Further Study of Aldosterone Secretion-Inhibitory Factor and Brain Natriuretic Peptide on Cortisol Production of Guinea Pig Zona Fasciculata Cells

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Abstract

The suppressive effect of aldosterone secretion-inhibitory factor (ASIF) and brain natriuretic peptide (BNP-32) on the basal and ACTH-stimulated cortisol production in a primary culture enriched with guinea pig *Zona Fasciculata* (ZF) cells was further studied. The binding of ^{125}I-labeled ACTH(1-24) and ASIF to ZF cells was found to be displaced by ACTH(1-24), [Phe^2, Nle^4 and Ala^{24}]-ACTH(1-24), ASIF, and BNP in a concentration-dependent manner. The binding of ^{125}I-labeled [Phe^2, Nle^4 and Ala^{24}]-ACTH(1-24) to two transformed clones of mammalian cells expressing the guinea pig ACTH receptor was also competitively inhibited by ASIF and BNP. ASIF and BNP significantly suppressed ACTH-stimulated cAMP production in ZF cells. The 10- and 30-min cellular changes in cAMP induced by ASIF and BNP did not correlate in the rank order with the ultimate magnitude of cortisol suppression observed in ZF cells after a 24-hour treatment with these peptides. Nevertheless, the results did conform to the signaling mechanism of their action. Overall, the findings clearly demonstrated that ASIF and BNP suppressed the adrenocortical function and inhibited ACTH for their antagonistic action against ACTH primarily at the ACTH receptor site. These results support the notion that a physiological role of adrenal medulla in regulating the adrenocortical function may be mediated by the neuropeptides through a paracrine pathway.

Key Words: ACTH, ACTH receptor, aldosterone secretion-inhibitory factor, brain natriuretic peptide, adrenal zona fasciculata cells, guinea pig

Introduction

Atrial natriuretic peptide (ANP), since its discovery (1), has been extensively studied for its physiological role in volume regulation. It has been found to exert natriuretic and diuretic effects through the renal, renin-angiotensin-aldosterone, and cardio-vascular systems. A great deal of research effort has been devoted to the pathophysiology of ANP in congestive heart failure and essential hypertension (for review, see ref. 2,3). These previous studies have discovered that the sites of ANP biosynthesis, not limited to the cardiac atrium, included various nervous tissues, so ANP should be semantically categorized as a neuropeptide. Consistent with this notion, an analogous ANP peptide originally isolated from porcine brain, has been called BNP (4). Furthermore, it has been proven that ANP biosynthesis occurs also in adrenal chromaffin granule (5).

On the other hand, a current view of the

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regulatory control of the adrenocortical function emphasizes the stimulatory role of the adrenocorticotropic hormone (corticotropin, ACTH) and the negative feedback inhibition on the hypothalamus and pituitary by glucocorticoids. There is, however, increasing evidence indicating that the adrenal sympathetic nerve and the adrenal medulla may significantly modulate adrenocortical activity and related physiological function (6). In addition to catecholamines, this peripherally regulatory pathway of adrenocortical functions depends on a large number of neuropeptides including ANP and BNP, which have been identified in the medulla tissue. The inhibitory effect of ANP on the adrenal response to ACTH has been confirmed in different laboratories (7-9), although its physiological impact has not been substantiated (10). We studied the structure-activity relationships in an in vitro culture system of guinea pig adrenal *zona fasciculata* (ZF) cells, and found that two neuropeptides, aldosterone secretion-inhibitory factor (ASIF) and brain natriuretic peptide (BNP-32), possessed the most potent inhibitory action on cortisol production (11). Their mechanism of action remains unknown. In the present series of experiments, we investigated the site of their action in isolated ZF cells with a specific aim at determining where the inhibitory action may take place.

Materials and Methods

Peptides

Human ACTH(1-24) was supplied by Phoenix Pharmaceuticals, Inc., Belmont, CA, USA. Guinea pig ACTH differs from human ACTH(1-39) by Ala^{24} and Glu^{29} substituting Pro^{24} and Asp^{29} (12). Based on the structural modification of human ACTH proposed by Buckley et al. (13), we custom-synthesized [Phe^{2}, Nle^{4} and Ala^{24}]-ACTH(1-24) as a stabilized molecule of the bioactive guinea pig ACTH peptides, which can be iodinated without losing a slightly stronger steroidogenic activity with guinea pig adrenal ZF cells than human ACTH(1-24) and ACTH(1-39). ASIF (1-35) was purchased from Sigma Chemical Co. St. Louis, MO, USA. Human BNP-32 was purchased from Peninsula Laboratories, Inc., Belmont, CA, USA. Radioactive human ACTH(1-24), [Phe^{2}, Nle^{4} and Ala^{24}]-ACTH(1-24), and ASIF were labeled in our own laboratory with isotopic ^{125}I, using chloramine-T as an oxidant. The ^{125}I-tagged peptides were purified on a Sephadex G-10 column immediately after iodination. The specific radioactivity of labeled products was calculated by the amount of radioactivity retained by the hormone peak in the column chromatogram and was at least 1,000 mCi/mmol.

Cell Preparation and Culture

Primary cultures of guinea pig adrenocortical preparation enriched with ZF cells have been described in detail previously (14). The isolated ZF cells in aliquot 6- or 12-well plates needed at least two days to adapt in a humidified incubator, 37 °C, under 95% air and 5% CO_2 . Then the steroidogenic function of the cultured ZF cells recovered, as seen by an increased amount of cortisol released into the culture medium. Starting the 3rd or 4th day, an established culture of cells showed the characteristic feature of a further increase in the basal cortisol production rate, which lasted for the ensuing three days. Ultimately the steroidogenic property declined, probably due to the overwhelming growth of the contaminated fibroblasts, whereas the ZF cells did not divide themselves.

Cloned ACTH Receptor cDNA

A partial sequence of the guinea pig ACTH receptor (ACTH-R) gene has been determined in our laboratory and deposited with Genbank, under the access number AF104058. ACTH-R cDNA consists of 894 nucleotides (from nc # 117 to # 1110), encoding a receptor protein of 297 amino acids prior to a stop codon. ACTH-R cDNA was ligated to a pcDNA3.1/V5-His-TOPO vector purchased from Invitrogen Corp., Carlsbad, CA, USA. The plasmid expression vector was used to transfect two types of mammalian cells: Cloudsman S91, a strain of mouse melanoma cells, and COS-7, a strain of monkey kidney mesangial cells, both of which were purchased from the Cell Bank of the National Health Research Institute, Taiwan. The successfully transformed clones were selected with geneticin (G-418) in culture. RT-PCR evidence confirmed the incorporation of ACTH-R cDNA into the plasmid vector and cell chromosomes. A subsequent ligand binding technique demonstrated the expression of ACTH-R.

Studies of Cortisol Production

The ZF cells, after four days in culture, would respond to a 24-hr exposure of ACTH with a concentration-dependent stimulation not only in the amount of cortisol produced but also in the time of cortisol production sustained. Therefore, in our experimental design for testing the action of peptides, we simply added the peptides in the culture medium for a 24-hr treatment on day 5. Media collected on days 5, 6, and 7 was for cortisol measurement by a radioimmunoassay technique using kits purchased from Diagnostic Products Corp., Los Angeles, CA, USA.

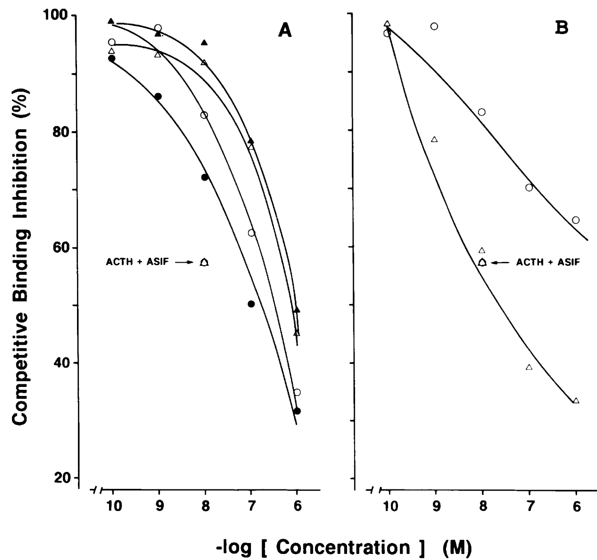

Fig. 1. (A) Displacement of ^{125}I-ACTH(1-24) binding to ZF cells by ACTH(1-24) (❍), [Phe^2, Nle^4, Ala^{24}]-ACTH(1-24) (●), ASIF (Δ), and BNP-32 (▲) (B) Displacement of ^{125}I-ASIF binding by ACTH(1-24) (❍), and ASIF (Δ). In both panels, the displacement by a combined mixture of ACTH(1-24) and ASIF is shown by a symbol (Δ) for comparison. Each point represents a mean value of 3 wells with a variation below 10% of the mean. There is over 1.1×10^4 cpm in the control wells as 100% binding.

ACTH Receptor Binding Test

After four days in culture, the functioning ZF cells were washed with a serum-free medium (modified McCoy's 5A medium, supplied by Life Technologies, Grand Island, NY, USA) containing 0.1% BSA, at 37 °C, for 5-10 min. Then, removing the washing medium, we carried out the binding experiment by incubating the cells for 3 hrs at 4 °C with a cold serum-free medium containing isotopic ACTH(1-24) or ASIF ($1 - 2 \times 10^5$ cpm/well) alone or in combination with varied concentrations of unlabeled peptides. At the end, after the medium was removed, the cells were gently washed once with phosphate-buffered saline (PBS), and then solubilized in 10% sodium dodecyl sulfate (SDS) to count the radioactivity retained by the cells.

A similar experimental procedure was carried out to test the competitive binding of the more specific ^{125}I-[Phe^2, Nle^4 and Ala^{24}]-ACTH(1-24) than ACTH (1-24) by cold displacing [Phe^2, Nle^4 and Ala^{24}]-ACTH(1-24), ASIF, and BNP-32 in two transformed clones of mammalian cells, which express guinea pig ACTH-R.

Studies of Cyclic-AMP Production

The 4-day-old ZF cells in culture were treated with a serum-free medium containing 0.1% BSA and 0.5 mM isobutyl-1-methyl-xanthine (IBMX, a diesterase inhibitor) at 37 °C for 1 – 2 hr. Then, cells were exposed to the same medium containing ACTH (1×10^{-9} M) alone or in combinations with ASIF or BNP-32 (1×10^{-8} M), and incubation continued for exactly 10 or 30 min. At the end, the medium was removed and the plate was placed on ice. The cells were dissolved in cold 10% trichloroacetic acid (TCA), 0.5 ml/well. Each well was rinsed once with 0.5 ml of 10% TCA. TCA in the combined solution was extracted out four times with an equal volume of ether each time. The TCA-free aqueous layer was lyophilized and then reconstituted in assay buffer for cyclic AMP measurement using a RIA kit supplied by NEN Life Science Products, Inc., Boston, MA, USA.

Statistical Analysis of Data

Each group consisted of a minimum of 3 wells. The daily cortisol production, receptor binding, and cyclic AMP production of each group were expressed in Mean ± S.D. Comparisons between groups were accomplished by t-tests with a significant difference defined at $P < 0.05$. The inhibitory efficacy of a peptide on the 3-day cortisol production was also assessed by an one-way analysis of variance (ANOVA) for significant difference with the critical F-value set at $P < 0.05$.

Results

The results in Tables 1 and 2 consistently confirm the stimulatory effect of ACTH and the inhibitory effect of ASIF and BNP-32 on the basal and ACTH-stimulated cortisol production of ZF cells in culture. Previous studies (11) revealed that both ACTH stimulation and the inhibitory effect of ASIF and BNP-32 on cortisol production were concentration-dependent. In the present studies, we chose an ACTH concentration of 10^{-7} or 10^{-8} M and an ASIF and BNP-32 concentration of 10^{-7} M for the optimal effect on cortisol production. Furthermore, at this range of concentration, the inhibition of ASIF and BNP-32 on the steroidogenic function was not due to any cytotoxic effect, and ACTH stimulated the cell growth without mitogenesis (11).

The inhibition on binding of the cultured ZF cells by competitively displacing radioactive ACTH and ASIF is shown in Figure 1. In Fig. 1-A, the binding affinity of [Phe^2, Nle^4 and Ala^{24}]-ACTH(1-24) was higher than that of human ACTH(1-24), as its binding curve was left-shifted. ASIF and BNP-32 did not displace the binding of radioactive human ACTH (1-24) until their concentration reached 10^{-7} M. A significant inhibition on the binding of radioactive

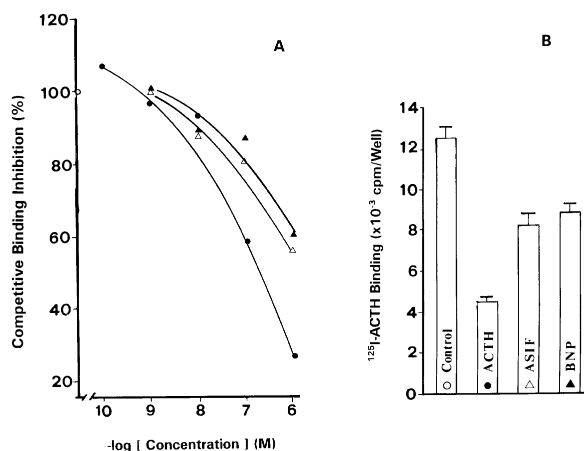

Fig. 2. (A) Displacement of ^{125}I[Phe^2, Nle^4, Ala^{24}]-ACTH(1-24) binding to the cloned ACTH-R in Cloudsman S91 cells by [Phe^2, Nle^4, Ala^{24}]-ACTH(1-24) (●), ASIF (Δ), and BNP-32 (▲) Each point represents a mean value of 3 wells with a variation below 8% of the mean. There is more than 1.2×10^4 cpm in the control wells as 100% binding. (B) Inhibition of ^{125}I- [Phe^2, Nle^4, Ala^{24}]-ACTH(1-24) binding to the cloned ACTH-R in COS-7 cells by [Phe^2, Nle^4, Ala^{24}]-ACTH(1-24), ASIF, and BNP-32, all at 1×10^{-6} M. Each column is the mean of 5 wells with the bar representing one S.D. Compared to the control, all peptides significantly suppressed the binding, $P < 0.001$.

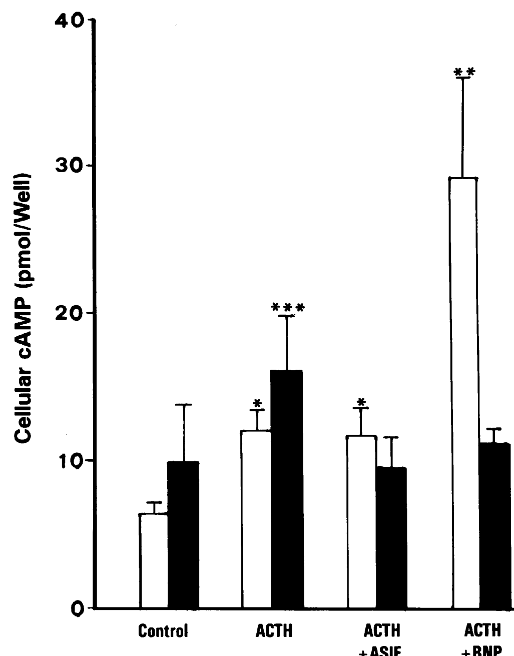

Fig. 3. Effects of ASIF and BNP-32 on ACTH-stimulated cAMP production of ZF cells after 10-min (open column) and 30-min (black column) exposure. Each column represents the mean value of 4 wells with the bar representing one S.D. $*P < 0.01$ compared to the 10-min control, $**P < 0.001$ and $***P < 0.05$ compared to the three respective columns.

[Phe^2, Nle^4 and Ala^{24}]-ACTH(1-24) by ASIF and BNP-32 required an even higher concentration of 1×10^{-6} M (data not shown). Fig. 1-B shows the displacement curves of radioactive ASIF binding to ZF cells. Cold ASIF shows a concentration-dependent inhibition. Human ACTH(1-24) was able to displace isotopic ASIF. However, the two binding curves do not appear in parallel up to a concentration of 10^{-6} M, indicating that different kinetics or more than one site may be involved in the binding of ASIF and ACTH(1-24). As shown in both panels, when a combined mixture of human ACTH(1-24) and ASIF of 1×10^{-8} M each was tested, an additive inhibition was observed.

Figure 2 shows that both ASIF and BNP-32 displace the binding of radioactive [Phe^2, Nle^4 and Ala^{24}]-ACTH(1-24) in two different clones of mammalian cells which express the guinea pig ACTH-R. In repeated experiments with one of the clones, we found that a low concentration of [Phe^2, Nle^4 and Ala^{24}]-ACTH(1-24), i.e., 1×10^{-10} M, resulted in a significantly increased receptor binding over the control (Fig. 2-A). The displacement curves indicate that the rank order of binding affinities was ACTH > ASIF > BNP-32. This difference in receptor-binding affinity was confirmed in the other clone, when the three peptides were compared at the same concentration of 1×10^{-6} M in Fig. 2-B.

Figure 3 shows the effect of the peptides on the speculated second messenger, cyclic AMP (cAMP) in cultured ZF cells. When cells were exposed to the testing concentrations, 10^{-9} M for ACTH and 10^{-8} M for ASIF and BNP-32, for 10 min, ACTH significantly stimulated cellular cAMP production, and ASIF showed a slight but insignificant suppression on cAMP stimulation by ACTH, but BNP-32 enhanced significantly the ACTH-stimulated cAMP production. Cellular cAMP content increased in the control wells from 10 to 30 min. When the exposure of cells to peptides continued to 30 min, ACTH alone still stimulated significantly more cAMP production, and both ASIF and BNP-32 sufficiently counteracted ACTH in the extended period, so that their cellular cAMP returned to the level of the control wells. Although ASIF and BNP-32 did not seem to reduce the initial ACTH-stimulated cellular cAMP production, they would interfere with the ACTH stimulatory action on cAMP after a prolonged treatment. However, these cAMP results do not correspond in proportion to the magnitude of changes in cortisol listed in Table 2. ASIF appeared to suppress more cAMP than BNP-32 in the presence of ACTH. Yet, BNP-32 showed slightly stronger anti-ACTH activity than ASIF in the suppression of cortisol production. Based on these results, we may conclude that the critical actions of ASIF and BNP occur after receptor binding but before cAMP production.

Table 1. Effect of Peptides (1×10^{-7} M) on Basal Cortisol Production*

Peptide	Day 5	Day 6	Day 7	%
Control	31.5 ± 5.4	87.5 ± 9.2	52.8 ± 3.6	100
ACTH	399.8 ± 17.4	1,589.0 ± 87.6	2,501.0 ± 471.8	2,550†
ASIF	23.7 ± 1.2	39.5 ± 15.9	56.0 ± 4.0	70.2†
BNP-32	23.5 ± 0.8	27.8 ± 1.8	26.9 ± 4.2	49.8†

* Data are daily production in ng/well, mean ± S.D., from 5 wells per peptide.

† Significant differences by ANOVA, as compared with the control, in which no peptide was added to the culture medium during day 5.

Table 2. Effects of ACTH (1×10^{-8} M) and Peptides (1×10^{-7} M) on ACTH-Stimulated Cortisol Production*

Peptide	Day 5	Day 6	Day 7	%
No ACTH	24.0 ± 2.0	82.4 ± 12.3	74.9 ± 16.6	
ACTH alone	233.0 ± 31.6	1,451.0 ± 72.0	1,079.0 ± 173.0	100
ACTH + ASIF	128.8 ± 6.9	1,165.6 ± 138.8	791.5 ± 76.0	77.5†
ACTH + BNP-32	123.5 ± 6.5	703.5 ± 13.1	447.1 ± 22.1	46.1†

* Data are daily production in ng/well, mean ± S.D., from 5 wells per treatment.

† Significant differences by ANOVA, compared to cells treated with ACTH alone.

Discussion

In our previous study (11), we clearly demonstrated a structural relationship between ASIF and natriuretic peptides with their inhibitory effect on the basal and ACTH-stimulated cortisol production of ZF cells. The primary structure of an effective inhibitor required an intact Cys-Cys loop of 17-amino acid residues with both N- and C-terminal extension from Cys residues of not less than 2 amino acids. We did not know how and where the inhibitory action took place in the cultured ZF cell. Nevertheless, for the antagonistic effect of ASIF against ACTH, we can safely rule out a direct protein-protein interaction between the two hormones as the cause, since their combination could produce an additive inhibition by either peptide's receptor binding alone (Fig. 1). Besides, both ASIF and BNP-32 significantly suppressed cortisol production in ZF cells cultured in a serum-free and thus ACTH-free medium.

Now we have at least two lines of evidence to pinpoint the receptor site upon which ASIF and BNP-32 primarily act. First, ASIF and BNP-32 can displace the ligand binding of ACTH receptors in a concentration-dependent manner, even though their binding affinity is 10- to 100-fold less than ACTH *per se*. Since both ASIF and BNP-32 are the natural substances occurring in the adrenal medulla (15,16), it is very likely that specific intrinsic receptors for these peptides, other than ACTH-R, may exist in adrenal cortical ZF cells. Indeed, the ASIF receptor was somewhat verified by the characteristic binding inhibition of radioactive ASIF to ZF cells (Fig. 1B). Then, surprisingly, ACTH also showed a concentration-dependent inhibition on the isotope-labeled ASIF binding. Furthermore, the combination of ACTH and ASIF showed an additive displacement in the binding of either isotopic ligand to ZF cells. These results may be interpreted that, although different intrinsic ACTH and ASIF receptors exist in ZF cells, ligands could somehow interfere with each other by cross-reacting with both types of receptor.

More convincing evidence, however, comes from the competitive inhibitions of ASIF and BNP-32 on ligand binding to the cloned ACTH-R. The two hormone peptides apparently can decrease ACTH activity in ZF cells by directly displacing ACTH from its receptor molecule binding. Since there is no cloned ASIF receptor available for testing, we took the finding that ACTH inhibits the radioactive ASIF binding in ZF cells to imply that ACTH and ASIF receptors may share some conformational domain in reacting with both ligand peptides. However, there is no obvious sequential analogy between ACTH and ASIF, nor do we know of any tertiary conformation of the epitopic region of their receptors. The present findings strongly challenge us for future studies in molecular modeling of the receptors.

Conceivably, the plasticity of receptor molecules in the same cell membrane may allow not only a

conformational change after ligand binding but also the kinetic alteration in signaling cascade mediating the ultimate bioactive properties of hormones. Whenever two different types of receptor are closely localized in a single strain of cells, there is always the possibility of physical or chemical interaction that may affect the functional activity of the cell. For an example, receptors of dopamine and somatostatin were reported to make a direct intramembrane association simply because they coexist in the same subgroup of neurons and allow a new level of molecular crosstalk to take place (17).

In addition to the mechanism of receptor binding, there are other possible sites of interfering action. The ACTH receptor is a single type of 7-transmembrane, G protein-coupled receptor mediating steroidogenic action (18). The level of the intracellular second messenger cAMP, which is regulated by the subunits of membrane G-protein with GTP as energy supplier, determines the ACTH activity. On the other hand, there are three subtypes of natriuretic peptide receptor (NPR), each recognizing the three known natriuretic peptides, ANP, BNP, and CNP, selectively (19). They are single transmembrane proteins whose extracellular binding and intracellular catalytic domains are homologous in 44% and 88% of the sequence, respectively. The action of natriuretic peptides is mediated by membrane guanylyl cyclase activation to generate intracellular cyclic GMP (cGMP) as a second messenger, also using GTP as energy substrate. Most recently, natriuretic peptides were found to rapidly inhibit G protein activation (20). Therefore, membrane G proteins seem to be a convergent point where ACTH and natriuretic peptides may crosstalk, affect the downstream signaling pathway, alter the intracellular level of cAMP, and ultimately determine the amount of cortisol produced.

Probably due to the difference in experimental designs, the cAMP results did not correlate very well with the cortisol data showing the relative potency between ASIF and BNP-32. Nevertheless, a definitively suppressing effect of the two peptides on ACTH binding to ACTH receptor provides a plausible explanation for the site and mechanism of their action. Our goal, however, is aimed at elucidating the significant effect of adrenal medulla on cortical function. Although ACTH is recognized to play the predominant role of controlling adrenocortical activity, the presence of abundant neuropeptides secreted from the adjacent medullar tissue can exert a modulating role in adrenal physiology. By defining the interference action of ASIF and BNP-32 with ACTH binding to its receptor, we conclude that the importance of a paracrine regulatory pathway from adrenal medulla to cortex shall not be overlooked.

Acknowledgments

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