Renal effects of nitric oxide synthase inhibition in conscious water-loaded dogs

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Renal effects of nitric oxide synthase inhibition in conscious water-loaded dogs. Am J Physiol Regulatory Integrative Comp Physiol 281: R584–R590, 2001.—The renal effects of the nitric oxide (NO) synthase inhibitor nitro-l-arginine methyl ester (L-NAME) were investigated in conscious dogs undergoing sustained water diuresis and replacement of urinary sodium losses. Experiments were performed with and without additional extracellular volume expansion (isotonic saline, 2% body wt). L-NAME (10 μg·kg⁻¹·min⁻¹) infused during water diuresis decreased urine flow (2.5 ± 0.2 to 1.5 ± 0.3 ml/min), free water clearance (1.9 ± 0.2 to 1.0 ± 0.2 ml/min), and sodium excretion (4.0 ± 1.7 to 2.1 ± 0.6 μmol/min). Arterial blood pressure increased from 112 ± 2 to 126 ± 3 mmHg, but creatinine clearance did not measurably change. Plasma endothelin and vasopressin concentrations increased (3.4 to 76.5 μg/kg/min). PRA decreased with control volume expansion but not during L-NAME. Urinary sodium excretion was also decreased with volume expansion. In addition, the experiments were performed using a system where each animal’s water losses (weight), which, of course, are primarily a result of urinary output, are servo-controlled and continuously replaced. Thus each animal received an equivalent amount of water and saline loading.

Water diuresis; volume expansion; sodium excretion; endothelin-derived relaxing factor

IN RECENT YEARS, an intense interest has developed in defining the role of nitric oxide (NO) in physiological regulation. A large body of investigative work has determined that this substance is involved in a wide variety of systems and mechanisms (18). As a result of these findings, defects in the NO system are being suggested as potential causative factors in certain clinical conditions, particularly hypertension and heart failure (6, 25).

Related to the above is the evidence that NO may modulate renal function through both vascular and tubular effects (10, 12). Much of the support for an important role of NO in control of renal hemodynamics, excretory function, and renin secretion stems from studies using l-arginine analogs that competitively inhibit the generation of NO (24). Use of these inhibitors has allowed investigations on the necessity of the NO system in basal renal function, assessed on both a short (14, 21)- and long-term basis (17, 27) as well as NO’s possible involvement during interventions that alter renal function. The latter includes the diuresis and natriuresis in response to increases in blood pressure (15, 16, 28) and extracellular fluid volume expansion (1, 13, 22, 27). With regard to most of these volume expansion studies, the nature of the protocols and variables measured required that anesthetized animal preparations be used. The exception is a study that one of us performed in conscious nonhuman primates in which it was demonstrated that inhibition of NO generation did attenuate intravenous volume expansion diuresis/natriuresis (22). However, the involvement of NO in the responses to an oral water load, as opposed to intravenous isotonic volume load, has never been investigated.

Therefore, the purpose of the present experiments was to investigate the effects of NO synthesis inhibition on the renal and hormonal responses of conscious dogs to 1) oral water loading and 2) oral water loading combined with intravenous isotonic saline volume expansion. In addition, the experiments were performed using a system where each animal’s water losses (weight), which, of course, are primarily a result of urinary output, are servo-controlled and continuously replaced. Urinary sodium excretion was also continuously measured and replaced. Thus each animal remained in a steady-state volume (water) and sodium condition throughout each experiment. This was also true for the isotonic volume expansion series. In these studies, the animals moved from a control steady state
with regard to volume and sodium to an expanded steady state.

**METHODS**

The experiments were performed on conscious female beagle dogs (n = 6) with a weight range of 9–14 kg. All animals had a daily intake of sodium of 70 mmol and free access to tap water. Before the study, the animals were subjected to aseptic surgery to place both common carotid arteries into skin loops for easier catheterization during the study and also to do an episiotomy to facilitate bladder catheterization. Animals were used in repeated treatments as described below but were allowed at least a week between studies.

**Experimental Preparation**

After the dog was brought to the lab, sterile catheters were inserted into an external jugular vein and saphenous vein for purposes of infusing solutions and obtaining blood samples. One of the carotid arteries was also catheterized to obtain arterial blood samples and measure blood pressure via a Statham P50 pressure transducer. Heart rate (HR) was monitored via an electrocardiogram. Both of these signals were displayed and digitized using a Danica patient monitor, and the numerical data were sampled every 10 s by computer. A modified Foley catheter was inserted into the bladder. The water load, equal to 2% body wt, was then administered by gavage. As the animal progressively went into water diuresis, the volume (weight) change due to urinary and evaporative losses was measured by means of a servo-control mechanism (2), which continuously replaced the water loss by infusing the correct amount of a solution of hypotonic glucose-urea (40 and 25 mM, respectively). Thus this represents a condition of sustained water diuresis. Urinary sodium concentration was also measured in every 15-min collection sample. The calculated sodium loss was returned to the animal over the next 15 min by changing the rate of infusion of a hypertonic (400 mM) NaCl solution. Each animal was, therefore, also in a type of steady-state condition with regard to sodium. Throughout each experiment, creatinine was also infused, and its clearance was used as an estimate of the glomerular filtration rate (GFR).

Each animal was studied under two experimental conditions: 1) basal servo-controlled sustained water diuresis with manual replacement of sodium and 2) sustained water diuresis in which a sustained isotonic saline volume expansion was induced. For both of these experiments, each animal was also studied under three different treatment conditions.

**Water diuresis.** Collection of control urine samples for complete analysis began 90 min after the administration of the water load and the beginning of the replacement of water and urinary sodium losses. Urine was collected at continuous 15-min samples throughout the experiment. For the control condition, this collection continued without any interventions for a total of 180 min. A second series was performed to test the effects of inhibition of NO synthesis. The NO synthase competitive inhibitor nitro-L-arginine methyl ester (L-NAME) was infused continuously at a dose of 10 μg·kg⁻¹·min⁻¹ beginning after 30 min when control urines were obtained. A third series was also performed to test the specificity and possible “reversibility” or abolition of L-NAME’s effects. This was accomplished by infusing L-NAME in the presence of a simultaneous infusion of NO substrate L-arginine (0.6 mg·kg⁻¹·min⁻¹). The L-arginine infusion began at the time when the water load was given or 90 min before the start of data collection. Each dog was subjected to each of these three treatments in a random fashion. Throughout each experiment, regular blood samples were obtained for measuring plasma vasopressin, endothelin, renin activity, creatinine, sodium, potassium, and osmolality.

**Volume expansion.** In this series, the water diuretic dogs were subjected to isotonic saline volume expansion (2% of body wt). This volume infusion was started following 30 min of control urine collection and was administered over the next 90 min. An additional 60 min of postexpansion samples were then collected. This was also a sustained volume expansion in that before infusion into the animal, the volume load was added to the servo-controlled weight load. Thus the infusion did not influence the signal driving the fluid replacement and, as such, any diuretic responses occurring were replaced by the system. Natriuretic responses were also measured and replaced with hypertonic saline infusion. Similar to the series described above, experiments were also performed during L-NAME and combined L-NAME/L-arginine infusion. Because the goal of this series was to examine the effects of volume expansion in the presence of these treatments rather than the treatments themselves, the L-NAME and/or L-arginine infusions were started at the time of the water loading, i.e., 90 min before data collection began.

**Analyses**

**Chemical analyses.** Sodium and potassium concentrations in plasma and urine were measured by flame photometry (Instrumentation Laboratories model 243) and osmolality by freezing-point depression (Advanced Instruments model 3MO). Creatinine concentration was determined by the colorimetric method of Bonsnes and Taussky (5).

**Hormone analyses.** Determination of plasma arginine vasopressin (AVP) was by a radioimmunoassay as previously described (7). Briefly, arterial plasma was acidified and then extracted using Sep-Pak C₁₈ cartridges (Waters, Millipore). The dried eluate was reconstituted in assay buffer, and the test samples and standards were incubated with a specific antibody (11). Bound and free antigen were separated with a charcoal-plasma suspension. The interassay coefficients of variation for 0.6 and 6.0 pg/tube were 13.6 and 4.3%, respectively. Intra-assay coefficient of variation for the middle sensitivity range of the assay was 7.8%. Recovery of synthetic AVP added to plasma was 85%. The detection limit of 0.1 pg/tube was used for all undetectable values when calculating means and standard errors of the data.

Plasma and urine endothelin immunoreactivity were also determined by an assay previously described (7). Samples were again acidified and extracted using Sep-Pak C₁₈ cartridges, and endothelin (ET-1) was measured with a specific antibody (Peninsula). The antibody is described to cross-react with ET-2 (7%), ET-3 (7%), and Big ET-1 (17%). For the analysis, the mean interassay coefficient of variation at 12 pg/tube was 7%. Intra-assay coefficient of variation as reported earlier (7) was 5%. Percent recoveries ranged from 96 to 100%. Detection limit was 0.6 pg/tube and was also used in mean calculations.

Plasma renin activity (PRA) was determined by radioimmunoassay using the antibody-trapping method of Poulsen and Jorgensen (23).

**Statistics.** All data within a particular treatment protocol were evaluated with a repeated-measures analysis of variance and Newman-Keuls multiple-range test. Differences between the three treatments within a series were also analyzed with an analysis of variance. Probability values less than 0.05 were considered statistically significant.
RESULTS

**Water Diuresis Experiments**

The urinary excretion results for this series are shown in Fig. 1. During the control water diuresis, urine flow (UV), sodium excretion (UNaV), and free water clearance (CH₂O) all remained constant, reflecting the steady-state conditions due to the urinary volume and sodium replacement. Infusion of L-NAME caused UV to decrease from $2.5 \pm 0.2$ to $1.5 \pm 0.3$ ml/min. Accompanying this was a fall in CH₂O from $1.9 \pm 0.2$ to $1.0 \pm 0.2$ ml/min. In this water diuresis series, control levels of UNaV were very low. Despite this, L-NAME did, however, still significantly decrease UNaV from $4.0 \pm 1.7$ to $2.1 \pm 0.6$ μmol/min. When L-NAME was infused simultaneously with L-arginine, all of these decreases in excretion rates occurring with L-NAME alone did not occur, i.e., the results were the same as under control conditions.

The hemodynamic results also showed significant effects of L-NAME (Fig. 2). Mean arterial blood pressure (MAP) increased from $112 \pm 2$ to $126 \pm 3$ mmHg, and HR decreased from $69 \pm 4$ to $51 \pm 5$ beats/min. Unlike the renal excretory effects, L-arginine did not abolish these changes due to L-NAME. Under this condition, there was still a significant rise in blood pressure ($111 \pm 2$ vs. $122 \pm 1$ mmHg) and bradycardia ($77 \pm 5$ vs. $63 \pm 5$ beats/min). The dose of L-NAME used did not cause any change in creatinine clearance (CCr). When L-arginine was added to the infusion, CCr also remained stable.

As expected, during water diuresis, plasma vasopressin concentrations were extremely low. They were also unaffected by L-NAME. At the end of the experiment, vasopressin levels in the control water diuresis, L-NAME infusion, and combined L-NAME and L-arginine infusion experiments were $0.29 \pm 0.12$, $0.27 \pm 0.17$, and $0.21 \pm 0.05$ pg/ml, respectively, values not significantly different from each other. L-NAME also had no effects on PRA (Table 1). Values were constant throughout the infusion. This was also true for the L-arginine series. There was a small, but significant, increase in PRA in the control water diuresis. However, these levels of PRA, similar to vasopressin, were extremely low as would be expected under the sodium intake and water-loading conditions, and they were not different from the L-NAME or L-arginine values.

![Fig. 1. Effects of N⁵-nitro-L-arginine methyl ester (L-NAME) and combined L-NAME plus L-arginine infusion on urine flow (UV), sodium excretion (UNaV), and free water clearance (CH₂O) in conscious water-loaded dogs. *Significantly different from preinfusion values at P < 0.05.](image1)

![Fig. 2. Effects of L-NAME and combined L-NAME plus L-arginine infusion on mean arterial blood pressure (MAP), heart rate (HR), and creatinine clearance (CCr) in conscious water-loaded dogs. *Significantly different from preinfusion values at P < 0.05.](image2)
Table 1. Effects of nitric oxide synthase inhibition on PRA in conscious water-loaded dogs

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Group</th>
<th>C</th>
<th>L-NAME</th>
<th>L-NAME</th>
<th>L-NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>Control</td>
<td>1.1 ± 0.2</td>
<td>1.4 ± 0.2</td>
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</tr>
<tr>
<td>120</td>
<td>L-NAME</td>
<td>1.3 ± 0.2</td>
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<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>150</td>
<td>L-NAME + L-arginine</td>
<td>1.2 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>1.2 ± 0.1</td>
<td>1.4 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. Plasma renin activity (PRA) units = ng ANG 1-1 ml⁻¹ h⁻¹; C, after 30 min of control; nitro-L-arginine methyl ester (L-NAME) 60, 120, and 150 min of L-NAME or vehicle infusion. *Significantly different from C value at P < 0.05.

Urine endothelin concentration remained constant during control conditions (3.54 ± 0.64 pg/ml at beginning vs. 3.24 ± 0.31 pg/ml at end) but increased during L-NAME infusion (3.42 ± 0.76 vs. 6.22 ± 0.70 pg/ml). However, the accompanying decrease in UV resulted in there being an unchanged rate of urinary excretion of endothelin. This L-NAME effect on urine endothelin concentration was reversed by L-arginine with urinary endothelin excretion again being constant (3.94 ± 0.59 vs. 4.54 ± 0.78 pg/ml). Additionally, the L-NAME-induced change in urine endothelin concentration did not reflect any effects on plasma endothelin levels. Blood samples taken 120 min after L-NAME was started had plasma endothelin concentrations of 3.3 ± 0.3 pg/ml, not significantly different from control water diuresis conditions (3.6 ± 0.2 pg/ml) or L-NAME plus L-arginine (4.0 ± 0.4 pg/ml).

Volume Expansion Experiments

Figure 3 depicts the results for the renal excretory changes during this series. Under the control condition, isotonic saline volume expansion caused a marked diuresis and natriuresis. UV increased from 2.5 ± 0.4 to 5.7 ± 0.5 ml/min and UNaV from 4 ± 2 to 77 ± 15 μmol/min. Volume expansion in the presence of L-NAME gave responses that were greatly attenuated compared with control. With L-NAME, the diuresis only increased from 1.6 ± 0.3 to 2.8 ± 0.7 ml/min and the natriuresis from 4 ± 2 to 34 ± 17 μmol/min. Unlike the basal water diuresis series, addition of L-arginine did not return the UV and UNaV responses to volume expansion completely back to the control condition. Although there was some reversal of the L-NAME results, this was only partial in nature, being most apparent only for the early phases of the diuresis and natriuresis.

The hemodynamic results for this series are shown in Fig. 4. MAP remained constant in the control group, even after the volume was infused, although this infusion did result in a significant rise in HR from 68 ± 4 to 95 ± 6 beats/min. This latter observation was consistent in that it occurred in every animal. The mechanism could not be determined from these experiments, although it could be a volume-induced reflex tachycardia often seen in conscious dogs with a low control preinfusion HR (4). The volume load also significantly increased Ccr (41 ± 4 to 50 ± 4 ml/min). As in the other series, pressor and bradycardia effects of L-NAME occurred. Because L-NAME infusion began 90 min before data collection was started, these effects were reflected by MAP and HR being different from control during the entire sampling portion of the protocol. The effects on HR and Ccr were essentially reversed by L-arginine, but the latter condition was still associated with a significant rise in MAP after volume loading. Unlike the control condition, Ccr was stable throughout the experiment in both the L-NAME alone and combined L-NAME and L-arginine conditions.

Similar to the basal water diuresis series, plasma vasopressin (AVP) levels were low and unaffected by L-NAME. Samples taken 30 min after the end of volume expansion had AVP values of 0.10 ± 0.01, 0.16 ± 0.05, and 0.12 ± 0.02 pg/ml in the control, L-NAME, and L-NAME plus L-arginine series, respectively.

PRA levels (Table 2) reflected a volume-induced fall in the control and L-arginine conditions. However, these values were again quite low probably because of the initial and servo-controlled water loading. There-
fore, despite there being no fall in PRA with volume expansion in the L-NAME group, there were no significant differences among any of the three experimental conditions. Urinary endothelin concentration was higher in the L-NAME group compared with the control group and decreased in both with volume expansion. During control, values fell from 5.28 ± 0.68 to 2.84 ± 0.50 pg/ml; and with L-NAME present, the decrease was from 7.63 ± 1.28 to 5.41 ± 0.55 pg/ml. With L-arginine present, the values were similar to control (4.23 ± 0.45 pg/ml) with no decrease after volume expansion. For all three conditions, as with the water diuresis series, urinary excretion of this substance was unaffected by any of the treatments. Plasma endothelin levels were unchanged under all three conditions: control 2.9 ± 0.3 pg/ml preexpansion vs. 3.1 ± 0.4 pg/ml at end-infusion; L-NAME 3.2 ± 0.3 pg/ml preexpansion vs. 3.5 ± 0.3 pg/ml after expansion; and combined L-NAME and L-arginine 3.6 ± 0.3 pg/ml preexpansion vs. 3.9 ± 0.4 pg/ml after expansion. None of these values was significantly different.

**DISCUSSION**

The results of our experiments have shown that in conscious dogs, inhibition of NO synthesis reduces the normal renal excretory responses to water loading. In the presence of a constant infusion of the NO synthase inhibitor L-NAME, the steady-state water diuresis could not be sustained but tended to decrease in magnitude as characterized by a progressive fall in UV and rise in urine osmolarity, i.e., a decrease in C\textsubscript{H\textsubscript{2}O}. In addition, the excretory responses to isotonic saline volume expansion were also affected by NO synthesis inhibition. Under this condition, both the diuretic and natriuretic effects were significantly blunted. As further evidence that these results were due to inhibition of NO, rather than any nonspecific effects of the L-NAME compound, simultaneous infusion of L-NAME together with NO substrate L-arginine caused reversal of the effects seen with L-NAME alone, although this reversal was only partial in the volume expansion series. These findings clearly demonstrate that NO may be an important factor in control of renal function under the above conditions of a water load and extracellular fluid volume expansion.

These results agree with previous work done in this area (reviewed in Ref. 12). However, there are a number of differences between our experimental preparation and others that have assessed the effects of NO synthesis inhibition on basal excretory function (14, 17, 21, 27). Our experiments examined the role of NO in water-loaded dogs. Furthermore, to accurately assess the contribution of NO during this water-loaded state, a servo-controlled sustained water diuresis preparation was used. Otherwise, a transient water diuretic response would have resulted from the water load and, under this condition, it would be difficult to define and separate out an effect of L-NAME. Thus a constant water diuresis was required as the control condition response, and this, in turn, required a servo-controlled hypotonic volume-replacement system.

Our results do not allow us to definitively state the mechanisms for L-NAME causing the fall in UV and C\textsubscript{H\textsubscript{2}O}. Plasma vasopressin levels remained low and were not increased by the L-NAME infusion. PRA and endothelin were also unchanged. L-NAME was associ-
ated with an increase in the urinary concentration of endothelin. However, the functional significance of this is unclear, because plasma endothelin was unaffected. GFR, as assessed by \( C_{\text{Cr}} \), was also unchanged during L-NAME infusion. Although others have shown an effect of NO synthesis inhibition to decrease glomerular filtration (12), this is probably related to the dose and duration of the inhibitor infusion and the nature of the experimental preparation. The dose employed in our studies was moderate because it caused only a 14-mmHg rise in blood pressure. This may have, however, reflected an increase in renal vascular resistance, a parameter that we did not measure. Others have shown effects of NO synthesis inhibition to decrease renal blood flow without affecting GFR (14, 21).

Our other series of experiments examined the effects of NO synthesis inhibition on the diuretic and natriuretic response to isotonic saline volume expansion. The results were also supportive of a possible important role for NO in mediating the renal effects of blood volume changes. Although other studies have also shown blunted excretory responses to volume loading in the presence of L-NAME (1, 13, 22, 26), all except the one study using monkeys (22) were performed in anesthetized animal preparations. Our results demonstrate that this effect of NO synthesis inhibition also occurs with volume expansion in conscious dogs. Moreover, our dogs were also water loaded with continual replacement of volume and sodium losses enabling us to determine the effects of L-NAME under sustained hypervolemic conditions.

Of additional interest with regard to this hypervolemia series is that, unlike the basal water diuretic series of experiments, L-arginine did not cause a complete reversal of the effects seen with L-NAME. There was almost complete reversal of L-NAME’s effects on blood pressure, HR, and the hormones measured but not its effects on UV and UNaV. The reason for this is not apparent but could, perhaps, be related to the dose of L-arginine used. It may not have been sufficient to provide enough excess NO substrate to effectively reverse all of the L-NAME excretory effects in this series, although it appears to have been adequate for complete reversal in the basal water-load series.

The mechanisms of L-NAME’s effects during volume expansion cannot be conclusively determined from the design of our experiments, although there were some group differences that are worth mentioning. For example, GFR (\( C_{\text{Cr}} \)) did increase with volume loading in the control experiments but not with L-NAME so these effects could be involved in the excretory differences. Similar to the basal water load series, blood pressure increased with L-NAME and actually increased further as the volume was infused. However, this should not cause blunted renal excretion but, presumably, an exaggerated response of a pressure diuresis/natriuresis instead, unless it reflects an increased renal vascular resistance, which is countering the direct effects of pressure on the kidney. PRA levels did not decrease with volume loading in the L-NAME experiments as they did in the control and L-NAME plus L-arginine experiments. Although the PRA levels were low to begin with, a recent report from this laboratory has shown that small changes in ANG II may have marked effects on renal UNaV and may be necessary for volume expansion natriuresis (3). However, as pointed out in the RESULTS section, these group differences may need to be interpreted cautiously because, despite there being no fall in PRA with volume expansion in the L-NAME group, there were no significant differences among the PRA levels in any of the three experimental conditions. With respect to endothelin, similar to the basal water diuresis series, urine endothelin concentration was elevated with L-NAME, but any connection between this and the renal excretory differences is difficult to explain when plasma endothelin levels and urinary endothelin excretion were unaffected.

Previous studies done with volume loading in anesthetized animals are of interest here as far as possible mechanisms for the effects of L-NAME. Atucha et al. (1) demonstrated that in rats the increase in renal papillary blood flow during saline loading is attenuated by L-NAME. Associated with this was less of a volume-induced increase in renal interstitial hydrostatic pressure, which has been shown by others to be linked to changes in UNaV (19, 20). Salazar et al. (26) and Krier and Romero (13) have also suggested that there is an interaction between NO and prostaglandins in mediating excretory responses to volume expansion in that the blunting of the response with L-NAME was accentuated with simultaneous prostaglandin synthesis inhibition. The former study (13) also showed that L-NAME caused less of a volume-induced increase in fractional excretion of lithium, an index of proximal tubular sodium reabsorption (30). This implies that NO may have effects on tubular transport in this nephron segment, although there is also evidence that it may affect sodium reabsorption in the collecting duct as well (29). Although it is not possible to definitively state that these possible mechanisms explain our findings in conscious water-loaded dogs, they are of interest and may be applicable.

**Perspectives**

The results of our study add further support to the experimental evidence implicating NO as an important substance with regard to various aspects of renal and cardiovascular function. Our findings have shown that an unimpeded NO system is required for appropriate renal excretory responses to a water load and isotonic saline volume load. This could be due to a permissive action of NO where a certain low rate of NO synthesis is necessary as a background for other physiological regulatory systems (e.g., humoral) to exert their effects either by increasing or decreasing their influence. On the other hand, NO could indeed be regulatory in nature and directly change renal water and salt excretion and thus work parallel with these other mechanisms. In any event, it appears that NO is a necessary factor in the above responses. Whether this has clinical
implications with respect to alterations in renal handling of water and sodium remains to be determined. It is of interest, however, that plasma concentrations of endogenous inhibitors of NO such as asymmetrical dimethyl-L-arginine are elevated in renal failure (31), implying that, in this condition, the NO system is operating at a depressed level of activity. The significance of this awaits further investigation, particularly to ascertain if defects in NO synthesis are a cause or a consequence of the associated renal dysfunction.

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