Induction of Prostaglandin Endoperoxide Synthase-2 by Serine-Threonine Phosphatase Inhibition

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ABSTRACT

Regulation of prostaglandin endoperoxide synthase-2 (PGHS-2) mRNA levels by serine-threonine phosphatases was examined in murine fibrosarcoma methylcholanthrene-101 cells. Okadaic acid (OA), a serine-threonine phosphatase inhibitor, induced PGE$_2$ production and a significant increase in PGHS-2 immunoreactive protein. A specific PGHS-2 inhibitor, N-(2-cyclohexyloxy-4-nitrophenyl) methanesulphonamide, completely abolished the OA-mediated increase in PGE$_2$ production, which suggests that the PGE$_2$ formation in response to OA was derived from PGHS-2. OA-mediated PGHS-2 mRNA accumulation was observed at 1 hr, remained elevated for 24 hr and was blocked by actinomycin D, which indicates that OA increases PGHS-2 gene transcription. A significant post-transcriptional mechanism also contributed to the increased PGHS-2 mRNA accumulation, because the mRNA half-life was approximately 4 to 5 h in OA-stimulated cells. Tumor necrosis factor-$\alpha$, but not OA, activated transcription factor nuclear factor-$\kappa$B in methylcholanthrene-101 cells, as demonstrated by translocation of the nuclear factor-$\kappa$B complex to the nucleus and disappearance of the cytoplasmic inhibitory protein, I$\kappa$B-$\alpha$. We conclude that inhibition of serine-threonine phosphatases contributes to the up-regulation of PGHS-2 expression in an NF-$\kappa$B-independent manner.

PGHS is the enzyme that converts arachidonic acid to prostanoids. Expression of the inducible isoenzyme of this enzyme, PGHS-2, is highly regulated by inflammatory cytokines and growth factors (DeWitt and Meade, 1993; Evett et al., 1993; Fletcher et al., 1992; Ristimaki et al., 1994). We have previously shown that TNF-$\alpha$ increases PGHS-2 mRNA accumulation in fibrosarcoma MCA-101 cells and that transcriptional and post-transcriptional mechanisms contribute to the increase in PGHS-2 mRNA accumulation induced by this cytokine (Mahboubi et al., 1997, in press). Moreover, PGE$_2$ production in response to TNF-$\alpha$ is mainly dependent on PGHS-2 and is regulated by protein tyrosine kinases and phosphatases (Mahboubi et al., 1997, in press). The importance of serine-threonine protein phosphatases in TNF-$\alpha$ signal transduction pathways is suggested by the similarity in the pattern of phosphorylation of cellular proteins induced by TNF-$\alpha$ and OA, a noncompetitive inhibitor of PP2A and PP1 serine-threonine phosphatases (Guy et al., 1992; Fujiki and Sugaanuma, 1994). Moreover, Guy et al. reported that a serine/threonine phosphatase of primary human fibroblasts is inactivated by TNF-$\alpha$ and OA (Guy et al., 1993). OA acid inhibits PP1 about 50 to 100 times less than PP2A, leading to an increase in the phosphorylation of many cellular proteins (Guy et al., 1992), and induces expression of mRNA for many genes by enhancing gene transcription and/or increasing stability of mRNA (Cao et al., 1992; Xia et al., 1993; Kim et al., 1990; Pshenichkin and Wise, 1995). The contribution of serine-threonine phosphatases to the expression of PGHS-2 has not been determined.

Increased protein phosphorylation by OA activates several transcription factors, including NF-$\kappa$B (Menon et al., 1995; Rieckmann et al., 1992; Suzuki et al., 1994; Menon et al., 1993), AP-1 (Rieckmann et al., 1992; Thevenin et al., 1991), Sp1 (Vlach et al., 1995) and CRE (Wadzinski et al., 1993), all of which are regulated by phosphorylation. NF-$\kappa$B is a transcription factor that regulates the expression of a variety of genes and exists in the cytoplasm of resting cells as a heterodimer composed of two polypeptides of 50 kDa (p50) and 65 kDa (p65), which are associated with a cytoplasmic inhibitory protein, I$\kappa$B-$\alpha$ (Siebenlist et al., 1994). Upon stimulation with a variety of inducers, including TNF-$\alpha$ (Miayamoto et al., 1994; Finco et al., 1994; Beg et al., 1993) and OA (Menon...
et al., 1995; Rieckmann et al., 1992; Suzuki et al., 1994; Menon et al., 1993), IκB-α is phosphorylated and subsequently degraded, which makes possible the translocation of NF-κB to the nucleus, where it binds to κB consensus sequences and initiates gene transcription. A binding site for transcription factors may differentially contribute to regulation of Biomol (Plymouth Meeting, PA). The PGHS-2 cDNA probe was obtained from the American Type Culture Collection (ATCC). Recombinant mouse TNF-α was purchased from Genentech, Inc. (Palo Alto, CA) at 1 µg/ml in blocking solution for 45 min and washed twice for 7 min with TBST. Membranes were incubated with HRP-conjugated goat-anti rabbit IgG (1:10,000 dilution) as the secondary antibody (Amersham, Arlington Heights, IL) for 1 hr at room temperature. Membranes were washed with TBST and washed for 30 min at room temperature. Membranes were washed with TBST, and IκB-α protein was detected by the ECL system.

Western blot analysis of IκB-α. Cell lysates and gel electrophoresis were performed as described in the Methods section. After blocking, membranes were incubated with rabbit anti-human IκB-α antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1 µg/ml in blocking solution for 45 min and washed twice for 7 min with TBST. Membranes were incubated with HRP-conjugated goat-anti rabbit IgG (1:10,000 dilution) as the secondary antibody (Amersham, Arlington Heights, IL) for 1 hr at room temperature. Membranes were washed with TBST, and IκB-α protein was detected by the ECL system.

Total RNA isolation and Northern blot analysis. Confluent, quiescent cells were incubated in the absence of presence of OA in media containing 0.5% serum. After various incubation periods, media were removed and the cell monolayers washed twice with ice-cold PBS. Total cellular RNA was isolated by lysing the cells in guanidine isothiocyanate sodium citrate buffer and extracting RNA with ethanol as described previously (Kamdar and Evans, 1992). Then 10 µg of RNA was electrophoresed in a 1% agarose/formaldehyde gel in 1X MOPS (3-[N-morpholino]propane sulfonic acid) buffer at 25°C for 1 hr. RNA was transferred to a nylon membrane (Genescreen, DuPont-New England Nuclear, Boston, MA) and hybridized to a randomly primed 32P-labeled cDNA probe in buffer containing 50% formamide, 10% dextran sulfate, 0.2% polyvinylpyrrolidone, 0.2% ficol, and 0.2% bovine serum albumin, 1.0 M NaCl, 1.0% SDS, 0.05 M Tris, pH 7.5 and 0.1% sodium phosphate at 42°C for 24 hr. After hybridization, the membrane was washed with 2X SSC (standard sodium citrate), 1.0% SDS at 65°C for 1 hr and with 0.1% SSC at 25°C for 1 hr. Then the probed blots were exposed at −70°C to XAR-5 X-ray film (Eastman Kodak, Rochester, NY).

Nuclear extraction and EMSA. Nuclear protein extracts were prepared following the method of Schreiber et al. (1989). Cells were washed twice in ice-cold PBS and then harvested in buffer A (20 mM HEPES, pH 8.0, 0.32 M sucrose, 2.0 mM CaCl2, 2.0 mM MgCl2, 0.1 mM EDTA, 0.5% Nonidet P-40, 1.0 mM DTT (dithiothreitol), 0.25 mM PMSF, 1 µg/ml leupeptin), and nuclei were pelleted by centrifugation at 1500 × g for 5 min at 4°C. Pelleted nuclei were resuspended in 50 µl of buffer B (20 mM HEPES, pH 8.0, 25% glycerol, 0.42 M NaCl, 2 mM MgCl2, 0.2 mM EDTA, 1.0 mM DTT, 0.25 mM PMSF, 1 µg/ml leupeptin). Nuclei pellets were gently mixed and incubated on ice for 15 min. Nuclear debris was removed by centrifugation for 15 min at 10,000 × g, and nuclear protein concentration was measured by the Bradford method.

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Materials and Methods

Cell lines and reagents. MCA-101 (a kind gift from Dr. Nicholas Restifo, NCI) is a fibrosarcoma cell line of B6 origin that was generated in 8-week-old female C57BL/6n (B6) mice by i.m. injection of OA. The tumor cell lines were passaged in B6 mice (Restifo et al., 1992). Tumors were harvested from mice, digested and maintained in monolayer culture in media consisting of RPMI 1640, 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 mg/ml), and gentamicin (1 µg/ml in blocking solution for 45 min and washed twice for 7 min with TBST). Membranes were incubated with HRP-conjugated goat-anti rabbit IgG (1:10,000 dilution) as the secondary antibody (Amersham, Arlington Heights, IL) for 1 hr at room temperature. Membranes were washed with TBST and washed for 30 min at room temperature. Membranes were washed with TBST, and IκB-α protein was detected by the ECL system.

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Five nanomoles of double-stranded NF-κB oligonucleotide (5’...AGTTGAGGGAGCTTCCAGGC, Promega, Madison, WI) was 5’-end-labeled using [γ-32P]ATP (specific activity 3000 Ci/mmol, Amersham, Arlington Heights, IL) and T4 polynucleotide kinase (Clontech, Palo Alto, CA). The unincorporated [γ-32P]ATP was separated from labeled probe by electrophoresis in a 15% polyacrylamide gel. The labeled NF-κB was extracted with phenol/chloroform followed by ethanol precipitation. The final pellet was dissolved in Tris-EDTA buffer, pH 7.4. Binding reaction mixtures containing 20 μg of protein of nuclear extract, 32P-labeled NF-κB probe and 2 μg of calf thymus DNA in binding buffer (20 mM HEPES, pH 8.0, 5 mM DTT, 0.2 mM EDTA, 0.2 mM PMSF, 2 mM MgCl2, 10% glycerol, 1 μg/ml leupeptin) were incubated at room temperature for 20 min. Samples were analyzed by using native 6% polyacrylamide gels followed by autoradiography.

Cytotoxicity assay. MCA-101 cells were cultured in 96-well plates (2.5 × 10^5 per well) overnight. The following day, media were removed, and new media containing various drugs or media were added for 4 hr. At the end of the incubation periods, the viability of the cells was determined by assaying their metabolic capacity using MTT (Mosmann, 1983). Briefly, MTT at 1 mg/ml was added to all the wells. After a 2-hr incubation at 37°C/5% CO₂ in the presence of MTT, acidified isopropanol was added to each well, and the plates were read using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Results

OA increases PGE₂ synthesis via PGHS-2. OA was used to investigate whether intracellular serine-threonine phosphatases regulate the expression of PGHS-2. Our first approach was to determine the effects of OA on PGHS-2 synthesis. Confluent, quiescent cells were incubated with media containing 0.5% serum (control) or 100 nM OA for 24 hr. OA at a concentration of 100 nM was selected because it inhibited approximately 80% of total phosphatase activity in MCA-101 cell lysates (data not shown). Treatment with OA significantly increased PGE₂ synthesis (fig. 1) and did not affect cell viability, as determined by MTT assay and trypan blue exclusion. We determined whether the OA-induced PGE₂ production was associated with increased PGHS-2 activity in experiments in which MCA-101 cells were incubated with OA in the presence of NS-398, a selective inhibitor of PGHS-2 (Futaki et al., 1994; Copeland et al., 1994). NS-398 (0.01 μM) completely abolished the OA-mediated PGE₂ production but had no effect on PGE₂ production by unstimulated cells (fig. 1). Thus, OA-induced PGE₂ synthesis may have occurred via a PGHS-2-dependent mechanism.

Time- and dose-dependent increase in PGHS-2 protein expression. Confluent, quiescent cells were incubated with media in the presence or absence of 100 nM OA for various times. After incubation with OA, cells were lysed and analyzed for PGHS-2 protein by Western blot analysis using a specific PGHS-2 antibody. As shown previously (Mahboubi et al., 1997, in press), low levels of PGHS-2 protein were detected in unstimulated cells (fig. 2A). After challenge with OA, increased levels of PGHS-2 protein were observed at approximately 4 hr and remained elevated up to 24 hr (fig. 2A). Dose-response experiments with OA revealed that 10 nM OA significantly induced PGHS-2 protein expression and that 0.1 nM OA did not increase PGHS-2 protein levels (fig. 2B). OA at 10 nM and 0.1 nM inhibited phosphatase activity in MCA-101 cell lysates by approximately 60% and 50%, respectively (data not shown). Expression of PGHS-2 was maximal in response to 50 nM OA (fig. 2B). OA increases PGHS-2 mRNA accumulation in a time-dependent manner. We investigated whether accumulation of PGHS-2 mRNA could contribute to the augmentation of PGE₂ synthesis and PGHS-2 protein expression observed after stimulation of MCA-101 cells with OA. Confluent, quiescent cells were incubated with OA, and total RNA was isolated at zero (control), 1, 5, 8, 12, and 24 hr after the addition of OA (100 nM). PGHS-2 mRNA (4.2 kb) was detected in unstimulated cells; mRNA accumulation was similar at each of the time-points tested (fig. 3A). PGHS-2 mRNA accumulation was evident within 1 hr after stimulation with OA, increased dramatically up to 12 hr and was followed by

![Fig. 1.](image1.png)

![Fig. 2.](image2.png)
a slight reduction at 24 hr (fig. 3A). Calyculin A, another inhibitor of PP1 and PP2A, increased PGHS-2 mRNA accumulation in MCA-101 cells (fig. 3B).

OA increases PGHS-2 mRNA accumulation by transcriptional and post-transcriptional mechanisms. The enhancement of PGHS-2 mRNA accumulation after OA treatment may be explained by enhanced gene transcription and/or stabilization of message. To test the first possibility, we arrested transcription with ACD treatment before the addition of OA and assessed PGHS-2 mRNA levels by Northern blot analysis using a specific 32P-labeled cDNA probe for PGHS-2. RNA quantity and integrity were verified by ethidium bromide staining of 28S and 18S ribosomal RNA. These figures are representative of three similar experiments.

OA inhibits OA-induced PGHS-2 mRNA accumulation in MCA-101 cells. Confluent, quiescent cells were preincubated with ACD (1.0 μM) for 30 min before the addition of 100 nM OA for 5 hr. After incubation, total RNA was isolated, and PGHS-2 mRNA levels were assessed by Northern blot analysis using a specific 32P-labeled cDNA probe for PGHS-2 (upper panel). RNA quantity and integrity were verified by ethidium bromide staining of 28S and 18S ribosomal RNA (bottom panel). This figure is representative of three similar experiments.

1.0 μM ACD. Cells were harvested at different times after the addition of ACD to assess mRNA half-life. The levels of PGHS-2 mRNA induced by OA decreased as a function of time in the presence of ACD (fig. 5). Because ACD blocks gene transcription, the amount of mRNA remaining at the various time-points is an index of mRNA half-life. PGHS-2 mRNA was quantified by scanning densitometry, using β-actin as an internal control. This figure is representative of three similar experiments.
mRNA half-life of OA-stimulated cells was approximately 4 to 5 hr (fig. 5). We previously showed that the half-life of PGHS-2 mRNA in phorbol 12-myristate 13-acetate-stimulated MCA-101 cells is approximately 1 hr (Mahboubi et al., 1997, in press). These data suggest that OA-dependent enhancement of the steady-state levels of PGHS-2 mRNA is associated with transcriptional activation and stabilization of the message.

**Effects of OA on NF-κB activation in MCA-101 cells.** OA activates NF-κB in a variety of cell types, including Jurkat T cells and fibroblasts (Menon et al., 1995; Suzuki et al., 1994; Menon et al., 1993; Sun et al., 1995). We evaluated the effects of OA on NF-κB activation in MCA-101 cells. Jurkat cells (positive control cell line) and MCA-101 cells were stimulated with 100 nM OA for 1, 2, or 4 hr. After incubation, cells were lysed, and NF-κB activity was measured by EMSA. TNF-α is a potent activator of NF-κB in a wide variety of cell types (Miyamoto et al., 1994; Fincs et al., 1994; Beg et al., 1993; Henkel et al., 1993), so nuclear extracts from TNF-α-stimulated MCA-101 cells were used as a positive control for NF-κB activation. OA and TNF-α increased NF-κB DNA binding activity in Jurkat T cells (fig. 6). In contrast, NF-κB binding activity was detected in nuclear extracts prepared from TNF-α, but not OA-stimulated MCA-101 cells (fig. 6). Moreover, no NF-κB binding activity was detected in MCA-101 nuclear extracts after challenge with 500 nM OA for a range of different time periods (data not shown). These results suggest that OA does not activate NF-κB in MCA-101 cells.

**OA does not stimulate IκB-α degradation in MCA-101 cells.** Activation of NF-κB from cytoplasmic pools should be associated with proteolytic degradation of the inhibitory IκB-α, according to known mechanisms of NF-κB activation. To confirm that OA does not activate NF-κB in MCA-101 cells, we determined its effects on IκB-α protein degradation. Confluent, quiescent MCA-101 cells were incubated with media (control) or 100 nM OA for various times. After incubation, cells were lysed, and IκB-α protein was assessed by Western blot analysis using a specific IκB-α protein antibody. IκB-α protein was present in unstimulated cells (control) (fig. 7A). There was no change in IκB-α protein levels after treatment with OA (fig. 7A). On the other hand, IκB-α protein levels were slightly or completely reduced, respectively, after a 5- or 10-min stimulation with TNF-α (fig. 7B). Complete degradation of IκB-α protein, after a 15-min treatment with TNF-α, resulted in the activation of NF-κB and the translocation of NF-κB heterodimer to the nucleus (fig. 7B; fig. 6). After a 30-min stimulation with TNF-α, IκB-α protein was again detected, because of its NF-κB-dependent synthesis. OA causes IκB-α protein degradation in Jurkat cells (fig. 7C) and induces translocation of NF-κB to the nucleus (fig. 6). These results are in agreement with the data obtained by EMSA and indicate that OA does not induce degradation of IκB-α protein, a prerequisite for activation of NF-κB.

**Discussion**

We demonstrated that the inhibition of PP1 and PP2A by OA increased PGHS-2-dependent PGE₂ production, PGHS-2 protein expression, PGHS-2 gene transcription and PGHS-2 mRNA stability. These data are the first to show that inhibition of serine-threonine phosphatases up-regulates the expression of PGHS-2. Moreover, inhibition of PP1 and PP2A is not sufficient to activate NF-κB in MCA-101 cells and there-
fore increases PGHS-2 by an NF-κB-independent mechanism.

Treatment of MCA-101 cells with 100 nM OA, a dose that inhibited phosphatase activity in vitro by approximately 80% (data not shown), markedly increased PGE_{2} synthesis by these cells. Ohuchi et al. (1989) showed that OA increased PGE_{2} production in macrophages, an effect that was inhibited in the presence of cycloheximide, which suggests that protein synthesis was needed for the stimulation of arachidonic acid metabolism (Ohuchi et al., 1989). These authors did not determine the relative contribution of PGHS-1 and PGHS-2 in OA-induced PGE_{2} synthesis. OA failed to increase PGE_{2} synthesis in the presence of NS-398, a result that indicates a possible role for PGHS-2 in the OA-mediated increase in PGE_{2} synthesis in MCA-101 cells. Interestingly, OA increased PGHS-2 protein synthesis and PGHS-2 mRNA levels without affecting PGHS-1 protein levels in MCA-101 cells (data not shown). Induction of PGHS-2 mRNA by OA was inhibited in the presence of ACD, which indicates that OA increases PGHS-2 mRNA by activating transcription of the PGHS-2 gene. These results suggest that inhibition of PP1 and PP2A may be important in regulating PGHS-2 gene transcription in MCA-101 cells. Because NF-κB was not activated by OA in MCA-101 cells, a role for this transcription factor in OA-induced PGHS-2 gene transcription in these cells seems unlikely. However, TNF-α caused degradation of IκB-α protein and activation of NF-κB and has previously been linked to the increase in PGHS-2 mRNA in MCA-101 cells (Mahboubi et al., 1997, in press). NF-κB is involved in the TNF-α-dependent induction of PGHS-2 in MC3T3-E1 cells (Yamamoto et al., 1995). Thus, although NF-κB may be involved in the TNF-α-mediated increase in PGHS-2 in MCA-101 cells, it is not required for the OA-mediated increase in PGHS-2 mRNA accumulation. OA has been shown to activate Sp1 (Vlach et al., 1995) and CRE (Wadzinski et al., 1993), two transcription factors that are present in the PGHS-2 promoter (Tazawa et al., 1994). CRE was shown to act as a positive regulatory element for PGHS-2 gene transcription (Xie et al., 1994; Inoue et al., 1994). Activation of these transcription factors by OA may be responsible for OA-induced PGHS-2 gene transcription in MCA-101 cells. The effects of OA on the activation of CRE and Sp1 and other transcription factors, which may play a role in regulating transcription of PGHS-2, remain to be determined.

The 3′-untranslated region of the mouse PGHS-2 mRNA has multiple AU repeats responsible for shortening the half-life of many unstable mRNAs (Kujubu et al., 1991; Malter, 1989). The stability of AU-rich mRNAs is regulated by the activity of proteins designated AUBF. Phosphorylation of AUBF results in its activation and binding to labile mRNAs and the formation of a complex that is resistant to degradation (Malter and Hong, 1991). Previously, we have shown that TNF-α increases PGHS-2 mRNA half-life in MCA-101 cells (Mahboubi et al., 1997, in press). Stephens et al. (1992) demonstrated that OA and TNF-α up-regulate AUBF activity in 3T3-L1 preadipocytes cells, which subsequently results in stabilization of glucose transporter mRNA for in these cells. As shown in the present study, OA enhances PGHS-2 mRNA stability, which suggests that an increase in total serine-threonine phosphorylation by OA decreases the rate of degradation of PGHS-2 mRNA and contributes to its accumulation. Therefore, increased activity of an unidentified AUBF by OA and TNF-α may increase PGHS-2 mRNA stability in MCA-101 cells.

OA activates NF-κB in several cell types, including Jurkat T cells (Suzuki et al., 1994; Sun et al., 1995). However, OA did not activate NF-κB or induce degradation of IκB-α in MCA-101 cells. Taken together, these data suggest that inhibition of serine-threonine phosphatases by OA is not sufficient for the release of NF-κB from its inhibitor, IκB-α, in MCA-101 cells. Serine phosphorylation of IκB-α, an event that is essential for its degradation (Miyamoto et al., 1994), is achieved by an increase in serine-threonine kinase activity and/or inhibition of serine-threonine phosphatase activity. However, inhibition of phosphatases increases IκB-α phosphorylation only if kinases are activated. Therefore, it is possible that the serine-threonine kinase responsible for phosphorylation of IκB-α is not active in unstimulated MCA-101 cells. Interestingly, previous reports have suggested that OA-induced activation of NF-κB is dependent on the endogenous redox status of cells. For instance, Menon and co-workers (1993) found that OA was unable to activate NF-κB in primary fibroblasts and that oxidizing agents made possible the induction of NF-κB by OA in these cells. Thus the inability of OA to activate NF-κB in MCA-101 cells may be due to high intracellular levels of antioxidant molecules. In contrast, OA-mediated NF-κB activation in Jurkat cells, which are highly susceptible to changes in the intracellular redox state, may have been due to low intracellular levels of antioxidants (Droge et al., 1994).

In this study, we illustrated the importance of serine-threonine phosphorylation to the regulation of PGHS-2 mRNA expression. We previously showed that signal transduction via protein tyrosine kinase(s) and protein tyrosine phosphatase(s) is required for TNF-α-mediated increases in PGHS-2 mRNA accumulation in MCA-101 cells (Mahboubi et al., 1997, in press). Thus more than one signaling pathway may initiate PGHS-2 gene transcription in MCA-101 cells. Moreover, Guy et al. suggested that TNF-α signal transduction may involve the tyrosine phosphorylation of PP2A, an event that causes inactivation of PP2A (Guy et al., 1995; Guy et al., 1993; Chen et al., 1992). Therefore, it is also possible that inhibition of PP2A by TNF-α is an important event in the TNF-α-mediated increase in PGHS-2 mRNA accumulation in MCA-101 cells.

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References


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