Salt-sensitive hypertension in ANP knockout mice: potential role of abnormal plasma renin activity


Salt-sensitive hypertension in ANP knockout mice: potential role of abnormal plasma renin activity. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R255–R261, 1998.—Atrial natriuretic peptide (ANP), a peptide hormone secreted by the heart, exerts a chronic hypotensive effect. Knockout mice with a homozygous disruption of the pro-ANP gene (−/−) are incapable of producing ANP and are hypertensive relative to their wild-type (+/+) siblings. Previous studies showed that arterial blood pressure (ABP) was further increased in conscious −/− mice kept for 2 wk on 2% salt, but not in anesthetized −/− mice after 1 wk on 8% salt. To determine whether inconsistencies in observed effects of salt on ABP of −/− mice are due to duration of increased salt intake and/or the state of consciousness of the animals, we measured ABP from an exteriorized carotid catheter during and after recovery from anesthesia with ketamine-xylazine in adult +/- and −/− mice kept on low (LS; 0.009% NaCl)- or high (HS; 8% NaCl)-salt diets for 3–4 wk. Conscious ABP ± SE (mmHg) of +/- mice did not differ significantly on either diet (HS, 113 ± 3; LS, 110 ± 5). However, on HS diet −/− mice had significantly higher ABP (135 ± 3; P < 0.001) than both −/− (115 ± 2) and +/- (110 ± 5) mice on LS diet. Anesthesia decreased ABP in all groups, but the genotype- and diet-related differences were preserved. Plasma renin activity (PRA, ng ANG I · ml−1 · h−1) in blood collected at termination of experiment was appropriately different on the 2 diets in +/- mice (HS, 4.9 ± 1.9; LS, 21 ± 2.8). However, PRA failed to decrease in −/− mice on HS diet (HS, 18 ± 2.9; LS, 19 ± 3.7). Independent of genotype, concentration of endothelin-1 (ET-1, pg/mg protein) and endothelial constitutive NOS (eNOS, density/100 µg protein) was significantly elevated in kidneys of mice fed on HS diet (ET-1 −/−, 31 ± 4.7 and +/-, 32 ± 4.1; eNOS −/−, 160 ± 19 and +/-, 156 ± 19) compared with mice fed on LS diet (ET-1 −/−, 19 ± 1.9 and +/-, 21 ± 1.8; eNOS −/−, 109 ± 13 and +/-, 112 ± 18). We conclude that, regardless of the state of alertness, −/− mice develop salt-sensitive hypertension after prolonged feeding on HS, in part due to their inability to reduce PRA, whereas the specific renal upregulation of eNOS and ET-1 in response to HS intake may be an ANP-independent adaptive adjustment aimed at improving kidney function and counteracting the pressor effect of salt.

ACUTE ADMINISTRATION of atrial natriuretic peptide (ANP) a peptide hormone secreted by the heart (4), lowers blood pressure (37) and stimulates diuresis and natriuresis by a combination of effects that include direct inhibition of sodium reabsorption in the medullary collecting duct (34), alterations in renal hemodynamics (36), and inhibition of renin (39) as well as aldosterone activities (22).

Recent work with genetic mouse models expressing alterations in ANP activity provides support for a physiological role of this hormone in chronic regulation of blood pressure. Transgenic mice characterized by lifelong 8- to 10-fold elevation in plasma ANP concentrations have markedly reduced arterial blood pressure (ABP) (35). These mice are capable of maintaining salt balance even when kept on reduced salt intake (38), suggesting that the chronic hypotensive effect of ANP is largely independent of its natriuretic and diuretic actions. In contrast, "knockout" mice, in which ANP production is prevented by targeted homozygous (−/−) disruption of the native gene (17), have higher blood pressures than the wild-type (+/+) siblings. Furthermore, knockout mice lacking the guanylate cyclase-A (GC-A) receptor (24), which is thought to mediate most of the biological actions of ANP (2), also are hypertensive with respect to their wild-type controls.

There is conflicting evidence whether ANP plays a functional role in the cardiovascular and renal adaptations to high salt intake. Plasma concentration of ANP increases in parallel with salt intake (31, 40), and high dietary salt content potentiates the vasorelaxant effect of ANP in the renal vasculature (1), suggesting that ANP may be involved in maintaining constancy of ABP during increased dietary salt intake. On the basis of these findings, it is expected that a decrease in endogenous ANP activity may predispose to development of sensitivity of ABP to increased dietary salt. Previous studies showed that ABP increased further in −/− mice kept for 2 wk on 2% salt (17), but not in anesthetized −/− mice after 1 wk on 8% salt (18). This discrepancy suggests that manifestation of salt sensitivity of ABP in
mice may be determined by the duration of increased salt intake and/or the state of alertness of the animals. On the other hand, GC-A receptor knockout mice fail to develop salt sensitivity of hypertension (24), indicating that a possible counteraction of the pressor effect of salt by ANP is not mediated by the GC-A receptor.

To resolve the inconsistencies in the observed effects of salt on ABP of −/− mice, we measured ABP during and after recovery from anesthesia in adult +/+ and −/− mice kept on low (LS; 0.008% NaCl)- or high (HS; 8% NaCl)-salt diets for 3–4 wk. On the basis of previously reported effects of ANP in counteracting the physiological activity of the renin-angiotensin-aldosterone system (RAAS) (19, 22, 39) and given that increases in local production of nitric oxide (NO) (25, 32, 33) and endothelin-1 (ET-1) (20) are considered essential for the chronic renal adaptation to high dietary salt intake, we measured plasma renin activity (PRA) and renal concentration of endothelial constitutive nitric oxide synthase (eNOS) and ET-1 to determine whether the potential sensitization of ABP to salt in −/− mice is associated with abnormal activation of the RAAS and/or failure to adequately upregulate renal synthesis of NO and ET-1.

METHODS

Animals. The production of ANP knockout mice has previously been described (15). In brief, a targeting construct was designed to replace 11 base pairs of exon 2 of the mouse proANP gene (Nppa) with the neomycin resistance gene in embryonic stem cells of mouse strain 129. Chimeras harboring the mutation were then mated to mice of strain C57BL/6J (B6). Matings between the resulting 129 × B6 heterozygotes (+/−) produced F2 offspring of all three genotypes (+/+, +/−, −/−) in Mendelian proportions.

F2 homozygous mutant (−/−) and wild-type (+/+) mice of both sexes, 20–24 wk old and weighing 20–35 g, were used in this study. The animals were obtained from our resident colony, which was founded with pathogen-free heterozygous (+/−) breeding pairs. The genotypes were identified by Southern blot analysis of EcoRI-digested genomic DNA from the tail (17) soon after weaning and were confirmed after the experiment. The animals were housed according to sex in groups of two to four per cage and kept at ambient 23°C and 40% humidity in a room with a 12:12-h light-dark schedule.

Dietary regimen. Two groups each of +/+ and −/− mice were maintained on a powdered Purina diet containing either low salt (0.008% NaCl; n = 10 +/+; n = 9 −/−) or high salt (8% NaCl; n = 9 +/+; n = 10 −/−) for 3–4 wk before beginning the study. Food and distilled drinking water were available ad libitum. Except for the sodium content, the LS and HS diets were of identical composition.

Blood sample collection. On the day of the experiment, the animals were anesthetized with 0.03–0.04 ml intramuscular injection of a 2:1 mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml) (Sigma Chemical, St. Louis, MO). A catheter fashioned from pulled-out PE-50 polyethylene tubing was tunneled subcutaneously to exit at the nape of the neck. The catheter was flushed with heparinized (20 U/ml) saline and secured in place with silk sutures. The beveled tip of the catheter (300–400 µm diameter) was then inserted into the previously dissected right common carotid artery and advanced to the junction at the aortic arch and firmly tied in position with silk sutures for measurement of blood pressure.

Blood pressure measurements. ABP was monitored continuously during the experiment using a small volume displacement pressure transducer (model RP 1500, Narco Systems) connected to a MacLab/4e data acquisition system. Measurements of blood pressure were taken at 30-min intervals. Two measurements were obtained while the mice were still under anesthesia. After these measurements, the mice were returned to their individual cages and allowed to recover under an open-bottomed box (12.5 cm × 10 cm × 5 cm) with a slot in the top for passage of the catheter. On average, recovery was complete within 3 h after induction of anesthesia. After recovery, an additional four measurements of blood pressure at 30 min apart were taken from the conscious mice.

Blood sample collection. At termination of the experiment, the mice were reanesthetized with an intraperitoneal injection of pentobarbital sodium and quickly exsanguinated. Blood was collected in chilled tubes containing 1 mg/ml EDTA and spun at 3,000 revolutions/min (rpm) in a centrifuge for 10 min at 4°C. Nonhemolized plasma samples were stored at −70°C until assayed for PRA.

PRA. PRA was measured in unextracted plasma with the RIAEN angiotensin I (ANG I) 125I radioimmunoassay kit (DuPont-NEN, Boston, MA), according to the instructions provided by the manufacturer. All reactions were prepared in an ice bath. Briefly, 500 µl of plasma were mixed with 5 µl of dimercaprol, 5 µl of 8-hydroxyquinoline, and 500 µl of maleate buffer. This mixture was then split equally into two separate aliquots. One aliquot was incubated at 37°C for 1 h. The other aliquot was kept on the ice bath (4°C) for the same length of time. At the end of incubation, the 37°C samples were transferred to the ice bath and paired with the corresponding 4°C samples. For the radioimmunoassay, 100 µl of each sample was incubated with 100 µl of 125I-ANG I tracer (12,000 counts/µl) and 100 µl of ANG I antiserum (rabbit) for 2 h at room temperature (≈23°C). The immunocomplexes were then precipitated by incubation with 500 µl of normal rabbit serum (secondary antibody) for 30 min at room temperature. The precipitates were centrifuged at 3,000 rpm for 4°C for 20 min. The supernatants were removed by aspiration, and the radioactivity of the pellets was counted. The concentration of ANG I (ng/ml) was obtained from a standard curve of ANG I in the range of 0.1–10 ng/ml. Only concentrations falling in the linear range of the curve (0.25–6.0 ng/ml) were used, and samples beyond this range were diluted with maleate buffer and reassayed. PRA (ng ANG I · ml−1 · h−1) was calculated by subtracting the concentration of ANG I in the 4°C sample from the concentration of ANG I in the corresponding 37°C sample after application of a correction factor for sample dilution.

Tissue concentration of ET-1 and eNOS. ET-1 and eNOS immunoreactivity was measured by radioimmunoassay (RIA) and Western blot, respectively (8), in supernatants cleared at 10,000 g from whole organ homogenates of kidney and heart. For the ET-1 RIA, 100 µl of sample was incubated with a rabbit polyclonal anti-ET-1 serum (Peninsula Labs, Belmont, CA) and 125I-ET-1 tracer (NEN-DuPont, Markham, ON, Canada) according to the instructions provided by the supplier. The sensitivity of the assay was 5.8 pg ET-1/100 µl. Cross-reactivity with big ET-1, ET-2, and ET-3 was 35, 7, and 7%, respectively. For eNOS Western blot, 100 µg of total protein extract was electrophoresed in 6% sodium dodecyl sulfate-polyacrylamide gels under reducing and denaturing conditions, and transferred to Hybond-C nitrocellulose membranes (Amersham, Oakville, ON, Canada) by electroblotting. The membranes were blocked overnight at 4°C with 4%
bovine serum albumin in tris(hydroxymethyl)aminomethane-buffered saline (0.1% Tween 20 (pH 7.5) and incubated with 1:2,000 dilution of mouse anti-ecNOS monoclonal antibody (Transduction Labs, Lexington, KY) for 1 h and horseradish peroxidase anti-mouse immunoglobulin G (1:5,000) for 2 h. The ecNOS signal was detected by enhanced chemiluminescence (Amersham) and quantified with NIH Image 1.52 (National Institutes of Health, Bethesda, MD). All values for ET-1 and ecNOS were normalized for total protein concentration in the sample, as determined by the Bradford method (Bio-Rad, Mississauga, ON, Canada).

Statistical analysis. All results are presented as means ± SE. The data were analyzed by two-way analysis of variance (ANOVA) to test for separate and combined effects of genotypes and diet on ABP and PRA. One-way ANOVA followed by Bonferroni multiple-comparison test was used to compare differences in ABP between groups at each timed measurement and between individual measurements in each experimental period (anesthesia, conscious) within the groups. Inasmuch as no differences were found in the individual measurements of ABP within the groups, the average ABP (mmHg) during the conscious period (Fig. 2) was used to compare genotype- and diet-related differences in means were further compared by one-way ANOVA. The effect of genotype and diet on PRA was also considered to indicate statistically significant differences.

RESULTS

The time course patterns of ABP during and after recovery from anesthesia are shown in Fig. 1. ABP was uniformly and significantly higher (P < 0.05) in −/− mice maintained on HS diet compared with −/− mice on LS diet and +/+ mice on either diet (P < 0.001, genotypes, P < 0.001, diets, 2-way ANOVA). The average ABP (mmHg) during the conscious period (Fig. 2A) did not differ significantly between +/+ mice on either diet (HS = 113 ± 9, LS = 110 ± 5). However, −/− mice on HS diet had significantly elevated ABP compared with −/− mice kept on LS diet (HS = 135 ± 3, LS = 115 ± 2) (P < 0.01) and +/+ mice on either diet (P < 0.01) (Fig. 2A). The ABP of −/− mice on LS diet did not significantly differ from that of +/+ mice on either diet. Anesthesia lowered ABP slightly, but not significantly in all groups (HS −/− = 134 ± 6, +/+ = 97 ± 7; LS −/− = 106 ± 5, +/+ = 100 ± 6) and did not affect the genotype- and diet-related differences in ABP observed in the conscious state (Fig. 2B).

PRA (ng ANG I·mil−1·h−1) for all groups is given in Fig. 3. On the LS diet, the +/+ and −/− mice had comparable and appropriately elevated PRA values (+/+ = 21.1 ± 2.8, −/− = 19.1 ± 3.7). On the HS diet, PRA decreased significantly (P < 0.05) in +/+ mice to 4.9 ± 1.9, as expected, but not in −/− mice (17.7 ± 2.9).
Indeed, there was no difference in PRA of the −/− mice over the 1,000-fold difference in dietary salt content. The concentrations of ET-1 and ecNOS in kidneys and hearts of +/+ and −/− mice are shown in Table 1. There were no statistically significant differences in ET-1 and ecNOS concentrations between +/+ and −/− mice on either diet. However, ET-1 and ecNOS concentrations were significantly increased (P < 0.05) in kidneys of both genotypes fed on HS diet.

**DISCUSSION**

The principal finding of this study is that mice rendered genetically incapable of synthesizing ANP develop sensitivity of arterial blood pressure to prolonged high (8% NaCl) dietary salt intake. Taken together with our previous observations that 1 wk of feeding on the same diet failed to exacerbate hypertension in this model (18), the present results indicate that development of salt-sensitive hypertension in ANP knockout mice is time dependent, with at least 1 wk of latency.

The hypertensive effect of salt in the ANP knockout mice is associated with a failure to downregulate PRA in response to the excessive salt intake (Fig. 3). This is suggested by the observation that +/+ mice responded to the HS diet with an appropriate decrease in PRA and remained normotensive for the duration of the dietary regimen. There is evidence that the chronic hypertensive effect of ANP in animals fed on high-salt diet is partly mediated by suppressing the activity of RAAS (19). ANP inhibits renin (39) and aldosterone secretion (22) and opposes ANG II-mediated vascular and renal effects (9, 12). These observations suggest a functional role of ANP as a physiological antagonist of the RAAS. Considering that plasma ANP increases adaptively with elevated salt intake (31, 40), the failure of RAAS to respond to changes in dietary salt content in the −/− mice may be caused, in part, by the absence of such antagonism.

The nature of the derangement in PRA in the −/− mice is not known. Dietary salt loading is accompanied by a compensatory increase in delivery of NaCl to the distal tubule, an adaptation that is potentiated by ANP (14). Because the inhibitory effect of ANP on renin release is mediated by increasing NaCl delivery to the macula densa (30), the initiating defect in renin release in the −/− mice may be the inability to increase distal NaCl delivery in the face of increased dietary intake. This would lead to inappropriate activation of the macula densa. The antinatriuresis that is triggered by the resultant increase in ANG II production (11) could then operate as a positive feedback signal to the macula densa, thereby overriding any direct inhibitory effect of ANG II on the juxtaglomerular cells and sustain the chronic activation of renin.

The derangement in PRA in the −/− mice could be implicated in sensitization of ABP to high salt through, at least, two nonmutually exclusive mechanisms. First, the persistent direct antinatriuretic effects of chronically elevated ANG II concentration may sensitize ABP to salt by causing excessive salt retention and expansion of the extracellular fluid volume (ECFV) (11). Exogenous ANG II at subpressor concentrations raises ABP when administered in combination with high dietary salt intake (21). This salt-sensitive hypertension is triggered by a sequential mechanism that is initiated by an increase in cardiac output, consequent to sodium retention and expansion of the ECFV, and later maintained by a sustained increase in total peripheral resistance (21). The elevation in ABP and renal perfusion pressure then increases salt excretion by the pressure natriuresis mechanism, thereby bringing salt balance and normalization of ECFV (21). Interestingly, the temporal increase in ECFV in ANG II-induced salt-sensitive hypertension is accompanied by a parallel increase in release of ANP, suggesting that

![Graph showing plasma renin activity (PRA) for ANG II production in ANP knockout mice.](http://ajpregu.physiology.org/)

**Table 1. Concentration of ET-1 (RIA) and ecNOS (Western blot) in kidneys and hearts of +/+ and −/− mice**

<table>
<thead>
<tr>
<th>GROUP</th>
<th>ET, pg/mg protein</th>
<th>ecNOS, density/100 µg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>Heart</td>
<td>Kidney</td>
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<tr>
<td>Low-salt diet</td>
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<tr>
<td>+/+ (n = 6)</td>
<td>21.4 ± 1.8*</td>
<td>8.2 ± 1.1</td>
</tr>
<tr>
<td>−/− (n = 6)</td>
<td>19.3 ± 1.9*</td>
<td>9.8 ± 2.1</td>
</tr>
<tr>
<td>High-salt diet</td>
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<td></td>
</tr>
<tr>
<td>+/+ (n = 6)</td>
<td>31.9 ± 4.1</td>
<td>8.7 ± 2.3</td>
</tr>
<tr>
<td>−/− (n = 6)</td>
<td>31.3 ± 4.7</td>
<td>8.0 ± 0.5</td>
</tr>
<tr>
<td>2-Way ANOVA</td>
<td></td>
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</tr>
<tr>
<td>P, genotypes</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>P, diets</td>
<td>0.0048</td>
<td>0.0143</td>
</tr>
</tbody>
</table>

Values are means ± SE. NS, not significant; +/+, −/−, normal, absent atrial natriuretic peptide (ANP) respectively; ET-1, endothelin-1; ecNOS, endothelial constitutive nitric oxide synthase. *#Statistical difference between diets (*P < 0.05, #P < 0.10 by unpaired t-test).
the escape from the salt-retaining effects of ANG II may, in part, be mediated by ANP (21). Indeed, ANP increases the sensitivity of the pressure-natriuresis relationship partly by inhibiting ANG II-stimulated proximal tubular sodium reabsorption (12, 29). Thus the absence of this ANP-mediated counteraction of ANG II effects in the −/− mice could result in a state of antinatriuresis. We have some indirect evidence that the −/− mice may have relative salt-retention when chronically maintained on high salt, because hematocrits of −/− mice are significantly reduced after 2 wk on high-salt diet compared with wild-type littermates (17) and sodium reabsorption is comparatively greater in −/− mice than in +/+ mice after 2 wk on 8% NaCl (U. Honrath and H. Sonnenberg, unpublished observations). We suggest that salt balance in −/− animals can only be maintained by counteracting the antinatriuretic effects of elevated ANG II levels by raising ABP and thus renal perfusion pressure.

In addition to the direct salt-retaining effects of ANG II, a potential contribution of aldosterone to salt sensitivity in the −/− mice cannot be discounted. Although the high dietary salt is expected to exert a direct suppressive effect on aldosterone synthesis (13) and to reduce the sensitivity of the adrenal glomerulosa to ANG II (13, 23), the tonic elevation in ANG II may increase adrenal output of aldosterone (23). The attendant secondary aldosteronism would then compound the direct antinatriuretic effects of ANG II. The extent to which this mechanism may be operative in the −/− mice is not known. ANP reduces aldosterone secretion both by directly inhibiting its synthetic pathway and by preventing the agonist effect of ANG II (22). Furthermore, the elevation in ANP release that occurs coincident with expansion of the ECF has been shown to be temporally (10, 43) and causally (41) implicated in mediating the escape from aldosterone action. Also, in experimental ANG II-induced salt-sensitive hypertension, the inhibition of aldosterone release is mirrored by an increase in ANP release (21). Because these counterregulatory effects of ANP are absent in the −/− mice, it is plausible to speculate that sensitization of ABP to salt in these mice may at least in part result from failure to appropriately overcome the antinatriuretic effect of aldosterone.

The absence of ANP-mediated counteraction of aldosterone activity could also partially account for the contradictory effects of high salt intake on ABP of ANP-deficient mice and GC-A knockout mice (17, 24). These two genetic models are expected to share some functional similarities, specifically in relationship to the biological actions of ANP that are mediated by the GC-A receptor. However, they differ with respect to the ANP effects that are mediated by non-GC-A receptors. Because ANP-dependent inhibition of aldosterone synthesis by the adrenal glomerulosa is not mediated by the GC-A receptor (8), the GC-A knockout mice would be expected to have normal plasma aldosterone and respond to changes in dietary salt intake with appropriate adjustments in adrenal output of aldosterone. This has indeed been confirmed (24). Thus the failure of GC-A knockout mice to develop salt sensitivity of ABP may be partially related to their ability to properly regulate aldosterone action by non-GC-A, ANP-dependent mechanism(s), whereas the absence of such mechanism(s) in the ANP-deficient mice could incapacitate regulation of aldosterone and sensitize ABP to elevated salt intake.

It has previously been shown that an increase in local production of (NO) (25, 32, 33) and ET-1 (20) is essential for the chronic renal adaptation to high dietary salt intake. This is likely related to the ability of these factors to promote natriuresis and diuresis by their dual actions on the renal vasculature (5, 26, 27) and tubular function (6, 28, 42). On the basis of these premises, we suggest that the genotype-independent increase in content of ecNOS and ET-1, specifically in the kidneys of both +/+ and −/− mice fed on HS diet (Table 1), may be an adaptive counterregulatory adjustment unrelated to endogenous ANP activity aimed at improving kidney function and counteracting the pressor effect of salt.

In conclusion, this study shows that ANP knockout mice develop sensitivity of ABP to increased dietary salt in a time-dependent fashion, in association with failure to downregulate PRA. We postulate that the sensitization of ABP to salt in the ANP knockout mice may partly be due to the inability to escape from the persistent, salt-retaining effect of an hyperactive RAAS and the consequent expansion of the extracellular volume.

Perspectives

Previous work from our laboratory has shown that mice overexpressing an ANP transgene have lifelong hypotension (38). These animals are capable of maintaining salt balance on a very low salt diet (0.008%) without evidence of salt depletion, suggesting independent actions of ANP on blood pressure and renal function. A similar conclusion may be reached based on results in the ANP-deficient mouse (18), because relative hypertension was observed after 1 wk of high-salt feeding (8%) without evidence of sodium accumulation. However, as the present study indicates, with time, the lack of ANP action, apparently via inability to properly regulate PRA, results in development of an additional component of salt-sensitive hypertension.

Whether deficiencies in endogenous ANP activity play a contributory role in hypertensive diseases, salt-sensitive variants of hypertension in particular, remains controversial. The present study provides evidence that chronic lack of ANP impairs the ability of regulatory system(s) to maintain constancy of ABP in the face of increased salt intake. Similar dependency on ANP has been observed in other salt-sensitive animal models (15, 16) and human populations (3). It is likely that sensitization of ABP to dietary salt develops as a consequence of physiologically inappropriate functional alterations in salt- and pressure-regulating mechanisms, among which a deficiency in ANP synthesis may play a contributory role.
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