

Salt Inactivates Endothelial Nitric Oxide Synthase in Endothelial Cells^{1,2}

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Abstract

There is a 1–4 mmol/L rise in plasma sodium concentrations in individuals with high salt intake and in patients with essential hypertension. In this study, we used 3 independent assays to determine whether such a small increase in sodium concentrations per se alters endothelial nitric oxide synthase (eNOS) function and contributes to hypertension. By directly measuring NOS activity in living bovine aortic endothelial cells, we demonstrated that a 5-mmol/L increase in salt concentration (from 137 to 142 mmol/L) caused a 25% decrease in NOS activity. Importantly, the decrease in NOS activity was in a salt concentration-dependent manner. The NOS activity was decreased by 25, 45, and 70%, with the increase of 5, 10, and 20 mmol/L of NaCl, respectively. Using Chinese hamster ovary cells stably expressing eNOS, we confirmed the inhibitory effects of salt on eNOS activity. The eNOS activity was unaffected in the presence of equal milliosmol of mannitol, which excludes an osmotic effect. Using an ex vivo aortic angiogenesis assay, we demonstrated that salt attenuated the nitric oxide (NO)-dependent proliferation of endothelial cells. By directly monitoring blood pressure changes in response to salt infusion, we found that in vivo infusion of salt induced an acute increase in blood pressure in a salt concentration-dependent manner. In conclusion, our findings demonstrated that eNOS is sensitive to changes in salt concentration. A 5-mmol/L rise in salt concentration, within the range observed in essential hypertension patients or in individuals with high salt intake, could significantly suppress eNOS activity. This salt-induced reduction in NO generation in endothelial cells may contribute to the development of hypertension. *J. Nutr.* 139: 447–451, 2009.

Introduction

The contribution of high salt intake to hypertension has been debated for decades, despite epidemiological, clinical, and experimental studies strongly indicating high salt intake as an independent risk factor for hypertension (1–3). One pertinent detail to this debate is that the molecular mechanisms of how salt induces high blood pressure are not fully understood. Studies in humans and animals indicate that high salt intake can cause a small (2–4 mmol/L) rise in plasma sodium (4). Moreover, there is evidence that those who develop high blood pressure have an underlying defect in the ability of the kidney to excrete salt and a slight increase in plasma sodium (1–3 mmol/L) has indeed been observed in individuals with hypertension (4,5). Whether such a small increase in plasma sodium per se contributes to the development of hypertension is not clear.

In addition to the kidneys and central nervous system, the vascular endothelium constitutes an important system to regu-

late blood pressure (6–9). Endothelial nitric oxide synthase (eNOS)⁶ catalyzes the generation of nitric oxide (NO) in endothelial cells. NO diffuses from the endothelium to vascular smooth muscle cells where it activates soluble guanylate cyclase, leading to the relaxation of the vascular smooth muscle cells and to vasodilatation (9). Administration of arginine increases NO production, which prevents salt-sensitive hypertension in salt-sensitive patients and rats (10–14), and inhibition of NOS increases blood pressure and produces salt sensitivity in salt-resistant animals (15–18). Mice deficient in eNOS exhibited increased blood pressure in response to high salt intake and had a 2.5-fold increase in salt sensitivity compared with wild-type controls (19), indicating that eNOS is an important regulator of salt sensitivity and NO generated by eNOS is required for protection against salt-induced hypertension.

Several lines of evidence suggest that high salt intake is related to impaired NO generation (20–23). For example, Fujiwara et al. (21) reported that high salt intake is negatively correlated with total nitrite and nitrate concentrations in human plasma. Bragulat et al. (22) reported that high salt intake promotes a

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⁶ Abbreviations used: BAEC, bovine aortic endothelial cell; CHO, Chinese hamster ovary cell; eNOS, endothelial nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester; MAP, mean arterial pressure; NO, nitric oxide.

significant decrease in urinary nitrate excretion and exhibits significantly lower maximal acetylcholine-induced vasodilation in salt-sensitive patients compared with salt-resistant persons. Nitrite and nitrate are metabolic products of NO (24). Although nitrite and nitrate are often used as an indicator of NO generation, the nitrite/nitrate assay has a number of limitations. It does not fully represent the generation of NO, because the nitrite/nitrate concentrations are not only determined by the generation of NO but are also affected by the metabolism of NO and the clearance of nitrite/nitrate; the assay cannot elucidate the acute effect of salt on NO generation, because the low sensitivity of the assay requires hours of treatment to accumulate measurable amount of nitrite/nitrate.

In this study, we took 3 independent approaches to test our hypothesis that a small rise in salt per se suppresses eNOS activity and contributes to the increase in blood pressure.

Methods

Materials. [^3H]L-arginine was from PerkinElmer Life and Analytical Sciences. Sodium chloride, mannitol, calcimycin (A23187), acetylcholine chloride, N^G-nitro-L-arginine methyl ester (L-NAME), DETA NONOate, and Dowex AG50WX-8–200 were from Sigma-Aldrich. Dulbecco's PBS containing 137 mmol/L of sodium was from Invitrogen (catalog no. 14040). Cell survival assay reagent WST-1 was from Roche Diagnostics. The anti-eNOS IgG was from Santa Cruz Biotechnology.

Animals. C57BL/6J mice were from Jackson Laboratories. Sprague Dawley rats were from Animal Center of Shandong University. The mice and rats were fed standard rodent diet containing 18% protein, 6% fat, 50% carbohydrate, and 0.23% sodium from Harland (no. 2918). Animal care and experiments were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

Cell culture. Primary bovine aortic endothelial cells (BAEC) were from Cell Applications. The cells were cultured in bovine endothelial cell growth medium. Chinese hamster ovary cells (CHO) stably expressing eNOS (CHO-eNOS) were generated as we described previously (25).

Living cell NOS activity assay. NOS activity was measured in living cells as previously described (26). Briefly, the cells were plated into 12-well plates at 50,000 cells per well and grown to 80–90% confluence. The cells were preincubated in serum-free medium for 16 h and in PBS for 2 h at 37°C. After the preincubation period, the medium was replaced with 400 μL of PBS containing 27.75 MBq/L [^3H]L-arginine in the presence of extra NaCl (5, 10, or 20 mmol/L) or equal milliosmol of mannitol and incubated at 37°C for 15 min. Then the reaction was terminated and the [^3H]L-citrulline converted from [^3H]L-arginine was quantified. Three wells were used for each treatment group. The data were from at least 3 independent experiments.

[^3H]L-arginine uptake assay. The cells were plated into 12-well plates at 50,000 cells per well and grown to 80–90% confluence. The cells were cultured in serum-free medium for 16 h and in PBS for 2 h at 37°C. The cells were incubated in PBS buffer containing [^3H]L-arginine in the presence of extra salt (5, 10, or 20 mmol/L) for 15 min at 37°C. The cells were quickly washed with ice-cold PBS 3 times. The cells were then lysed in 1 mmol/L NaOH overnight and the total [^3H] in cell lysate was counted with a liquid scintillation counter. Three wells were used for each treatment group. The data were from at least 3 independent experiments.

Cell viability assay. The cell survival assay reagent WST-1 was used to determine the cell viability following the manufacturer's instructions. The data were from at least 3 independent experiments.

Aortic ring angiogenesis assay. The growth of mouse aortic endothelial cells was performed as we previously described (25). Aorta were isolated from 10- to 12-wk-old C57BL/6J mice ($n = 4$ per group).

Rat blood pressure measurement. Experiments were performed on 3-mo-old male Sprague Dawley rats ($n = 3$). The rats were anesthetized with thiobutobarbital sodium (100 mg/kg, intraperitoneal). A polyethylene tube was inserted into the right femoral artery for the administration of salt solution and a pressure transducer was inserted into the left femoral artery for measuring mean arterial pressure (MAP). A total of 200 mL of PBS was infused over 3 s and MAP was recorded. Ten minutes later, 200 μL of high concentration of salt in PBS was infused to acutely increase the plasma salt concentration by 5 or 20 mmol/L based on a calculation that the blood volume is 7% of the body weight (27). Finally, 200 μL of equal milliosmol of mannitol in PBS was infused. MAP was measured with a pressure transducer (MP150, Biopac Systems).

Immunoblot. Cells or tissues were lysed at 4°C for 20 min in lysis buffer containing 25 mmol/L 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5, 137 mmol/L NaCl, 1% (v:v) Triton X-100, 60 mmol/L octyl glucoside, and 0.1% (wt:v) SDS. The immunoblot was performed as we previously described (26).

Statistical analysis. Significance in experiments comparing 2 groups was determined by 2-tailed Student's *t* test. Significance in experiments comparing more than 2 groups was evaluated by 1-way ANOVA followed by post hoc analysis using Tukey's test. Means were considered different at $P < 0.05$.

Results

We used living cell NOS activity assay to determine whether a small rise in salt concentrations per se has a direct effect on NOS activity in primary BAEC cells. The cells were incubated in PBS buffer containing 137, 142, 147, and 157 mmol/L of NaCl and the NOS activity was determined by quantifying the conversion of [^3H]L-arginine to [^3H]L-citrulline. A small increase in salt concentrations, as small as 5 mmol/L (from 137 to 142 mmol/L), caused a 25% decrease in NOS activity. The NOS activity was suppressed in a salt concentration-dependent manner. In the presence of 142, 147, and 157 mmol/L of NaCl, the NOS activity was decreased by 25, 45, and 70% compared with 137 mmol/L of NaCl, respectively (Fig. 1B). These data indicate that

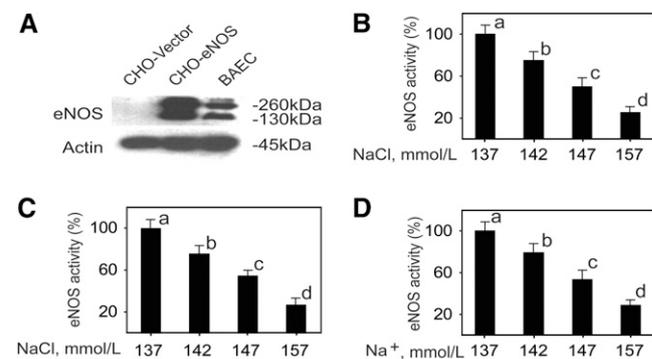


FIGURE 1 Effect of salt on eNOS activity in endothelial cells. (A) Western blot of eNOS. The expression of eNOS in CHO-eNOS or primary BAEC was detected by immunoblot using anti-eNOS IgG as primary antibody. (B,C) The effect of salt on eNOS activity. Primary BAEC (B) or CHO-eNOS (C) were incubated with [^3H]L-arginine in PBS buffer containing 137, 142, 147, and 157 mmol/L of NaCl and the eNOS activity were measured. In PBS buffer, the eNOS activity obtained was 16 ± 3 fmol of [^3H]L-citrulline per well in BAEC cells and 28 ± 4 fmol of [^3H]L-citrulline per well in CHO-eNOS cells. (D) The effect of sodium phosphate on eNOS activity. Primary BAEC were incubated with [^3H]L-arginine in PBS buffer containing 0, 5, 10, and 20 mmol/L of extra Na⁺ in the form of sodium phosphate and the eNOS activity were measured. The data are presented as a percentage of eNOS activity in PBS buffer. Values are means \pm SD, $n = 3$. Means without a common letter differ, $P < 0.05$.

the NOS activity is sensitive to changes in salt concentrations. In another set of experiments, the BAEC cells were incubated in PBS with extra salt for 2 h and then [^3H]L-arginine was added to measure the NOS activity. Similar inhibitory effects of salt on NOS were observed (data not shown). The effect of salt on eNOS activity was determined using CHO cells stably expressing eNOS. This CHO-eNOS system was widely used for the eNOS activity assay (26). Similar to BAEC cells, eNOS activity was significantly suppressed in a salt concentration-dependent manner in CHO-eNOS cells (Fig. 1C). We employed sodium phosphate to test whether sodium is the active agent. Similar to sodium chloride, an increase in sodium phosphate suppressed eNOS activity in a dose-dependent manner (Fig. 1D).

To exclude the possibility that the salt-induced reduction in eNOS activity is caused by an osmotic effect, we performed a NOS activity assay in the presence of equal milliosmol of mannitol. The eNOS activity was unaffected in the presence of 10, 20, and 40 mmol/L mannitol, which excludes the osmotic effect (data not shown). The availability of arginine is a rate-control step for NOS activation. Suppression of arginine uptake will result in decreased NO generation. We performed an arginine intracellular uptake assay to determine whether an increase in salt concentrations affects arginine uptake. The uptake of arginine was unaffected by the increase in salt concentrations (data not shown). It is important to point out that the uptake of arginine is a membrane transporter-mediated process (28). It requires a cationic amino acid transporter system y^+ . The fact that salt suppresses eNOS activity but does not affect the activity of the arginine transporter indicates that salt specifically suppresses eNOS activity but does not universally impair functions of the cell. We next determined whether the increase in salt concentrations affects the viability of cells. We treated the cells with salt for 15 min and the cell viability was determined by WST assay. Treatment of cells with salt did not affect the viability (data not shown). Furthermore, we treated the cells with salt for 15 min, washed out the salt with PBS, and then performed the eNOS activity assay in PBS. The eNOS activity was fully recovered after PBS wash, indicating that the acute inhibition of eNOS activity by salt is not caused by cell death (data not shown). It should be mentioned that even though we did not detect any salt-induced acute damage to the cells by the WST assay, it is possible that a long-term decrease in eNOS activity may cause endothelium dysfunction considering that NO generated by eNOS is required for maintaining the normal function of endothelial cells (24).

We used an ex vivo aortic angiogenesis assay to further determine the effect of salt on eNOS activity using aorta isolated from mice. The aortic angiogenesis assay measures the NO-dependent endothelial cell proliferation. This assay is widely used as an ex vivo method to determine the eNOS activity (29,30). We isolated aorta from C57BL/6J mice and cultured the aortic segments in matrigel in the presence of 5 or 20 mmol/L of extra NaCl. A large number of outgrowing endothelial cells were observed by d 8 when the aortic segments were cultured in normal concentrations of salt. However, when the aortic segments were cultured in the presence of extra salt, the outgrowth of endothelial cell was significantly attenuated. The outgrowing endothelial cells were significantly decreased in the presence of 5 mmol/L extra NaCl and almost completely inhibited in the presence of 20 mmol/L extra NaCl, compared with cells cultured in medium only (Fig. 2). To determine whether salt inhibits endothelial cell proliferation via suppressing NO generation, we treated aortic segments with 1 $\mu\text{mol/L}$ L-NAME. The outgrowth of cells was dramatically suppressed.

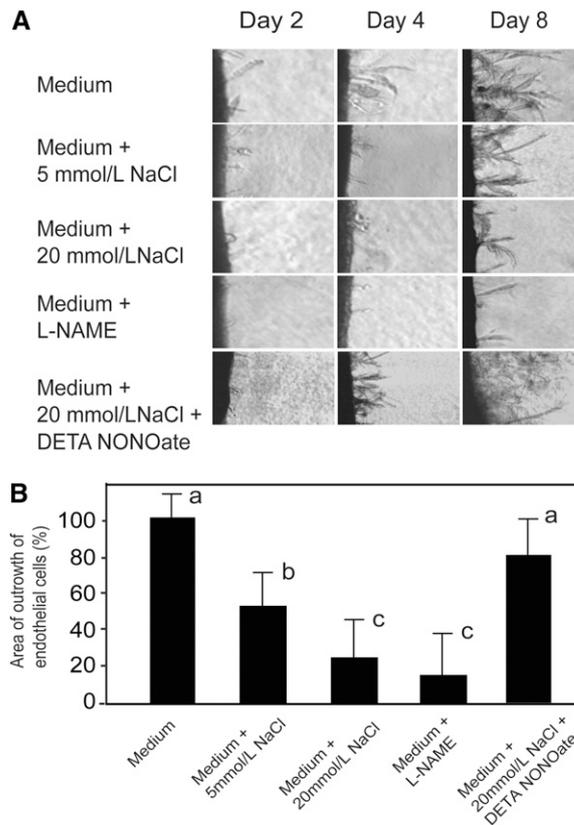


FIGURE 2 Effect of salt on endothelial cell proliferation in aorta from 10- to 12-wk-old C57BL/6J mice. The aorta were cut into 3- to 4-mm segments and cultured in matrigel for up to 8 d in complete medium in the presence/absence of 5 or 20 mmol/L of extra NaCl, 1 $\mu\text{mol/L}$ L-NAME, or 20 mmol/L of extra NaCl plus 0.5 $\mu\text{mol/L}$ of DETA NONOate. The growth of endothelial cells was monitored with a phase contrast microscope (A) and quantified on d 8 by measuring the area of outgrowing (B). The data are presented as a percentage of the area of outgrowing cells in complete medium. Values are means \pm SD, $n = 4$. Means without a common letter differ, $P < 0.05$.

We also added 0.5 $\mu\text{mol/L}$ DETA NONOate in the presence of 20 mmol/L of extra NaCl. DETA NONOate can slowly release NO. The presence of NO donor completely restored endothelial cell proliferation. These data indicate that salt inhibits endothelial cell proliferation via suppressing NO generation. We would like to point out that although the aortic angiogenesis assay has been widely used as an ex vivo method to determine eNOS activity, the assay is an indirect method to elucidate the effects of salt on eNOS. The data here support the conclusion obtained from the living cell NOS assay.

We then determined the effect of an acute rise in plasma salt concentrations on blood pressure by directly monitoring the changes in MAP in response to salt infusion. Infusion of salt to rats induced an acute and significant increase in MAP in a salt concentration-dependent manner (Fig. 3). Compared with infusion of PBS, infusion of 5 mmol/L salt increased the MAP from 102 ± 3 mm Hg to 115 ± 3 mm Hg ($P < 0.05$, vs. PBS) and infusion of 20 mmol/L salt increased the MAP to 135 ± 5 mm Hg ($P < 0.05$, vs. PBS). After salt infusion, the kidney-volume system rapidly rebalanced the plasma salt to normal concentrations so that there was only a temporary increase in blood pressure. Infusion of equal milliosmol of mannitol tended to increase MAP ($P = 0.11$ vs. PBS). Our data are consistent with Zerbe and Robertson's (31) report that infusion of salt to human volunteers induced an acute increase in MAP.

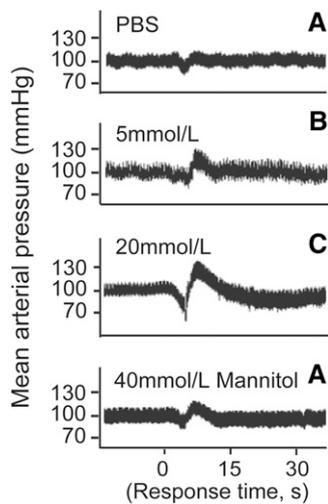


FIGURE 3 Effect of salt infusion on MAP. Sprague Dawley rats were infused with 200 μ L of high concentration of salt in PBS over 3 s to acutely increase the plasma salt concentration by 5 or 20 mmol/L. Infusion of equal volume of PBS or mannitol/PBS was used as control. MAP was measured with a pressure transducer. Values are means \pm SD, $n = 3$. Different letters in the upper right corner of panels indicate that MAP values differ, $P < 0.05$.

Discussion

eNOS in endothelial cells plays a primary role in regulating vascular tone (8). In this study, we assessed our hypothesis that a small rise in sodium concentrations, within the range in individuals with high salt intake or in essential hypertension patients, impairs eNOS activity and contributes to the development of hypertension. We used 3 independent assays to test our hypothesis. By directly measuring NOS activity in living endothelial cells, we demonstrated that NOS activity is sensitive to changes in salt concentration. A 5-mmol/L increase in salt concentrations (from 137 to 142 mmol/L) causes a 25% decrease in NOS activity. Because the reduction in NOS activity is in a salt concentration-dependent manner, we could expect that the small rise in sodium concentrations, observed in essential hypertension patients or in individuals with high salt intake, will impair NOS activity. Similar findings were obtained with CHO-eNOS cells, which confirms the inhibitory effects of salt on eNOS activity. Control experiments using equal milliosmol of mannitol revealed that the eNOS activity was unaffected by an increase in mannitol concentrations, which excludes the possibility that the suppression of eNOS activity by salt is caused by osmolality. Importantly, the transporter-dependent intracellular uptake of arginine was unaffected by salt, indicating that salt specifically suppresses eNOS activity but does not universally impair functions of the cells. Using an aortic angiogenesis assay, we demonstrated that the NO-dependent proliferation of endothelial cells was significantly attenuated by the increases in salt concentrations. By directly monitoring blood pressure changes in response to the acute rise in plasma salt concentrations, we found that *in vivo* infusion of salt induced an acute increase in blood pressure in a salt concentration-dependent manner.

Oberleithner et al. (23) recently reported that a 5- to 15-mmol/L increase in sodium concentration has a significant effect on the stiffness and deformability of endothelial cells. Using a nitrite assay, the authors showed that, at high concentrations (15

mmol/L), sodium reduces the generation of nitrite in cultured endothelial cells. Although Oberleithner et al. (23) provided convincing evidence demonstrating that a small increase in salt concentrations (5 mmol/L) affects the stiffness and deformability of endothelial cells, the authors did not provide evidence showing whether such a small increase in salt concentrations could reduce the generation of NO in cultured endothelial cells. In the current study, we utilized the living cell NOS activity assay to elucidate the effect of salt on NOS activity. NOS catalyzes the oxidation of arginine to generate an equal number of NO and citrulline molecules. In the living cell NOS activity assay, the generation of citrulline is quantified and used to determine the NOS activity so that this assay can directly measure NOS activity. With this sensitive assay, we were able to demonstrate that a small increase in salt concentrations (5 mmol/L) had a large effect on NOS activity.

Using recombinant eNOS, Schrammel et al. (32) reported that high salt (>200 mmol/L) suppresses eNOS activity. The authors further demonstrated that the effect of different ions followed the Hofmeister series, which suggests that salt inhibits eNOS activity by altering the status of eNOS solvation. Compared with our living cell eNOS assay, purified eNOS is much less sensitive to increased salt concentration. eNOS is a membrane protein, which prefers a nonpolar environment. It is not surprising that in living cells, eNOS is more sensitive to changes in salt concentrations and even a small increase in salt concentrations may have a profound effect on the status of eNOS solvation. Whether salt-induced changes in eNOS solvation are responsible for the reduction in eNOS activity *in vivo* remains to be clarified.

Changes in eNOS expression directly affect eNOS activity. We tested the acute effect of salt on eNOS expression in primary BAEC cells by immunoblot. Treatment of the primary BAEC cells with different amounts of salt for 15 min or 2 h failed to induce any change in eNOS expression (data not shown). We also determined the effect of salt on eNOS expression in rats. Acute infusion of high salt did not induce any change in eNOS expression in aorta 5 min following salt infusion (data not shown). Although we did not observe any acute effect of salt on eNOS expression in BAEC cells or in rats, a number of studies showed that long-term high salt intake affects eNOS expression, which depends on the duration of salt intake and the tissue distributions (18,33–40). For example, Mattson et al. (35) reported that high salt intake for 7 d induced significant increases in the expression of eNOS, inducible NOS, and neuronal NOS in the medulla of kidneys in rats but the expression of eNOS in the renal cortex and aorta remained unchanged. Herrera et al. (39) reported that high salt intake for 7 d increased eNOS expression in thick ascending limb by 125%. Ortiz et al. (37) reported similar findings. Paradoxically, despite the marked increase in eNOS expression, the generation of NO by eNOS was not increased, implying that the eNOS activity is impaired. Interestingly, Ni et al. (40) reported that high salt intake for a longer time (3 wk) induced significant downregulation of eNOS expression in the kidneys and aorta in rats. Combined with our findings, these data suggest that an elevation in salt concentrations suppresses eNOS activity, which induces compensative eNOS expression in endothelial cells. However, a long-term defect in NO generation due to impaired eNOS activation causes endothelial cell damage that results in the loss of eNOS expression. Indeed, Barton et al. (41) reported that long-term high salt intake caused a significant reduction in aortic relaxation in response to acetylcholine in salt-sensitive rats, which provides support to this hypothesis.

In summary, the results of this study provide direct evidence demonstrating that eNOS is sensitive to the rise in salt concentrations. A 5-mmol/L increase in salt concentration, within the range in individuals with high salt intake or in essential hypertension patients, could significantly suppress eNOS activity. This salt-induced reduction in NO generation in endothelial cells may contribute to the development of hypertension.

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