mini-pumps at day -4 and had access to pelleted rat chow and water ad libitum. On day 0, one group was water loaded by substituting daily feedings of a liquid formula for the pelleted chow, while the controls continued to receive pelleted rat chow and water ad libitum. Values are expressed as mean ± S.E. *p<0.05 compared to control group.

Fig. 2. Effects of L-NAME on daily urinary NO₂ and NO₃ excretion after water loading. All rats were implanted with dDAVP osmotic mini-pumps at day -4 and had access to pelleted rat chow and water ad libitum. On day 0, one group was water loaded by substituting daily feedings of a liquid formula for the pelleted chow, while the controls continued to receive pelleted rat chow and water ad libitum. One group of the water-loaded rats was implanted with an osmotic mini-pump that delivered L-NAME (20 mg/kg/day) one day before the start of water loading. Values are expressed as mean ± S.E. *p<0.05 compared to control group. †p<0.05 compared to WL group.

Fig. 3. Effects of L-NAME on urine volume (A) and urine osmolality (B) after water loading. All rats were implanted with dDAVP osmotic mini-pumps at day -4 and had access to pelleted rat chow and water ad libitum. On day 0, two groups of rats were water loaded by substituting daily feedings of a liquid formula for the pelleted chow, while the controls continued to receive pelleted rat chow and water ad libitum. One
group of the water-loaded rats was implanted with an osmotic mini-pump that delivered L-NAME (20 mg/kg/day) one day before the start of water loading. Values are expressed as mean ± S.E. *p<0.05 compared to water-loaded group without L-NAME.

**Fig. 4.** Effects of L-NAME on sodium (A) and potassium (B) balances during water loading. WL: water-loaded; N: water-loaded treated with L-NAME. All rats were implanted with dDAVP osmotic mini-pumps at day -4 and had access to pelleted rat chow and water ad libitum. On day 0, rats were water loaded by substituting daily feedings of a liquid formula for the pelleted chow. One group was implanted with an osmotic mini-pump that delivered L-NAME (20 mg/kg/day) one day before the start of water loading. Daily sodium and potassium balances were calculated from dietary intake and urinary losses, ignoring fecal losses which were assumed to be small and constant across groups. Values are expressed as mean ± S.E. There are no significant differences in sodium intake and excretion as well as potassium between two groups on each day.

**Fig. 5.** Effects of L-NAME treatment on aquaporin-2 protein expression after water loading. C: non-water-loaded control; WL: water-loaded; WL + L-NAME: water-loaded treated with L-NAME. (A) Western immunoblots of whole kidney homogenates from Sprague-Dawley rats after water loading for 5 days (all rats received dDAVP by mini-pumps). L-NAME (20 mg/kg/day) was administered via osmotic mini-pumps implanted one day before the start of water loading. Each lane was loaded with sample from a different rat (7 µg total protein/lane). Blots were probed with anti-aquaporin-2 antibody (120 ng/ml). The 29-kD band is the nonglycosylated form and 40-kD band is the
glycosylated form of aquaporin-2. (B) Summary of densitometry data (sum of both 29- and 40-kD bands) from the immunoblots. Values represent the mean value of each group expressed as a percentage of the average value of the non-water-loaded control group. *p<0.05 compared to control group; †p<0.05 compared to WL group. Water loading markedly decreased kidney aquaporin-2 protein expression, and this effect was significantly blunted by L-NAME treatment.

Fig. 6. Effects of L-NAME and/or diclofenac on urine volume (A) and urine osmolality (B) after water loading. All rats were implanted with dDAVP osmotic mini-pumps at day -4 and had access to pelleted rat chow and water ad libitum. On day 0, all rats were water loaded by substituting daily feedings of a liquid formula for the pelleted rat chow. Experimental groups were implanted with osmotic mini-pumps that delivered L-NAME (10 mg/kg/day) and/or diclofenac (2.5 mg/kg/day) one day before the start of water loading. Values are expressed as mean ± S.E. *p<0.05 compared to water-loaded group.

Fig. 7. Effects of L-NAME and/or diclofenac on sodium (A) and potassium (B) balances during water loading. WL: water-loaded; N: water-loaded with L-NAME; D: water-loaded with diclofenac; N+D: water-loaded with L-NAME and diclofenac. All rats were implanted with dDAVP osmotic mini-pumps at day -4 and had access to pelleted rat chow and water ad libitum. On day 0, rats were water loaded by substituting daily feedings of a liquid formula for the pelleted chow. Experimental groups were implanted with osmotic mini-pumps that delivered L-NAME (10 mg/kg/day) and/or diclofenac (2.5 mg/kg/day) one day before the start of water loading. Daily sodium and potassium balances were calculated from dietary intake and urinary losses, ignoring fecal losses.
which were assumed to be small and constant across groups. Values are expressed as mean ± S.E. There are no significant differences in sodium intake and excretion as well as potassium among each group on any days.

**Fig. 8.** Effects of L-NAME and/or diclofenac on aquaporin-2 protein expression after water loading. WL: water-loaded; L-NAME: water-loaded with L-NAME; diclofenac: water-loaded with diclofenac; L-NAME + diclofenac: water-loaded with L-NAME and diclofenac. **(A)** Western immunoblots of whole kidney homogenates from Sprague-Dawley rats after water loading for 5 days (all rats received dDAVP by mini-pumps). L-NAME (10 mg/kg/day) and/or diclofenac (2.5 mg/kg/day) were administered via osmotic mini-pumps implanted one day before the start of water loading. Each lane was loaded with sample from a different rat (7 µg total protein/lane). Blots were probed with anti-aquaporin-2 antibody (120 ng/ml). The 29-kD band is the nonglycosylated form and 40-kD band is the glycosylated form of aquaporin-2. **(B)** Summary of densitometry data (sum of both 29- and 40-kD bands) from the immunoblots. Values represent the mean value of each group expressed as a percentage of the average value of the water-loaded control group. *p<0.05 compared to water-loaded group. Water loading markedly decreased aquaporin-2 protein. When administered separately, neither L-NAME nor diclofenac had significant effects on aquaporin-2 protein expression, but when the same doses of L-NAME and diclofenac were given in combination, the decrease in aquaporin-2 expression induced by water loading was significantly blunted.
Table 1.

Food intake after L-NAME treatment.

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<td>WL (ml)</td>
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<td>17 ± 4</td>
<td>13 ± 4</td>
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<td>L-NAME (ml)</td>
<td>59 ± 1</td>
<td>11 ± 2</td>
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WL (n = 4): water-loaded; WL + L-NAME (n = 4): water-loaded treated with L-NAME. Food intake was expressed as volume of liquid diet (ml). L-NAME (20 mg/kg/day) was infused via osmotic mini-pumps from one day before the start of water loading. Values are presented as mean ± S.E. There are no significant differences between WL and L-NAME group on each day. Since non-water-loaded rats were not fed with liquid diet, they were not included here.
**Table 2.**

**Effects of L-NAME on plasma sodium after water loading.**

<table>
<thead>
<tr>
<th></th>
<th>Control (4)</th>
<th>Water-loaded (4)</th>
<th>Water-loaded + L-NAME (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma sodium (mEq/l)</td>
<td>137.4 ± 0.3</td>
<td>98.5 ± 0.4*</td>
<td>101.8 ± 0.2*†</td>
</tr>
</tbody>
</table>

Plasma samples were collected after 5 days of water loading. L-NAME (20 mg/kg/day) was infused via osmotic mini-pumps from one day before the start of water loading. Values are expressed as mean ± S.E. *p<0.05 compared to non-water-loaded control. †p<0.05 compared to water-loaded control. Numbers of rats for each group are indicated in parenthesis.
Table 3.

Effects of L-NAME on sodium and potassium balances during water loading.

<table>
<thead>
<tr>
<th></th>
<th>Na⁺ (mEq)</th>
<th>K⁺ (mEq)</th>
<th>Na⁺ and K⁺ (mEq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WL</td>
<td>-2.26 ± 0.15</td>
<td>-4.15 ± 0.24</td>
<td>-6.41 ± 0.18</td>
</tr>
<tr>
<td>L-NAME</td>
<td>-1.85 ± 0.14</td>
<td>-3.49 ± 0.13*</td>
<td>-5.34 ± 0.20*</td>
</tr>
</tbody>
</table>

WL: water-loaded, L-NAME: water-loaded treated with L-NAME (20 mg/kg/day). Sodium and potassium balances were calculated from sum of dietary intake and urinary losses between day 1 and day 5. Values are presented as mean ± S.E. *p<0.05 compared to water-loaded group.
Table 4.

Food intake after L-NAME and/or diclofenac treatment.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>WL (ml)</td>
<td>60±5</td>
<td>11±4</td>
<td>10±1</td>
<td>15±2</td>
<td>29±3</td>
</tr>
<tr>
<td>L-NAME (ml)</td>
<td>58±6</td>
<td>8±2</td>
<td>10±1</td>
<td>14±1</td>
<td>28±2</td>
</tr>
<tr>
<td>diclofenac (ml)</td>
<td>56±5</td>
<td>15±2</td>
<td>9±2</td>
<td>13±1</td>
<td>25±4</td>
</tr>
<tr>
<td>L-NAME+diclofenac (ml)</td>
<td>58±6</td>
<td>9±3</td>
<td>9±1</td>
<td>14±1</td>
<td>27±4</td>
</tr>
</tbody>
</table>

WL (n = 4): water-loaded; L-NAME (n = 3): water-loaded with L-NAME; diclofenac (n = 4) water-loaded with diclofenac; L-NAME + diclofenac (n = 3): water-loaded with L-NAME and diclofenac. Food intake is expressed as a volume of liquid diet (ml). Values are presented as mean ± S.E. There are no significant differences among each group on any days.
Table 5.

Effects of L-NAME and/or diclofenac on plasma sodium after water loading.

<table>
<thead>
<tr>
<th></th>
<th>WL (4)</th>
<th>WL + L-NAME (3)</th>
<th>WL + diclofenac (4)</th>
<th>WL + L-NAME +diclofenac (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma sodium</td>
<td>98.5 ± 1.4</td>
<td>100.4 ± 0.7</td>
<td>99.2 ± 1.1</td>
<td>99.6 ± 2.0</td>
</tr>
<tr>
<td>(mEq/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All groups were water-loaded (WL) for 5 days. L-NAME (10 mg/kg/day) and/or diclofenac (2.5 mg/kg/day) were infused via osmotic mini-pumps from one day before the start of water loading. Values are expressed as mean ± S.E. There are no significant differences among each group. Numbers of rats for each group are indicated in parenthesis.
Table 6.

Effects of L-NAME and/or diclofenac on sodium and potassium balances during water loading.

<table>
<thead>
<tr>
<th></th>
<th>Na(^+)</th>
<th>K(^+)</th>
<th>Na(^+) and K(^-) (mEq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WL</td>
<td>-1.96 ± 0.27</td>
<td>-4.12 ± 0.33</td>
<td>-6.08 ± 0.60</td>
</tr>
<tr>
<td>L-NAME</td>
<td>-1.46 ± 0.49</td>
<td>-3.83 ± 0.26</td>
<td>-5.28 ± 0.49</td>
</tr>
<tr>
<td>diclofenac</td>
<td>-1.96 ± 0.03</td>
<td>-3.86 ± 0.16</td>
<td>-5.82 ± 0.19</td>
</tr>
<tr>
<td>L-NAME + diclofenac</td>
<td>-1.46 ± 0.18</td>
<td>-3.20 ± 0.32</td>
<td>-4.65 ± 0.44</td>
</tr>
</tbody>
</table>

WL: water-loaded; L-NAME: water-loaded with L-NAME (10 mg/kg/day); diclofenac: water-loaded with diclofenac (2.5 mg/kg/day); L-NAME + diclofenac: water-loaded with L-NAME and diclofenac. Sodium and potassium balances were calculated from sum of dietary intake and urinary losses between day 1 and day 5. Values are presented as mean ± S.E. There are no significant differences among each group on any days.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8