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The role of nitric oxide in bradycardia of rats with obstructive cholestasis

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Abstract

Nitric oxide (NO) has an important role in controlling heart rate and contributes to the cholinergic antagonism of the positive chronotropic response to adrenergic stimulation. Based on evidence of NO overproduction in cholestasis and also on the existence of bradycardia in cholestatic subjects, this study aimed to evaluate the chronotropic effect of epinephrine in isolated atria of cholestatic rats and determine whether alterations in epinephrine-induced chronotropic responses of cholestatic rats are corrected after systemic inhibition of NO synthase (NOS) with N^G-nitro-L-arginine (L-NNA). Male Sprague–Dawley rats were used. Cholestasis was induced by surgical ligation of the bile duct under general anesthesia and sham-operated animals were considered as control. The animals were divided into three groups, which received either L-arginine (200 mg/kg/day), L-NNA (10 mg/kg/day) or saline. One week after the operation, a lead II ECG was recorded from the animals, then spontaneously beating atria were isolated and chronotropic responses to epinephrine were evaluated in a standard oxygenated organ bath. The results showed that plasma γ-glutamyl transpeptidase and alanine aminotransferase activity was increased by bile-duct ligation, and that L-aginine treatment partially, but significantly, prevented the elevation of these markers of liver damage. The results showed that heart rate of cholestatic animals was significantly less than that of sham-operated control rats in vivo and this bradycardia was corrected with daily administration of L-NNA. The basal spontaneous beating rate of atria in cholestatic animals was not significantly different from that of sham-operated rats in vitro. Meanwhile, cholestasis induced a significant decrease in chronotropic effect of epinephrine. These effects were corrected by daily administration of L-NNA. Surprisingly L-arginine was as effective as L-NNA and increased the chronotropic effect of epinephrine in cholestatic rats but not in sham-operated animals. Systemic NOS inhibition corrected the decreased chronotropic response to adrenergic stimulation in cholestatic rats, and suggests an important role for NO in the pathophysiology of heart rate complications in cholestatic subjects. The opposite effect of chronic L-arginine administration in cholestasis and in control rats could be explained theoretically by an amelioration of cholestasis-induced liver damage by chronic L-arginine administration in bile duct-ligated rats.

Keywords: Cholestasis; Nitric oxide (NO); Chronotropism; Bradycardia; Epinephrine; Isolated atrium, rat

1. Introduction

Sinus bradycardia bas been linked to cholestatic liver disease among other systemic, metabolic and central nervous conditions (Bashour et al., 1985; Kluger et al., 1995). Accumulation of bile acids in plasma of cholestatic subjects was traditionally used as explanation of the bradycardia in cholestasis (Song et al., 1983). Involvement of the parasympathetic system was also supposed to have a role in this condition because intravenous atropine temporarily abolished sinus pauses in severe sinus node dysfunction in obstructive cholestasis (Bashour et al., 1985). Furthermore, attenuation of β-adrenergic inotropic responses has been shown in jaundiced patients as well as in animal models of cholestasis (Binah et al., 1985; Lumlertgul et al., 1991) but

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the precise mechanism(s) of these cholestasis-induced cardiac complications is not understood.

The mRNA of endothelial type nitric oxide synthase (NOS) has been shown to be expressed abundantly in atria as well as ventricular cardiomyocytes (Seki et al., 1996), supporting the role of nitric oxide (NO) in the regulation of cardiac function. Han et al. (1994) found that inhibition of NOS could block the carbachol-induced inhibition of L-type Ca^{2+} current that had been augmented by β-aderenoceptor stimulation in isolated spontaneously beating sino-atrial node cells (Han et al., 1994). Thus, it has been suggested that a NO-mediated signaling pathway might be involved in the cholinergic inhibition of cardiac function (Balligand et al., 1993). Based on evidence of NO overproduction in cholestasis (Dehpour et al., 1998; Nahavandi et al., 1999a; Sadr et al., 1999), we have shown that chronic inhibition of NOS with N^G-nitro-L-arginine could correct the decreased inotropic response of cholestatic heart to adrenergic stimulation (Nahavandi et al., 1999b). The present study was carried out to evaluate the chronotropic effect of epinephrine in isolated atria of cholestatic rats and to determine whether alterations in epinephrine-induced chronotropic responses of cholestatic rats are corrected after systemic inhibition of NO synthesis.

2. Methods

2.1. Animal manipulation

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by US National Institute of Health (NIH Publication No. 85-23, revised 1985). Male Sprague–Dawley rats weighing 200–250 g were used in the experiments. All animals were given free access to food and water. Laparotomy was performed under general anesthesia induced by an intraperitoneal injection of ketamine HCl (Gedoon Richter, Hungary), 50 mg/kg and xylazine HCl (Bayer, Germany), 10 mg/kg. The bile duct was isolated and double-ligated as previously described (Dehpour et al., 1999). Sham-operated age-matched rats served as controls. Sham operation consisted of laparotomy and bile duct identification and manipulation without ligation.

2.2. Drug administration

The animals were divided into three experimental groups, which received either 200 mg/kg/day of L-arginine (Fluka, Switzerland), 10 mg/kg/day of L-NNA (Fluka) or an equivalent volume of saline subcutaneously 1 day after the operation (Nahavandi et al., 1999b) for 6 days. One week after the operations, the rats were anesthetized with 50 mg/kg of sodium pentobarbital (Merck, Germany) and the heart of each animal was removed. Blood collection was also done for the measurement of plasma enzyme activity and determination of plasma concentration of $NO_2^- + NO_3^-$ and L-nitro-tyrosine.

2.3. Isolated whole atrium study

The auricles were dissected out from isolated hearts in oxygenated modified Krebs solution and suspended vertically under isometric conditions under 500 mg tension in a 50-ml glass chamber. The temperature of the bathing solution was 37°C and pH was 7.4. The composition of modified Krebs solution in mM was as follows: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.6; $MgCl_2$, 1.2; NaH_2PO_4, 1; $NaHCO_3$, 25; Glucose, 11.1; EDTA, 0.004; and ascorbic acid, 0.11 (Dehpour et al., 1995). The solution was oxygenated with a gas mixture of 97% O_2 and 3% CO_2. Isometric spontaneous contractions were recorded with an isometric transducer and displayed on a DMR-4B Physiograph (Narco Biosystem). To avoid artifacts evoked by dissection, an equilibration period of 30 min was allowed before evaluation of the chronotropic response of isolated atrium to epinephrine. The basal spontaneous rate of each atrium was recorded and then epinephrine (Fluka) was added to the organ chamber in concentrations from 1 to 1000 nM.

2.4. In vivo measurement of heart rate

Another series of rats was operated as described above. One week after the operation, the animals were anesthetized with ketamine (Sikuler et al., 1991) and a lead II electrocardiogram (ECG) was recorded for 15 min using a cardiac coupler connected to a DMR-4B Physiograph (Narco Biosystem).

2.5. Plasma enzyme activities

Plasma was obtained for determination of γ-glutamyl transpeptidase and alanine aminotransferase as markers of liver damage (kits from Zist-Shimi, Iran).

2.6. $NO_2^- + NO_3^-$ determination in plasma

Plasma $NO_2^- + NO_3^-$ levels of 24-h fasted animals were determined using the Griess reaction (Green et al., 1982). Briefly, after passing the samples through a copper plated cadmium column for nitrate reduction, nitrite was measured by the absorbance at 540 nm after mixing with a reagent consisting of 0.2% naphthylethylendiamine dihy-

drochloride, 0.4% procaine in 6% trichloreacetic acid (Griess reagent, Sigma, USA). The value obtained expressed the total amount of plasma NO end products, namely $NO_2^- + NO_3^-$.

2.7. L-nitro-tyrosine determination in plasma

L-nitro-tyrosine was determined in plasma as a marker of exposure to reactive nitrogen species (mainly peroxynitrite). It was measured using its characteristic spectral shift in alkaline solution (Herce-Pagliai et al., 1998). The pK_a of the phenolic group in L-nitro-tyrosine is 7.5, which is considerably lower than that for L-tyrosine. Both compounds have an absorbance maximum at 280 nm (pH = 3.5), but L-nitro-tyrosine has an additional secondary peak situated at 430 nm under basic conditions (pH = 9.5). Thus, L-nitro-tyrosine concentration was calculated from the increased absorbance at 430 nm.

2.8. Statistical analysis

All data are presented as the means ± S.E.M. Statistical evaluation of the data was done with the analysis of variance (ANOVA) followed by the Newman–Keuls test for multiple comparisons, and a P value less than 0.05 was considered statistically significant.

3. Results

One day after laparotomy, bile duct-ligated rats showed manifestations of cholestasis (jaundice, dark urine and steatorrhea). After the animals were killed, plasma γ-glutamyl transpeptidase and alanine aminotransferase activity was significantly higher in bile duct-ligated rats than in sham-operated rats ($P < 0.01$ and $P < 0.01$, respectively, Table 1), showing that bile duct-ligation induced cholestasis. Chronic L-arginine administration partially, but significantly ($P < 0.05$) prevented the elevation of plasma γ-glutamyl transpeptidase and alanine aminotransferase activity in bile duct-ligated rats, both markers of severity of cholestasis-induced liver damage. Plasma levels of $NO_2^- + NO_3^-$ were significantly higher in the bile duct-ligated rats than in sham-operated animals ($P < 0.01$) and L-NNA (10 mg/kg/day) reduced plasma levels of $NO_2^- + NO_3^-$ in the groups mentioned (Table 1). Chronic L-arginine administration did not increase plasma levels of $NO_2^- + NO_3^-$ in bile duct-ligated rats and there was no significant difference in plasma $NO_2^- + NO_3^-$ concentrations between bile duct-ligated/L-arginine and sham-operated/saline animals. The results for plasma L-nitro-tyrosine levels were very similar to those for $NO_2^- + NO_3^-$ and are shown in Table 1.

Table 1 also shows the basal spontaneous rate of isolated atria in the experimental groups. The basal spontaneous beating rate of cholestatic animals was not significantly different from that of sham-operated rats in vitro ($F = 0.91$; $P = 0.47$). Furthermore, administration of L-NNA or L-arginine did not induce a significant difference in basal rate of bile duct-ligated and sham-operated animals in vitro (Table 1).

Fig. 1 shows the chronotropic effect of epinephrine in isolated atria of sham-operated animals. Daily administration of L-NNA significantly increased, and L-arginine decreased, the chronotropic effect of epinephrine in sham-operated rats (Fig. 1). As shown in Fig. 2, the responses of isolated atria of bile duct-ligated/saline rats to adrenergic stimulation were significantly decreased compared to that of sham-operated/saline animals. This effect was corrected by daily administration of L-NNA, and there was no significant difference between sham-operated/saline and bile duct-ligated/L-NNA animals for the chronotropic effect of epinephrine (Fig. 2). Surprisingly, L-arginine acted as effectively as L-NNA and increased the chronotropic

Table 1
Comparison of basal heart rate, γ-glutamyl transpeptidase, alanine aminotransferase, $NO^{2-} + NO^{3-}$ and L-nitro-tyrosine in bile duct-ligated (cholestatic) and sham-operated (sham) rats that received L-arginine, N^G-nitro-L-arginine (L-NNA) or saline

Group (treatment)	Basal heart rate (beat/min)	γ-Glutamyl transpeptidase (μM/l min)	Alanine aminotransferase (μM/l min)	$NO_2^- + NO_3^-$ (μM)	L-nitro-tyrosine (μM)
Cholestatic (saline)	212 ± 9	45 ± 3[a]	81 ± 8[a]	75 ± 5[a]	2.7 ± 0.2[a]
Cholestatic (L-NNA)	197 ± 10	46 ± 4[a]	79 ± 6[a]	35 ± 7[b]	1.3 ± 0.2[b]
Cholestatic (L-arginnie)	204 ± 11	32 ± 4[c]	50 ± 8[c]	46 ± 8[b]	1.0 ± 0.2[b]
Sham (saline)	214 ± 9	21 ± 3	27 ± 6	39 ± 6	0.7 ± 0.1
Sham (L-NNA)	226 ± 13	23 ± 5	29 ± 7	26 ± 7	0.8 ± 0.2
Sham (L-arginine)	201 ± 12	22 ± 3	27 ± 8	40 ± 7	0.9 ± 0.1

Data are shown as means ± S.E.M.; six to eight rats were used in each group.
[a] $P < 0.01$ in comparison with Sham (saline) group.
[b] $P < 0.01$ in comparison with cholestatic (saline) group.
[c] $P < 0.05$ in comparison with cholestatic (saline) and cholestatic (L-NNA) animals.

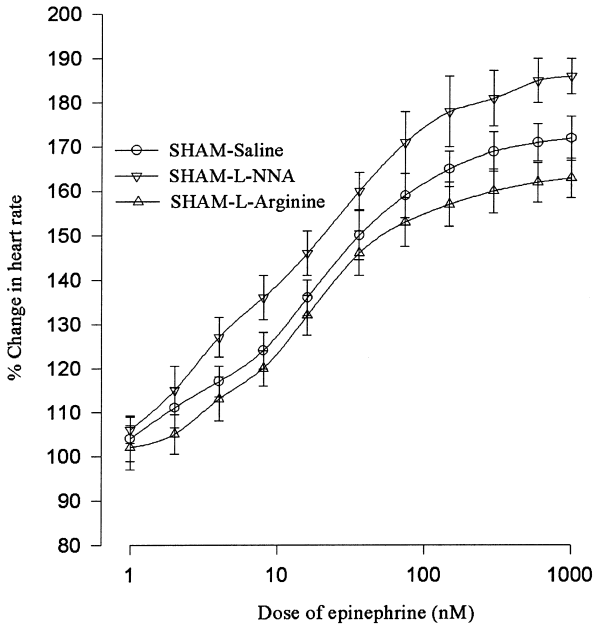

Fig. 1. Chronotropic effect of epinephrine in isolated atrium of sham-operated rats given saline, L-arginine or N^G-nitro-L-arginine (L-NNA); six to eight rats were used in each group.

effect of epinephrine in bile duct-ligated rats but not in sham-operated animals (Fig. 1 and Fig. 2).

Results of the in vivo study (lead II ECG) showed that the heart rate of cholestatic animals was significantly ($P < 0.05$) less than that of sham-operated control rats and

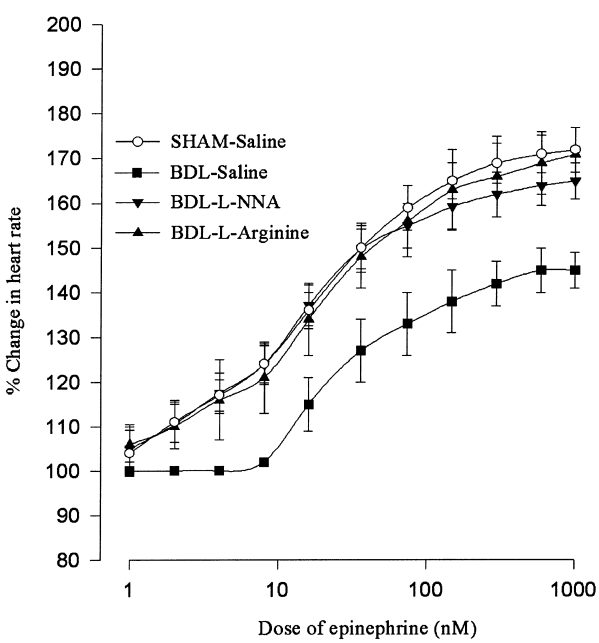

Fig. 2. Comparison of the chronotropic effect of epinephrine in isolated atrium of bile duct-ligated rats given saline, L-arginine or N^G-nitro-L-arginine (L-NNA) with sham-o operated rats given saline; six to eight rats were used in each group.

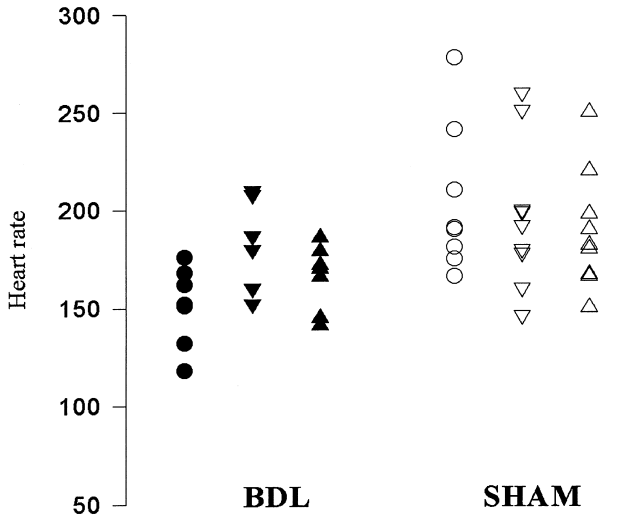

Fig. 3. Comparison of heart rate (beat/min) between bile duct-ligated (BDL) and sham-operated (SHAM) anesthetized rats given saline (circles), L-arginine (triangles-up) or N^G-nitro-L-arginine (triangles-down); six to eight rats were used in each group.

that this bradycardia was significantly corrected by daily adminstration of L-NNA (Fig. 3).

4. Discussion

The sino-atrial node is richly innervated by both sympathetic and parasympathetic fibers. At rest, there is a continuous efferent vagal nerve traffic to this region and relatively little resting sympathetic activity. However changes in both sympathetic and parasympathetic tone may increase or decrease heart rate (Levy and Zieske, 1969). In the present experiments, we have shown that bradycardia is present in anesthetized bile duct-ligated rats. But our in vitro study could not show the bradycardia for the basal spontaneous heart rate of bile duct-ligated animals. In the in vitro study, disconnection of autonomic innervations in isolated atria diminished the dominant effect of parasympathetic tone via the vagus nerve and it is well known that vagal nerve stimulation antagonizes the positive cardiac chronotropic effects of adrenergic agents to decrease heart rate. Muscarinic acetylcholine receptor agonists can reduce heart rate by direct G protein activation of the potassium current ($I_{K\ ACh}$) (Pfaffinger et al., 1985) and inhibition of the cAMP-stimulated hyperpolarization-activated current (I_f) (DiFrancesco and Tromba, 1988). Indirectly, acetylcholine receptor agonists also decrease heart rate by inhibition of the β-adrenoceptor-activating pathway that leads to phosphorylation of the L-type Ca^{2+} channel ($I_{Ca\ L}$) (Hescheler et al., 1986), thus, any defect in the β-adrenoceptor-activating pathway can increase the responsiveness of the heart to a negative chronotropic effect of parasympathetic innervation. Our results showed that the chronotropic response of isolated atria to adrenergic stimu-

lation diminished in a rat model of cholestasis while the spontaneous basal heart rate was not changed in vitro. In view of the dominant effect of parasympathetic tone and also considering the cholinergic antagonism of the β-adrenoceptor-activating pathway in the control of heart rate, a possible explanation of the bradycardia in cholestasis is that decreased adrenergic chronotropism could increase the responsiveness of cholestatic heart to the dominant vagal stimulation. The report of Bashour et al. (1985) is consistent with our hypothesis, which showed that intravenous atropine temporarily abolished severe sinus node dysfunction in obstructive cholestasis. Further experiments must be done to confirm this explanation.

As mentioned above, cholinergic agonists indirectly decrease heart rate by inhibition of the β-adrenoceptor-activating pathway that leads to inhibition of $I_{Ca\ L}$ (Hescheler et al., 1986). Furthermore, the NO-cGMP pathway has been implicated in the parasympathetic control of heart rate by indirect inhibition of $I_{Ca\ L}$ (Han et al., 1996). Sears et al. (1998) showed that inhibition of NOS slows heart rate recovery from cholinergic activation and they concluded that NO contributes to the cholinergic antagonism of the positive cardiac chronotropic effect of adrenergic stimulation. Our in vitro study showed that chronic inhibition of NOS activity with L-NNA corrected the decreased responsiveness of atria to adrenergic stimulation in cholestatic animals. This finding suggests that NO overproduction may have a role in this condition. In the sino-atrial node and in cardiac myocytes NO is synthesized by NOS III (constitutive endothelial isoform) (Balligand et al., 1995). Ferraz and Wallace (1997) reported an increase of NOS III mRNA expression in bile duct-ligated rats and this finding is consistent with NO overproduction in cholestasis.

Since Vallance and Moncada (1991) proposed that NO could be responsible for hyperdynamic circulation in cirrhosis, there has been increasing evidence that NO is implicated in the pathophysiology of liver diseases such as cholestasis (Heinemann and Stauber, 1995; Ghafourifar et al., 1997; Inan et al., 1997, Marley et al., 1999; Nahavandi et al., 1999a). Several studies have suggested overproduction of NO in cholestasis as well as in animal models of cirrhosis (Heinemann and Stauber, 1995; Niedergerger et al., 1995; Inan et al., 1997). According to Vallance and Moncada's (1991) hypothesis, NO overproduction may be due to increased incidence of endotoxemia after bile duct obstruction, and endotoxemia may induce NO overproduction directly or indirectly through cytokines. Wardle and Wright (1971) first suggested the association between endotoxemia and cholestasis, and thereafter results of many studies have suggested that gut-derived endotoxins are implicated in the pathophysiology of cholestasis (Raynolds et al., 1995; Inan et al., 1997). For example, Inan et al. (1997) showed that endotoxemia in cholestasis may induce overproduction of NO that may lead to impairment of cGMP-associated vasodilatation and disrupt autoregulation of the vascular bed. However, some studies did not support Vallance and Moncada's (1991) hypothesis (Fernandez et al., 1995; Zimmermann et al., 1996). For example Fernandez et al. (1995) could not show any significant increase in NOS II (inducible isoform) activity in bile duct-ligated rats. It seems that an increase of NOSIII activity is responsible for NO overproduction in cholestasis (Gadano et al., 1999) but the reason for this effect is not completely understood. It is well known that overproduction of NO in biological systems leads to the formation of reactive nitrogen species such as peroxynitrite which reacts avidly with L-tyrosine residues in proteins to form L-nitro-tyrosine (Herce-Pagliai et al., 1998). Since peroxynitrite has a very short half-life, the presence of L-nitrotyrosine has been used as a marker of reactive nitrogen species. The results of the present study showed that plasma levels of $NO_2^- + NO_3^-$ and L-nitro-tyrosine are significantly increased in bile duct-ligated animals and these results are consistent with NO overproduction in cholestasis.

Surprisingly, the results showed that L-arginine acted as well as L-NNA and increased the chronotropic effect of epinephrine in bile duct-ligated rats but not in sham-opertated animals. Furthermore, we showed that L-arginine treatment significantly prevented the elevation of plasma γ-glutamyl transpeptidase and alanine aminotransferase activity, which are markers of liver damage (Table 1). These results are in agreement with the Muriel and Gonzalez (1998) report, which showed that cholestasis-induced liver damage in rats is ameliorated by L-arginine. It has been reported that bile-duct ligation doubles liver lipid peroxidation and L-arginine completely prevents this change (Muriel and Gonzalez, 1998). Although L-NNA treatment failed to induce changes in any markers of liver injury, chronic L-arginine administration had a protective effect against liver damage induced by ligation of bile duct. This difference suggests that correction of cholestasis-induced bradycardia by L-arginine and by L-NNA is due to different mechanisms. L-arginine has some important NO-independent functions such as stimulation of insulin secretion (Giugliano et al., 1997). Giugliano et al. (1997) demonstrated that the effect of L-arginine on vasodilation is mediated in part by stimulation of insulin secretion. On the other hand, insulin itself is one of factors responsible for hepatotrophic regeneration (Chen et al., 1997) and the oxidative phosphorylation of liver mitochondria is regulated by insulin (Chen et al., 1999). Chronic L-arginine treatment in cholestatic rats can inhibit liver damage via this NO-independent mechanism but further investigations are needed to prove this hypothesis. Decrease of plasma levels of $NO_2^- + NO_3^-$ and L-nitro-tyrosine following L-arginine administration can be interpreted as decreases in the severity of cholestasis in L-arginine-treated groups but further experiments using measurements of specific markers of liver injury as well as histopathologic evaluations are necessary to prove these explanations.

Systemic NOS inhibition corrected the decreased chronotropic response to adrenergic stimulation in cholestatic rats and suggests an important role for NO in the pathophysiology of heart rate complications in cholestatic subjects. The opposite effect of chronic L-arginine administration in rats with cholestasis and in control rats could be explained as an amelioration of cholestasis-induced liver damage by chronic L-arginine administration in bile duct-ligated rats.

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