Differential display PCR reveals increased expression of 2′,3′-cyclic nucleotide 3′-phosphodiesterase by lithium

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Abstract Differential display PCR was used to study the effects of lithium on gene expression. Four candidate genes were isolated and verified by Northern hybridization after 1 week treatment of C6 glioma cells with therapeutically relevant concentrations of LiCl (1 mM). Sequencing analysis revealed three previously unidentified cDNA fragments in addition to a sequence with 99% homology with the cDNA for 2′,3′-cyclic nucleotide 3′-phosphodiesterase type II (CNPaseII). Since CNPaseII is important in myelinogenesis and possibly neuronal growth and repair, the present findings suggest that lithium treatment may regulate these processes.

Key words: Differential display PCR; Lithium; 2′,3′-Cyclic nucleotide 3′-phosphodiesterase; Bipolar disorder; Gene expression

1. Introduction

Bipolar affective disorder (BD) is a common psychiatric disorder in which biological factors are important in the development of symptoms and response to drug treatment [1]. Specific pathophysiological processes operative in BD, however, are still being investigated. One of the strongest clues to the biological basis of BD is the large body of evidence suggesting a genetic predisposition to the disorder [1,2]. Recent research in our laboratory and others indicates that G-proteins and possibly other components of the signal transduction pathway play an important role in the mechanism of BD although the precise defect is far from clear [3-6].

Lithium is a highly effective treatment for BD [1,7]. It is widely acknowledged that in BD, chronic treatment with lithium (i.e. at least 1 week) is needed for its acute effects against mania and that long-term (i.e. several years or longer) treatment can markedly improve the course of the illness and prevent relapses of either mania or depression [7]. Furthermore, long-term changes in neuronal synaptic function are related to changes of gene expression in brain which may underlie the effects of psychotropic drugs [8]. It has therefore been hypothesized that, among other effects, lithium treatment may act by altering gene expression in brain [9,10]. Lithium-induced changes in gene expression have been documented in cultured cells and animal models for several years [9,11]. Several studies have demonstrated that lithium alters the expression of the early response gene c-*fos* through a protein kinase C (PKC)-mediated mechanism [12,13]. For example, incubation of PC-12 cells for 16 h with lithium significantly potentiates c-*fos* expression induced by the muscarinic agonist, carbachol. Lithium pretreatment in these cells also potentiates c-*fos* expression in response to phorbol esters, which directly activate PKC [13]. Chronic lithium treatment (i.e. a week or longer) has also been shown to reduce the expression of G-protein α-subunit (α_s and α_i) but to increase expression of adenylyl cyclase types I and II [14-16]. It is also reported that lithium alters the mRNA levels of a number of neuropeptides [17,18]. For example, acute treatment with lithium enhances basal expression of the neuropeptide Y gene in rat hippocampus [17]. Chronic administration of lithium (3 weeks) increased the prodynorphin mRNA abundance in the rat striatum [18]. Considering these findings of lithium's effect on gene expression together, nonetheless, provides an incomplete picture of the underlying mechanism of action of this drug in BD. Therefore the isolation of other lithium-regulated genes (LiRGs) is very important, particularly if these genes can be shown to be involved in the development of BD or can be shown to be functionally relevant to central nervous system (CNS) functioning.

The differential display PCR (DD-PCR) technique was developed to identify differentially expressed genes and to detect differences in specific mRNAs in different cell lines or after specific pharmacological manipulations [19-21]. We employed this method to screen and clone differentially expressed genes after administration of lithium at therapeutically relevant concentration (1 mM) for 1 week in C6 glioma cells. This cell line was chosen since much of the earlier work on the molecular mechanism of mood stabilizing drugs had been carried out in this cell line [22,23]. This includes the recent convergence of findings which suggest that components of signal transduction pathways are regulated by lithium thereby increasing the likelihood of finding a result which is physiologically relevant [24-26]. We report here that the expression of four genes was increased by lithium including 2′,3′-cyclic nucleotide 3′-phosphodiesterase type II (CNPaseII), the latter of which may be important in neuronal growth and repair.

2. Materials and methods

2.1. Cell culture

C6 glioma cells were grown in medium containing Dulbecco's modified Eagle's medium, 15% horse serum, and 2.5% fetal calf serum. Cells were treated for 1 week in the presence or absence of different concentrations of LiCl (0.04, 0.2 and 1 mM) or NaCl (1 mM). In other experiments cells were treated with therapeutically relevant concentrations of two other mood stabilizers, carbamazepine (0.05 mM)

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Abbreviations: DD-PCR, differential display polymerase chain reaction; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; LiRG, lithium-regulated gene; MBP, myelin basic protein; G-protein, GTP binding protein; PKC, protein kinase C; CNS, central nervous system; BD, bipolar affective disorder

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[7,16] and sodium valproate (0.5 mM) [7,24], and as a control for the monovalent Li cation, NaCl (1 mM).

2.2. Differential display of mRNA by PCR (DD-PCR)

Total RNA was isolated using the Trizol reagent (Gibco Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. Chromosomal DNA was removed by digestion with RNase-free DNase I. Total RNA was extracted with phenol/chloroform and precipitated in 100% ethanol at −20°C overnight. The concentration of RNA in each sample was quantitated by measuring the OD at 260 nm and the purity determined by the 260/280 ratio. 3 μg of DNase-treated RNA was separated by electrophoresis on a denaturing 1% agarose gel to check the integrity of the RNA (18S and 28S bands). All reagents used in DD-PCR were from GenHunter Corporation (Nashville, TN) except Taq polymerase which was obtained from Perkin-Elmer Corp. (Branchburg, NJ). DNA-free RNA was transcribed to cDNA with reverse transcriptase using three different one-base-anchored primers: oligo-dTG, oligo-dTA and oligo-dTC. Primers for PCR reaction were one of eight arbitrary decamers and one of oligo-dTG, oligo-dTA or oligo-dTC. The PCR reaction mixture (20 μl) contained 2 μM of each primer, 25 μM dNTP, 0.5 μM [α-^{35}S]dATP (1200 Ci/mmol) and 1 unit of Taq polymerase, and buffer supplied by the manufacturer (GenHunter Corp.). The PCR reaction (40 cycles) consisted of sequential incubations for 30 s at 94°C, for 2 min at 40°C, for 30 s at 72°C, with a final extension for 5 min at 72°C. The amplified cDNAs were then separated on 6% denaturing polyacrylamide gel. Gels were dried and exposed to Kodak X-omat film for 2 days. Differentially displayed cDNAs were then recovered from gel and reamplified by PCR with the same primers. The reamplified cDNAs were extracted from a nondenaturing 1.5% agarose gel by QIAEX Gel Extraction Kit (QIAGEN Inc., Chatsworth, CA).

2.3. DNA cloning and sequence analysis

Differentially displayed PCR products were ligated into the pNoTA/T7 vector at 25°C for 30 min with T4 DNA ligase (5 primer - 3 primer, Inc., Boulder, CO). Competent bacterial cells were transformed with the ligated construct. Plasmid DNA from subclones containing inserts was then purified using a Wizard Miniprep DNA purification system (Promega, Madison, Wyoming). Inserts were sequenced by the dideoxy chain-termination method [27]. Sequences thus derived were compared for homology to the sequences present in the current GenBank database through the National Center for Biotechnology Information with the BLASTN program [28].

2.4. Northern hybridization and slot blot analysis

Total RNA from C6 glioma cells was applied to nylon membranes using either a slot blot apparatus or by transfer after electrophoresis in 1% agarose/formaldehyde gel, and ultraviolet-cross-linked. Membranes were then pre-hybridized at 42°C for 3 h in 5×SSC, 50% formamide, 5×Denhardt's, 1.0% SDS, 5% dextran sulfate, salmon sperm DNA (100 μg/ml) and hybridized at 42°C overnight in this buffer with differentially displayed cDNA probes derived as described above. cDNA probes were labeled with [^{32}P]dCTP by using a random prime labeling method [29,30]. Membranes were then washed at room temperature for 45 min in 1×SSC, 0.2% SDS twice and at 58°C for 15 min in 0.1×SSC, 0.2% SDS twice. Membranes were exposed overnight at −80°C to Kodak X-Omat film with an intensifying screen.

2.5. Analysis and interpretation of data

All sequence data were analyzed by DNASIS sequence analysis program and aligned with sequences available in GenBank. Data from slot blot studies were obtained by densitometric analysis of autoradiograms with the Northern Exposure program from ImagExperts, Inc. (Oakville, Ont.). Results were expressed as percent of control. Changes in gene expression after drug treatment were expressed as the mean ± SEM from three separate experiments. Statistical significance of differences between means was determined by Student's *t*-tests.

3. Results

Five bands were differentially expressed between control and 1 mM LiCl-treated cells (Fig. 1). These differentially expressed PCR products, i.e. LiRGs, were excised from the gels and were reamplified, purified and named LiRG1 (128 bp), LiRG2 (178 bp), LiRG3 (120 bp), LiRG4 (160 bp) and LiRG5 (155 bp). The reamplified displayed cDNAs were radioactively labeled for probing Northern blots containing total RNA from control and 1 mM LiCl-treated cells to confirm their expression in cells. The expression of LiRG1, LiRG2, LiRG3 and LiRG5 was increased by 1 mM LiCl whereas no signal was found for LiRG4 (Fig. 2). These four differentially expressed cDNAs were then directly cloned into the pNoTA/T7 cloning vector and DNA sequencing was performed. As shown in Fig. 3, the sequences of LiRG1, LiRG2, LiRG3 and LiRG5 revealed DNA fragments which included the arbitrary decamers and anchored oligo-dT primers in addition to AATAAA (i.e. the polyadenylation signal) upstream of the poly A sequence. These results suggest that the LiRG1, LiRG2, LiRG3 and LiRG5 cDNA fragments are from the 3′ ends of these specific genes. The clone named LiRG5 displayed 99% homology to rat CNPaseII [31]. LiRG1, LiRG2 and LiRG3 showed no significant homology (<85%) to any entry in existing nucleic acid databases available through the National Center for Biotechnology Information.

To further investigate possible similarities between lithium and other drugs which are commonly used for treatment of BD, we determined the effect of therapeutically relevant concentrations of sodium valproate and carbamazepine [7] on CNPaseII gene expression by slot blot analysis. As shown in Fig. 4, 1.0 mM sodium chloride, 0.5 mM sodium valproate, 0.05 mM carbamazepine and 0.04 mM LiCl had no effect on CNPaseII gene expression. LiCl 0.2 mM and 1.0 mM significantly increased CNPaseII gene expression by 25.7 ± 9.3% ($P<0.05$, $n=3$) and 62.0 ± 12.6% ($P<0.01$, $n=3$). There was no difference in level of hybridization of β-actin compared to control cells after any of these treatments.

4. Discussion

DD-PCR revealed four differentially expressed gene products after treatment with LiCl for 1 week at therapeutically relevant concentrations in C6 glioma cells. Although the identity of three of these gene products is at present unknown, the expression of a cDNA with greater than 99% homology to the gene for the enzyme CNPaseII was markedly increased after lithium treatment. The expression of this cDNA in cells was confirmed by Northern hybridization and quantitative slot blot analysis and was not increased by either sodium chloride

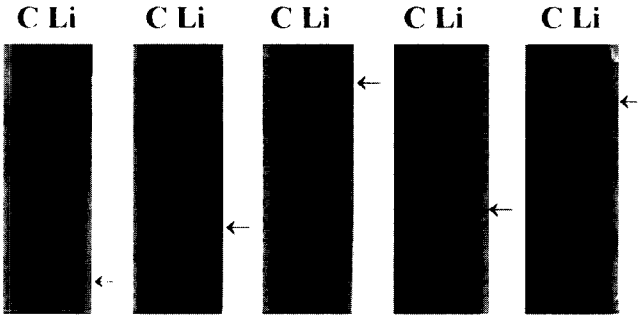

Fig. 1. Differential display of mRNA in the presence and absence of 1 mM LiCl using multiple anchored oligo-dT and random primer sets. Arrows point to differentially displayed products. Five products show differential display between control (C) and LiCl (Li)-treated cells.

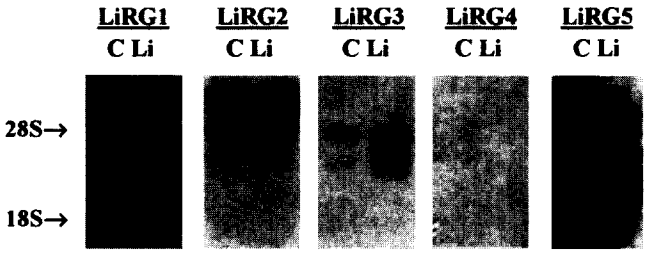

Fig. 2. Northern blot analysis of candidate LiRGs. Total RNA was isolated from C6 glioma cells grown with (Li) or without (C) 1 mM LiCl for 1 week. RNA (15 μg) was analyzed by Northern blot analysis using probes prepared from products of DD-PCR (LiRG1–LiRG5). The positions of 18S and 28S rRNA are indicated.

or the other drugs commonly used to treat BD (i.e. sodium valproate or carbamazepine at therapeutically relevant concentrations [7]). These results add to the growing body of evidence suggesting that lithium alters gene expression in brain after chronic treatment which may be important in its action as a long-term prophylactic treatment for BD [8,9,11]. These results are also consistent with the current notion that psychotropic drug treatment may act through long-term adaptational changes in the CNS to bring about their therapeutic effects [8].

CNPaseII, a myelin-associated enzyme, was among the four differentially expressed genes identified after lithium treatment in C6 glioma cells. Although the function of CNPaseII is not yet clear, much investigation has been done to understand its significance in the CNS. In vitro, CNPase hydrolyzes 2′,3′-cyclic nucleotides to form 2′-nucleotides [32,33], however, 2′,3′-cyclic nucleotides are not known to have physiological relevance in mammals. In the CNS, CNPase is highly localized to the myelin sheath and oligodendroglia [34-37]. It is reasonable to assume that CNPase function may be related to differentiation of oligodendroglial cells. In this respect, it has been shown that elevation of brain CNPase activity in rodent brain occurs in parallel with myelination [38,39]. CNPase begins to appear earlier than myelin basic protein (MBP), and in rat brain CNPase activity increases to 65% of the adult level 20 days after birth, whereas MBP reaches only 30% of the adult level. CNPase maintains its high activity for 1 year after birth. Thus CNPase may have an important role in differentiation of oligodendroglial cells and CNS myelination.

Molecular analysis of CNPase indicates the presence of several potential nucleotide- and phosphoryl-binding sites on the CNPase molecule [33]. For example, the amino acid sequence of CNPase includes a consensus sequence GXXX-XGK and DXXXG/A, which is found to comprise two of the three elements of the GTP-binding domain of G-protein α-subunits [40]. This suggests that CNPase may act as a kinase-related protein. This observation has led to the speculation that CNPase may play a role in glial differentiation by providing a binding site for ATP. It was reported that in brain myelin CNPase is phosphorylated by cyclic AMP-dependent protein kinase and PKC and that CNPaseII is far more highly phosphorylated than CNPaseI [41]. These latter results suggest that CNPaseII might be a target for CNS signal transduction pathways which regulate protein phosphorylation. Of further interest to this latter possibility is the extensive body of recent data which has suggested that lithium has important effects on such signal transduction pathways [9,11].

Since the exact role of CNPaseII in the CNS is not known, we can only speculate about the significance of increased CNPaseII expression after chronic lithium treatment. CNPase has been investigated in the pathophysiology of several disorders including multiple sclerosis. For instance, CNPase activity was reduced to 50% of control values in postmortem brain white matter samples obtained from subjects with multiple sclerosis [42]. The fact that CNPaseII is involved in cell differentiation and myelinogenesis raises the possibility that lithium treatment may lead to its therapeutic effects in BD by regulating one of these processes. Regulation of neuronal plasticity and long-term adaptational changes in CNS are currently under investigation as targets of psychotropic drugs, including lithium [8,43]. It is therefore interesting to consider the possibility that upregulation of CNPaseII in brain by lithium may result in long-term changes in CNS functioning which are in turn relevant for symptomatic improvement. A better understanding of the physiologic significance of CNPaseII may allow these interesting questions to be answered.

The relevance of an effect of lithium on CNPaseII is further demonstrated by the previous finding of white matter abnormalities in patients with BD. Patients with multiple sclero-

LiRG1 (Unidentified)

AAGCTTCTCAACGTATAAAGACACATTTCTGACTG
CTATTTTTACATCAAAGAATTCATACAAATAAGAT
GGAAAATAAAATAATAACGTTTAATAAAATTTAA
AGTTCACAGTTGTAAAAAAAAAAAAGCTT

LiRG2 (Unidentified)

AAGCTTCTCAACGAACACATAGCATTATGTAAACT
CTAATAATTCATTAAGTAGTGAATGAATCTTAACA
ATTTAATGCCTTTGGGAAATAAAAGTGAAAACTG
GGCAAGGGCAGGGGGCTGGGCGTGAACCGCTTAC
TAGATAATGTTCTCTAAAAATTGGCTCTGAAAAAA
AAAAAGCTT

LiRG3 (Unidentified)

AAGCTTCTCAACGCAGTATAAGAATGTGATATATT
GTATATTGGAAAGTATTTACCTTATTTATATACCT
GAATGTTCCTATTAAACAAATAAACATATACTAAA
TTCTAAAAAAAAAAAAGCTT

LiRG5 (99% homology to CNPaseII)

AAGCTTTTACCGCAATGGGAAAGTCTGCTCAGTAA
CAGGACCTTGCTAATTGGGTTGTCACCCAAGCATC
TGGGATTTATGTTCCTACCCTCACCCTGTGTTGCCC
ACCTGAGCAACAACTAAGACTGATACTGAAATAAA
TCGTGTTAATCCCAAAAAAAAAAAAGCTT

Fig. 3. Nucleotide sequence of LiRG1, LiRG2, LiRG3 and LiRG5. Anchored oligo-dT and arbitrary decamers are underlined. Polyadenylation signal is marked with *. DNA sequence identities were determined using the BLASTN program.

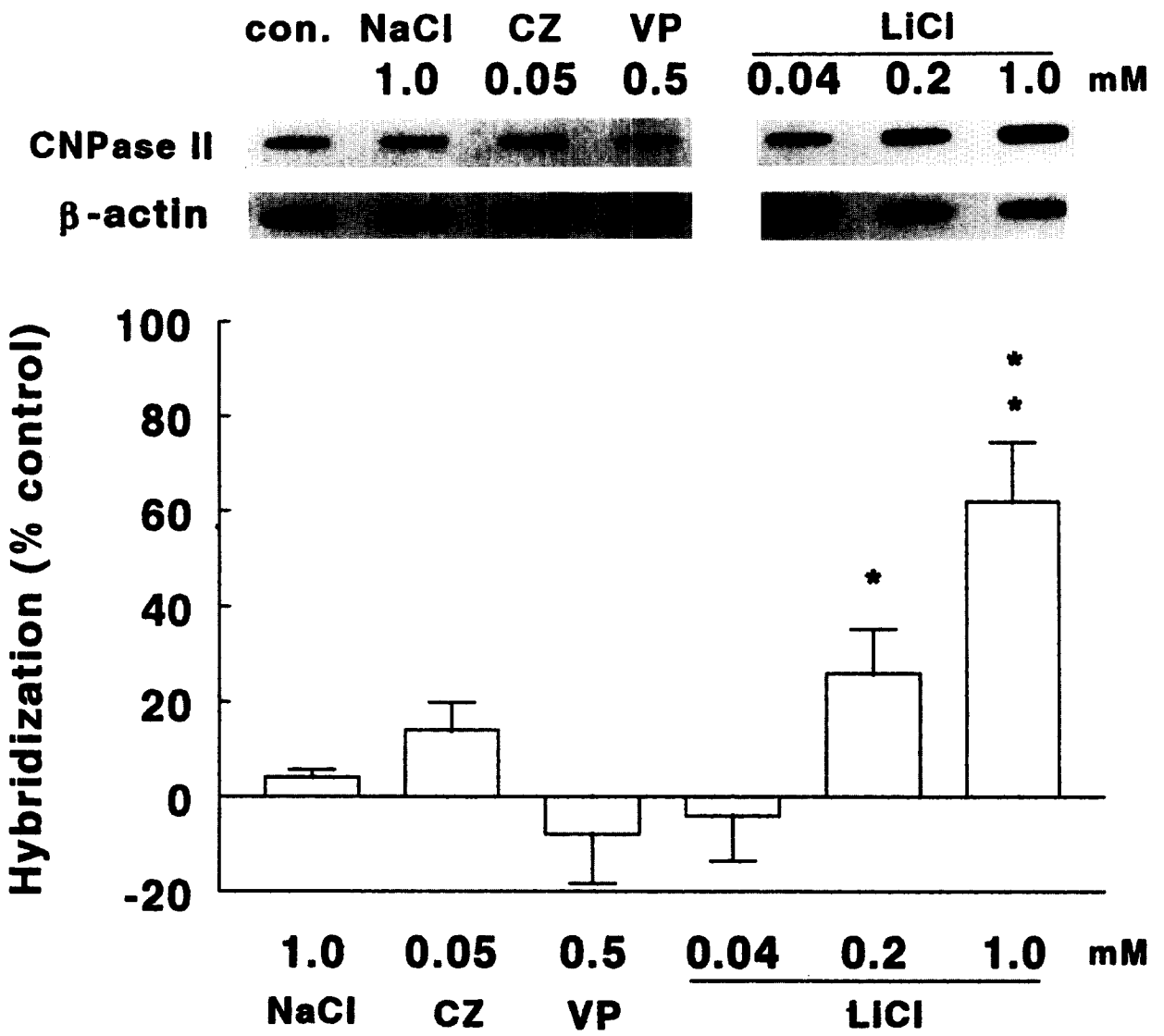

Fig. 4. Effect of sodium valproate (VP), carbamazepine (CZ) and lithium chloride (LiCl) on CNPaseII gene expression. C6 glioma cells were grown for 1 week under 1.0 mM NaCl, 0.5 mM sodium valproate, 0.05 mM carbamazepine and different concentration of LiCl (0.04, 0.2 and 1.0 mM). Control cells (con.) were treated identically but without addition of drugs. Total RNA (5 μg) was analyzed by slot blot hybridization using ^{32}P-dCTP labeled probe of CNPaseII. Autoradiograms were quantitated by densitometry. Results are the mean ± S.E.M. of triplicate determinations. $^{*}P<0.05$, $^{**}P<0.01$, when compared with control cells.

sis commonly present with mood instability reminiscent of BD, which in some cases may respond to mood stabilizing medications such as lithium [44,45]. Furthermore, a relationship between multiple sclerosis and depression or mania has been recognized in clinical and epidemiological studies [46,47]. Using magnetic resonance imaging several research groups have reported increased white matter signal intensity and decreased white matter volume in patients with BD compared with age-matched control subjects which illustrates the potential significance of white matter abnormalities to this illness [48-50]. The increased expression of CNPaseII by lithium treatment suggests that this treatment may have an effect on the process of myelination (and possibly neurodevelopment) which may have implications for BD in which white matter abnormalities may occur. It is not known at present what effect lithium treatment has on gene expression in neuronal cell lines or in mature brain, so the significance of the present results obtained in a glial cell line will await the result of future investigations. Further work clearly needs to be undertaken to clarify the potential significance of these novel findings.

In conclusion, chronic lithium treatment leads to the increased expression of several mRNAs in C6 glioma cells, of which one can at present be identified. The results of the present study demonstrate the potential utility of differential display PCR to study the mechanisms of action of psychotropic drugs and also in the identification of pathophysiologic changes in psychiatric disorders such as BD. Alterations in gene expression in brain by lithium are consistent with the current focus on the action of psychotropic drugs on long-term adaptational changes in brain due to alterations in gene expression rather than in changes in the level of specific monoamines or their metabolites.

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