Role of prostaglandins in collecting duct-derived endothelin-1 regulation of blood pressure and water excretion

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Ge Y, Strait KA, Stricklett PK, Yang T, Kohan DE. Role of prostaglandins in collecting duct-derived endothelin-1 regulation of blood pressure and water excretion. Am J Physiol Renal Physiol 293: F1805–F1810, 2007. First published October 3, 2007; doi:10.1152/ajprenal.00307.2007.—Collecting duct (CD)-derived endothelin-1 (ET-1) exerts natriuretic, diuretic, and hypotensive effects. In vitro studies have implicated cyclooxygenase (COX) metabolites, and particularly PGE2, as important mediators of CD ET-1 effects. However, it is unknown whether PGE2 mediates CD-derived ET-1 actions in vivo. To test this, CD ET-1 knockout (KO) and control mice were studied. During normal salt and water intake, urinary PGE2 excretion was unexpectedly increased in CD ET-1 KO mice compared with controls. Salt loading markedly increased urinary PGE2 excretion in both groups of mice; however, the levels remained relatively higher in KO animals. Acutely isolated inner medullary collecting duct (IMCD) from KO mice also had increased PGE2 production. The increased IMCD PGE2 was COX-2 dependent, since NS-398 blocked all PGE2 production. However, increased CD ET-1 KO COX-2 protein or mRNA could not be detected in inner medulla or IMCD, respectively. Inner medullary COX-1 mRNA and protein levels and IMCD COX-1 mRNA levels were unaffected by Na intake or CD ET-1 KO. KO mice on a normal or high-Na diet had elevated blood pressure compared with controls; this difference was not altered by indomethacin or NS-398 treatment. However, indomethacin or NS-398 did increase urine osmolality and reduce urine volume in KO mice on a normal or high-Na diet. In summary, IMCD COX-2-dependent PGE2 production is increased in CD ET-1 KO mice, indicating that CD-derived ET-1 is not a primary regulator of IMCD PGE2. Furthermore, the increased PGE2 in CD ET-1 KO mice partly compensates for loss of ET-1 with respect to maintaining urinary water excretion, but not in blood pressure control.

cyclooxygenase; inner medulla; sodium

THE COLLECTING DUCT (CD) is the predominant site of endothelin-1 (ET-1) production and binding in the kidney (6). Recent studies have implicated CD-derived ET-1 in the regulation of systemic blood pressure and renal Na and water excretion. CD-specific knockout of ET-1 causes impaired ability to excrete a Na load, as well as salt-sensitive hypertension (1). CD-specific knockout of the ETB receptor also results in hypertension and reduced Na excretion after an acute salt load (5). In addition, CD-specific knockout of ET-1 causes reduced ability to excrete an acute water load associated with heightened sensitivity to the cAMP-elevating effects of vasopressin (AVP) (4). Thus CD-derived ET-1 functions as a natriuretic, diuretic, and hypotensive factor.

Perhaps the most extensive studies on mediation of collecting duct ET-1 actions have involved analysis of the role of PGE2. ET-1 stimulates PGE2 accumulation by acutely isolated rat inner medullary collecting duct (IMCD), an effect that is mediated by activation of the ETB receptor (7). Similarly, ET-1 increases PGE2 levels in acutely isolated rabbit IMCD (12). ET-1 inhibition of ouabain-sensitive 86Rb uptake in acutely isolated rabbit IMCD was abolished by pretreatment with ibuprofen; taken together with the above observations, this finding suggested that ET-1 inhibition of Na/K ATPase activity in IMCD is PGE2 dependent (12). In contrast, the role of PGE2 in mediating ET-1 inhibition of CD water transport is less evident. ET-1 inhibition of AVP-dependent cAMP accumulation in acutely isolated rat IMCD, in the presence of phosphodiesterase blockade, was shown to be unaffected by indomethacin pretreatment (7). Furthermore, ET-1 inhibition of AVP-stimulated osmotic water permeability (Pf) in isolated, perfused rat IMCD was not affected by prior exposure to indomethacin, whereas PGE2 inhibition of AVP-stimulated Pf was unaffected by pretreatment with ET-1 (8). Although not related to a direct CD effect, PGE2 has been shown to reduce ET-1-induced vasoconstriction of medullary descending vasa recta in rat, thereby potentially increasing medullary blood flow (9). Thus, taken together, these in vitro studies suggest that PGE2 may mediate, at least in part, ET-1 inhibition of CD Na transport, as well as potentially modulating ET-1 effects on neighboring cells in the medulla (e.g., vasa recta). However, there is no in vivo evidence for a role of PGE2, or other cyclooxygenase (COX) metabolites, in mediating CD ET-1 actions. Consequently, the current study was undertaken, using mice with CD-specific knockout of ET-1, to assess the in vivo relevance of PGE2 in mediating CD-derived ET-1 actions.

MATERIALS AND METHODS

Transgenic mouse lines. All experiments were performed with approval from the Institutional Animal Care and Use Committees at the University of Utah. CD ET-1 knockout (KO) and littermate control mice were generated utilizing the Cre/loxP system, as previously described (1, 5). Briefly, mice with exon 2 of the ET-1 gene flanked by loxP-sites (floxed) (provided by Dr. Masashi Yanagisawa, Howard Hughes Institute, University of Texas Southwestern Medical Center) were mated with AQP2-Cre mice, containing 11 kb of the mouse aquaporin-2 (AQP2) gene driving expression of Cre recombinase. Female AQP2-Cre mice were mated with male floxed ET-1 mice; female offspring heterozygous for both AQP2-Cre and floxed ET-1 were bred with males homozygous for floxed ET-1. Animals homozygous for floxed ET-1 and heterozygous for AQP2-Cre (CD ET-1 KO) were used in this study. Littersates that were homozygous for the floxed ET-1 gene, but without Cre, were used as control mice. Genotyping was performed as previously described (1). All mice were

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studied at 3–4 mo of age. All reagents used in studies were obtained from Sigma Chemical (St. Louis, MO) unless stated otherwise.

**Chronic telemetry studies.** Mice had catheters inserted into the right carotid artery, tunneled subcutaneously, and the attached radio transmitter was localized to the back. Continuous recording of blood pressure (BP) and pulse was performed using telemetry (Data Sciences International, Arden Hills, MN). Two days after the surgery, mice were placed into Hatteras metabolic cages (Cary, NC) and acclimated for 3 days. Mice were fed 10 ml of a normal Na (0.3%) gelled diet that contained all nutrients and water as previously described (1). Hemodynamic values were not recorded during this conditioning period. After the 3-day acclimation period, BP and pulse were determined for 2 consecutive days. Daily gel intake and body weights were measured. Systolic, diastolic, and mean BP, as well as pulse rate, were averaged over the course of each day. At the end of this baseline period, mice were given vehicle alone, NS-398 (15 mg·kg⁻¹·day⁻¹ ip), or indomethacin (5 mg·kg⁻¹·day⁻¹ ip) while remaining on the gelled normal Na intake. After 3 days of NS-398, indomethacin, or vehicle treatment on a normal Na diet, mice were placed on a high-Na diet for 7 days. The high-Na diet consisted of 10 ml of gelled diet containing 1% Na plus normal saline to drink. Daily weights and telemetry were obtained.

**In vivo water and solute excretion studies.** Mice were acclimated to metabolic cages and a normal Na gelled diet and then given 2 more days of a normal Na diet. Urine was collected on **day 2** of the normal Na diet. Mice were then given vehicle alone, NS-398 (15 mg·kg⁻¹·day⁻¹ ip), or indomethacin (5 mg·kg⁻¹·day⁻¹ ip) for 3 days while being maintained on the normal Na diet. Urine was collected on the third day of NS-398 or indomethacin treatment. Urine volume was measured and urine osmolality determined using an Osmett II (Precision Systems, Natick, MA).

**In vivo PGE2 and COX studies.** Mice were acclimated to metabolic cages and a normal Na gelled diet and then given 2 more days of a normal Na diet. Urine was collected on **day 2** of the normal Na diet. Mice were then given vehicle alone, NS-398 (15 mg·kg⁻¹·day⁻¹ ip), or indomethacin (5 mg·kg⁻¹·day⁻¹ ip) for 3 days while being maintained on the normal Na diet. Urine was collected on the third day of NS-398 or indomethacin treatment. Urine volume was measured and urine osmolality determined using an Osmett II (Precision Systems, Natick, MA).

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**In vitro PGE2 determination.** CD ET-1 KO and control mice were treated with 2 days of a normal Na diet followed by 3 days of a high-Na diet (diets as described above). Mice were killed at the end of the normal or high-Na diet periods. IMCD were acutely isolated in a manner similar to that previously described (7). Briefly, inner medulla were minced in Krebs buffer with 1 mg/ml collagenase (Invitrogen, Carlsbad, CA) and 0.1 mg/ml DNase and then incubated at 37°C for 30 min. IMCD fragments were washed, resuspended in HBSS, and preincubated in vehicle alone or 25 μM NS-398 for 30 min at 37°C. Cells were gently pelleted by spinning at 3,000 rpm in a table-top microfuge for 1 min, and the cell pellets were gently resuspended in HBSS with vehicle alone, 25 μM NS-398, or 10 μM indomethacin and placed back in a 37°C water bath for an additional 30 min. Cells were pelleted at 6,000 rpm for 1 min, aliquots of the supernatant were removed, and PGE2 was measured as described above. The cell pellet was used for determination of protein by the Bradford assay (Bio-Rad, Hercules, CA). PGE2 levels were normalized to total cell protein.

**Western analysis.** Renal inner medulla from normal or high-Na diet CD ET-1 KO and control mice were homogenized in 50 ml Tris·Cl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitor cocktail tablets (catalog no. 1197498001; Roche Diagnostics, Indianapolis, IN). Samples were centrifuged at 4,000 g, and the supernatant was centrifuged at 17,000 g for 30 min. Protein concentration was determined using Coomassie

![Fig. 1. Urinary PGE2 excretion in control and collecting duct (CD) endothelin-1 (ET-1) knockout (KO) mice. Values are shown after 2 days on a normal Na diet and after 3 days on a high-Na diet (n = 6 for each data point). *P < 0.025 vs. control animals on a similar Na diet. #P < 0.001 vs. same genotype on a normal Na diet.](image_url)

**Table 1. Western blot and real-time PCR analysis of COX-1 and COX-2 protein and mRNA expression in inner medulla from control and CD ET-1 KO mice.**

<table>
<thead>
<tr>
<th>Condition</th>
<th>COX-1</th>
<th>COX-2</th>
</tr>
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<tbody>
<tr>
<td>Normal Na</td>
<td>1.15±0.14</td>
<td>1.03±0.09</td>
</tr>
<tr>
<td>High Na</td>
<td>1.37±0.24</td>
<td>1.03±0.05</td>
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Data for cyclooxygenase (COX)-1 and COX-2 protein and mRNA expression in inner medulla from control and collecting duct (CD) ET-1 knockout (KO) mice are normalized to β-actin (Western blot) or GAPDH (PCR) and expressed in relative densitometry (Western blot) or relative fluorescence units (PCR); n = 6 for each data point. *P < 0.01 vs. COX-2 values in mice with the same genotype on a normal Na diet.

![Fig. 2. PGE2 production by inner medullary collecting duct (IMCD) acutely isolated from control and CD ET-1 KO mice previously treated with 2 days of a normal Na diet or 3 days of a high-Na diet. Cells were treated for 30 min with vehicle alone, a cyclooxygenase-2 (COX-2)-specific inhibitor (NS-398; 25 μM), or indomethacin (10 μM; Indo), followed by measurement of PGE2 release over the next 30 min (n = 12 for each data point). *P < 0.025 vs. control animals on a similar Na diet. #P < 0.001 vs. same genotype on a normal Na diet. $P < 0.001 vs. vehicle alone in same genotype and on same Na diet.](image_url)
reagent. Samples were solubilized in Laemmli buffer containing 0.5% lithium dodecyl sulfate and run on a denaturing NUPAGE 4–12% Bis-Tris mini-gel using the MOPS buffer system (Invitrogen). Proteins were transferred to polyvinylidene difluoride plus nylon membranes by electroelution. Blots were incubated overnight at 4°C with primary antibody and then incubated with 1:2,000 horseradish peroxidase-conjugated donkey anti-rabbit IgG for 1 h (GE Healthcare, Piscataway, NJ). Antibody binding was visualized using the enhanced chemiluminescence system (Amersham International, Little Chalfont, UK). Primary antibodies utilized were 1:5,000 rabbit anti-mouse COX-1, 1:5,000 rabbit anti-mouse COX-2 (catalog nos. 160109 and 1601269, respectively; both from Cayman Chemicals), and 1:10,000 rabbit anti-human β-actin (Abcam, Cambridge, MA). All blots were reprobed for β-actin.

Real-time PCR. IMCD from CD ET-1 KO and control mice were isolated as described above. Total RNA was prepared using RNeasy minicolumns with on-column DNase I treatment (Qiagen, Valencia, CA). Samples were reverse transcribed, and real-time PCR was performed using a SmartCycler (Cephid, Sunnyvale, CA). Primer sequences for mouse COX-1 cDNA were forward, 5′-CAC TGG TGG ATG CCT TCT CT-3′ and reverse, 5′-CCG TAC AGC TCC TCC AAC TC-3′, which yielded a product size of 226 bp and amplified between bp 1405 and 1631 (GenBank NM_008969). Primer sequences for COX-2 cDNA were forward, 5′-CCC TGA AGC CGT ACA CAT CA-3′ and reverse, 5′-TGT CAC TGT AGA GGG CTT TCA ATT-3′, which yielded a product size of 80 bp and amplified between bp 1470 and 1550 (GenBank NM_011198). The primers for GAPDH were forward, 5′-TGG CCT CCA AGG AGT AAG AA-3′ and reverse, 5′-CTG GGA TGG AAA TTG TGA GG-3′, which yielded a product size of 110 bp. The ratio of COX-1 or COX-2 to GAPDH cDNA was determined for each sample.

Cyclic AMP studies. CD ET-1 KO and control mice were given 2 days of a normal Na diet and then killed. IMCD were prepared as described above; all incubations were done in Krebs buffer. IMCD were preincubated with vehicle alone, 10 μM indomethacin, or 25 μM NS-398 as described above, rinsed, and then incubated with 10 nM AVP with vehicle alone or with COX inhibitors at 37°C. Cells were extracted with ethanol, and cAMP was measured using ELISA (Assay Design, Ann Arbor, MI) using a SpectraMax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Total cell protein was measured using the Bradford assay. Cyclic AMP levels were normalized to total cell protein.

Statistics. Comparisons between floxed ET-1 and CD ET-1 KO mice were analyzed using one-way analysis of variance with Bonferroni correction. P < 0.05 was considered significant. Data are means ± SE.

RESULTS

CD ET-1 KO and control mice were analyzed at 3–4 mo of age. There were no differences between the two groups with respect to body weight or sex distribution (males females within groups). On a normal Na diet, urinary PGE2 excretion was significantly elevated in CD ET-1 KO animals compared with controls (Fig. 1). In mice placed on a high-Na diet for 3 days, urinary PGE2 excretion markedly increased in both

Fig. 3. Urine osmolality (A and B) and volume (C and D) in control and CD ET-1 KO mice on a normal Na diet and treated for 3 days with vehicle alone, Indo (5 mg·kg⁻¹·day⁻¹ ip; A and C) or NS-398 (10 mg·kg⁻¹·day⁻¹ ip; B and D) (n = 6 for each data point). *P < 0.01 vs. control treated with same COX inhibitor and vs. CD ET-1 KO treated with vehicle alone. **P < 0.025 vs. control treated with same COX inhibitor and vs. CD ET-1 KO treated with vehicle alone.
groups compared with a normal Na diet. However, in mice on a high-Na diet, urinary PGE2 excretion remained greater in CD ET-1 KO mice compared with controls. The 3-day high-Na diet time period was based on the time at which maximal Na retention was observed in CD ET-1 KO mice after the change to a high Na intake (1). To determine the mechanism by which urinary PGE2 is elevated in CD ET-1 KO mice compared with controls, we determined inner medullary COX-1 and COX-2 protein levels (Table 1). COX-1 protein expression was not detectably different between CD ET-1 KO and control mice maintained on a normal Na diet; furthermore, COX-1 protein expression was unchanged in either group by 3 days of a high-Na diet. COX-2 protein expression was also not different between CD ET-1 KO and control mice on a normal Na diet; 3 days of high Na intake increased COX-2 protein levels, but these were not different between CD ET-1 KO and control animals. Since failure to detect a difference in inner medullary COX protein expression could relate to the presence of non-CD cells, acutely isolated IMCD were studied. Despite combining inner medulla from five mice, COX-1 and COX-2 protein could not be detected in isolated IMCD. Consequently, COX-1 and COX-2 mRNA steady-state levels were determined in acutely isolated IMCD (Table 1). No differences in IMCD COX-1 mRNA levels were detected between CD ET-1 KO and control mice on a normal or high-Na diet; similarly, IMCD COX-2 mRNA levels were not different between CD ET-1 KO and control mice on either Na intake. Since mRNA levels do not necessarily reflect protein expression or enzyme activity, production of PGE2 by acutely isolated IMCD and the effect of specific COX isoform inhibition were assessed. PGE2 production was significantly increased in IMCD from CD ET-1 KO mice compared with controls when both groups had been maintained on a normal Na diet (Fig. 2). Similar to the urinary PGE2 excretion data, high Na intake increased IMCD PGE2 production (compared with a normal Na diet) in IMCD from both groups of animals. NS-398, a specific COX-2 inhibitor, abolished PGE2 production by IMCD from both groups to an extent comparable to that seen with indomethacin, thereby strongly supporting the notion that the elevated PGE2 production in CDs from CD ET-1 KO mice is COX-2 dependent.

Since PGE2 has been shown to antagonize the hydroosmotic effects of AVP, the effect of blocking cyclooxygenase activity, by using either nonspecific COX blockade or specific COX-2 inhibition, on urine osmolality and volume was assessed (Fig. 3). CD ET-1 KO and control mice had similar urine osmolality and volume on the normal Na gelled diet (fluid and solute intake precisely matched, since they consumed all the gel and were given no other food or water). After administration of either NS-398 or indomethacin, urine volume fell and urine osmolality increased in CD ET-1 KO mice. However, neither NS-398 nor indomethacin altered urine volume or osmolality in control animals. To determine whether the COX inhibitor-
impaired water excretion in CD ET-1 KO mice compared with controls was due to altered AVP-induced cAMP accumulation, we studied acutely isolated IMCD from animals on a normal Na diet. AVP increased IMCD cAMP accumulation to a greater extent in CD ET-1 KO IMCD than in control mice (Fig. 4). NS-398 or indomethacin, both of which abolished all PGE2 production (see above), markedly increased cAMP levels in IMCD from both groups of mice and to a comparable extent. However, AVP responsiveness remained greater in CD ET-1 KO mice than in controls. Notably, these studies were done in the absence of phosphodiesterase inhibition, thereby allowing any potential alteration in cAMP formation or degradation to be detected.

Since PGE2 can exert natriuretic and antihypertensive effects, the effect of blocking COX activity, using either non-specific COX blockade or COX-2-specific inhibition, on systemic blood pressure was determined (Fig. 5). Animals were maintained on the gelled diets to ensure comparable intakes. On a normal Na diet, systolic BP was ~13 mmHg greater in CD ET-1 KO compared with control mice. Indomethacin increased systolic BP in the two groups of mice and to a comparable degree. Similarly, NS-398 increased systolic BP in both groups of a mice by comparable amounts. Administration of a high-Na diet increased BP in CD ET-1 KO mice but not in control mice, as has been previously reported (1).

DISCUSSION

Based on the finding that ET-1 increases CD PGE2 production and that PGE2 can exert natriuretic and diuretic effects, it was expected that CD-specific knockout of ET-1 would result in relative CD PGE2 deficiency and that such deficiency would explain, at least in part, the hypertension and impaired water excretion in CD ET-1 KO animals. However, this proved to not be the case. Urinary PGE2 excretion and IMCD PGE2 production were unexpectedly increased in CD ET-1 KO mice. This finding indicates that CD-derived ET-1 is not the primary factor driving CD PGE2 production. In fact, the absence of CD-derived ET-1 apparently results in a compensatory increase in CD PGE2 production. Furthermore, this compensation seems to be mainly in response to ET-1 deficiency-induced impaired water excretion but not to CD-derived ET-1-dependent BP control.

Although changes in COX-1 and COX-2 protein and mRNA expression in CD ET-1 KO mice could not be detected, the current study suggests that the apparent compensatory elevation in IMCD PGE2 production in CD ET-1 KO mice is COX-2 dependent. This conclusion is supported by the findings that NS-398 caused similar increases in IMCD PGE2 production and AVP-induced cAMP accumulation compared with indomethacin, whereas NS-398 reduced water excretion to an extent comparable to indomethacin (see below). The biological significance of COX-2 in the CD is uncertain. COX-2 mRNA has been localized to mouse IMCD, although COX-1 mRNA levels are relatively greater (11). In addition, acutely isolated rat IMCD PGE2 production was more extensively inhibited by COX-2 than COX-1 blockade (10). COX-2 regulation of CD water transport has not been extensively investigated. In one study, bilateral ureteral obstruction increased COX-2 and decreased AQP2 protein expression in rat inner medulla; administration of NS-398 ameliorated the fall in AQP2 levels (3). Taken together, these studies support the notion that COX-2 may regulate water transport and that elevated COX-2-derived PGE2 may help counterbalance the antidiuretic effect associated with ET-1 deficiency in the CD. These studies do not prove that increased COX-2 per se is responsible for the compensatory elevation in PGE2 seen in CD ET-1 KO mice (changes in PGE2 synthase or prostaglandin receptors could be involved); rather, these studies show that the increase in PGE2 is through pathways that depend on COX-2.

The mechanisms by which increased CD PGE2 might ameliorate the relative antidiuretic effect of CD ET-1 KO are speculative. Our group has previously reported that AVP-induced cAMP accumulation, in the presence of phosphodiesterase inhibition, is augmented in IMCD from CD ET-1 KO animals (4). The current study demonstrates that such increased AVP responsiveness is not abolished by complete blockade of PGE2 production. Notably, blockade of PGE2 production markedly increased cAMP levels, confirming that PGE2 can reduce cAMP content in IMCD. Furthermore, these studies were done, for the first time, in the absence of phosphodiesterase inhibition, to help exclude a potential effect on AVP phosphodiesterase activity. Taken together, the current data suggest that the elevated IMCD PGE2 content in CD ET-1 KO mice likely impacts AVP-dependent water reabsorption at a point beyond regulation of cAMP levels. Such a finding is consistent with previous studies demonstrating that PGE2 may reduce cell surface AQP2 expression by stimulating retrieval from the plasma membrane (13).

PGE2, particularly in the renal medulla, has been shown to regulate BP; medullary infusions of COX-1 or COX-2 inhibitors in rats increase systemic BP (11). It is interesting, therefore, that inhibition of PGE2 formation, whether nonspecifically with indomethacin or through COX-2 inhibition, while raising BP in both groups of mice, did not result in a greater elevation in BP in CD ET-1 KO mice compared with controls. The reasons for this are speculative, however, one possibility is that the regulation of BP is more dependent on medullary interstitial cell, as opposed to CD, PGE2 production. Although it was not possible to examine, the increase in PGE2 levels seen in CD ET-1 KO mice may conceivably reflect CD, but not interstitial cell, PGE2 production. Interstitial cells produce and express relatively large amounts of PGE2 and COX isoenzymes, respectively (2), and have been implicated in control of systemic BP (reviewed in Ref. 14). Clearly, this is an area in need of further investigation.

In summary, although ET-1 can stimulate IMCD PGE2 production, the current study demonstrates that CD-derived ET-1 is not the primary determinant of CD PGE2 synthesis. Furthermore, elevated COX-2-dependent CD PGE2 production apparently partially compensates for the antidiuretic effect of CD ET-1 KO. This apparent compensation is likely mediated by a PGE2 effect distal to AVP-stimulated cAMP accumulation. Thus the diuretic, natriuretic, and hypotensive effects of CD-derived ET-1 are mediated by other pathways.

GRANTS

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