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Molecular Mechanism of NO-induced Cell Death of PC12 Cells by IFNγ and TNFα

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Abstract

Nitric oxide (NO) is a small, diffusible, and highly reactive molecule, which plays dichotomous regulatory roles under physiological and pathological conditions. NO promotes apoptosis in some cells, and inhibits apoptosis in other cells. In the present study, we attempted to characterize the NO signaling pathway and cellular response in PC12 cells treated with cytokines. IFN-γ/TNF-α treatment resulted in a synergistic increase of nitrite accumulation, with the induction of inducible nitric oxide synthase (iNOS) in the PC12 cells. Moreover, as nitrite concentration increased, cell viability decreased. In order to explore MAP kinase involvement in nitric oxide production resultant from IFN-γ/TNF-α stimulation, we measured the activation of MAP kinase using specific MAP kinase inhibitors. PC12 cells pretreated with SB203580, a p38 MAP kinase-specific inhibitor, resulted in the inhibition of iNOS expression and NO production. However, PD98059, an ERK/MAP kinase-specific inhibitor, was not observed to exert such an effect. In addition, Stat1 activated by IFN-γ/TNF-α was interacted with p38 MAPK. These data suggest that p38 MAP kinase mediates cytokine-mediated iNOS expression in the PC12 cells, and Jak/Stat pathway interferes with p38 MAPK signaling pathway.

Keywords: PC12 cells, nitrite oxide, p38 MAPK, Stat1, capillary electrophoresis

Nitric oxide (NO) is a diffusible free radical species that is both a signaling molecule and a cytotoxic agent, which has been demonstrated to modulate a variety of cellular and physiological processes[1-3]. Increased levels of NO can potentially lead to nitrosative stress, and it affect to normal cellular functions[4]. For example, excessive NO were produced by astroglia and microglia cells following bacterial infections for defending invading pathogens, but it exerts cytotoxic effects concurrently[5]. NO is synthesized in mammalian cells by family of NO synthases (NOSs)[6]. Three isoforms of NOS have so far been reported to exist. Endothelial NOS (eNOS) and a neuronal NOS (nNOS) are constitutively expressed and Ca^{2+}-dependent. However, inducible NOS (iNOS) is rapidly transcribed and expressed in macrophages, microglia, and astrocytes, preceded by stimulation with bacterial lipopolysaccharides (LPS) or pro-inflammatory cytokines, including tumor necrosis factor alpha (TNFα), interleukin-1beta (IL-1β), and interferon gamma (IFNγ). iNOS functions in a calcium independent manner, and generates large amounts of NO for prolonged periods[7,8].

Mitogen activated protein kinases (MAPKs) have been considered to be an important regulators of a wide range of genes involved in cellular responses to pro-inflammatory and other stress signals as well as iNOS up-regulation induced by extensive stimuli in various cell types. Two of most investigated MAPKs involved in pro-inflammatory cytokine-mediated responses are ERK and p38 MAPK[9-12]. On the other hands, the synergism of IFNγ and TNFα has been extensively pursued[13]. Recently, we reported that IFNγ and TNFα synergically induced neurite outgrowth of PC12 cells[14] and it was also suggested that the novel level of cross-talk between IFNγ and TNFα through control of Stat1 availability[15]. Based on these observations, we investigated the combined effects of IFNγ and TNFα on Stat1 activation, MAPK activation and NO production through iNOS expression in the PC12. The goal of this study was to evaluate interrelation of MAPK and Stat1 in the context of synergistic NO production by IFNγ/TNFα in the PC12 cells.

Relationship between Nitric Oxide Production and PC12 Cell Viability

To explore the possible involvement of nitrosative stress as an alternative mechanism of cytokine mediated PC12 cell death, NO production was asse-

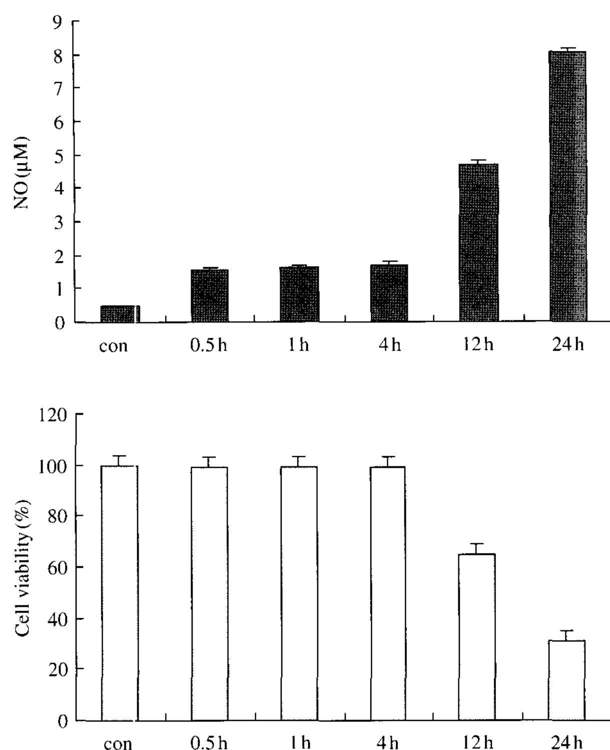

Fig. 1. IFNγ and TNFα induced nitrosative stress in PC12 cells. The cells were treated with 50 ng/mL IFNγ and 50 ng/mL TNFα for the indicated times. (A) Nitrite accumulation was measured with Griess reagents. (B) The PC12 cell viability was determined using MTT assay.

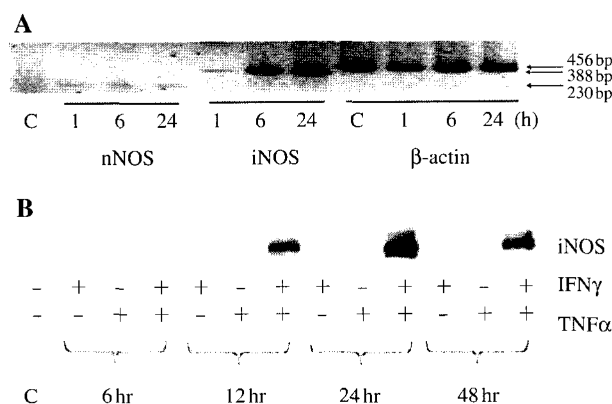

Fig. 2. IFNγ and TNFα induced iNOS expression in the PC12 cells. Cells were treated with 50 ng/mL IFNγ and 50 ng/mL TNFα and cultured for indicated times. (A) Total RNA was extracted and semi-quantitative RT-PCR was performed. The products were analyzed on 1% agarose gel electrophoresis. (B) Soluble protein was extracted from treated cells and western blotting was performed with iNOS antibody (I: IFNγ, T: TNFα).

ssed as the accumulation of NO_2^- in the culture supernatants, using a colorimetric reaction with Griess reagent. The PC12 cells were treated with a combination of 50 ng/mL IFNγ and 50 ng/mL TNFα for various time lengths. As shown in Figure 1-A, nitrite levels were enhanced with increasing incubation periods, and nitrites production was peaked at 12 hours. However, PC12 cell viability decreased to 35% at 12 hours (Fig. 1-B). The viability of the PC12 cells decreased with time, as well as the nitrite concentrations of cytokine mixtures. This decrease in cell viability is attributable to oxidative damage from the higher nitrite concentrations induced by cytokines.

Identification of iNOS Expression in PC12 Cells under Synergistic Stimulation

In order to clear the NO production mechanism by cytokines, expression of iNOS was measured at mRNA and protein levels by RT-PCR and Western blot analysis. When PC12 cells were treated with a combination of 50 ng/mL IFN-γ and 50 ng/mL TNFα, iNOS mRNA was induced profoundly 4 hours after treatment, and accumulated further thereafter. nNOS expression was constitutive in the PC12 cells, even though nNOS was rarely detectable (Fig. 2-A). In Fig. 2-B, iNOS protein expression became to appear by the mixture of these cytokines after 6 hours and then it was peaked at 24 hours. It is suggested that these cytokines induced nitrite production through iNOS induction at mRNA as well as protein level.

p38 MAPK has Important Role in iNOS Expression by Cytokines

According to above results, IFNγ and TNFα synergistically induced iNOS expression and NO production. In order to ascertain whether or not MAPK was involved in stimulation with IFNγ/TNFα, which induced iNOS expression, p38 MAPK inhibitor, SB203580, and ERK inhibitor, PD98059, were added prior to the addition of IFNγ/TNFα. SB203580 exerted a substantial preventive effect on iNOS expression (Fig. 3-A), and PD98059 did not exert an inhibitory effect on iNOS expression in response to costimulation with IFNγ and TNFα. Therefore, it has been suggested that p38 MAPK is the main target of IFNγ/TNFα with regard to iNOS expression, but this is not ERK mediated. On the other hand, IFNγ/TNFα activated Stat1 at both serine and tyrosine residue. Although both SB203580 and PD98059 could not inhibit stat1 phosphorylation, AG490, Jak inhibitor, interfered in p38 phosphorylation by these cytokines. It suggested that p38 MAPK pathway interacted with Jak/Stat pathway by IFNγ/TNFα in PC12 cells. In addition, we examined NO production in response to cytokines

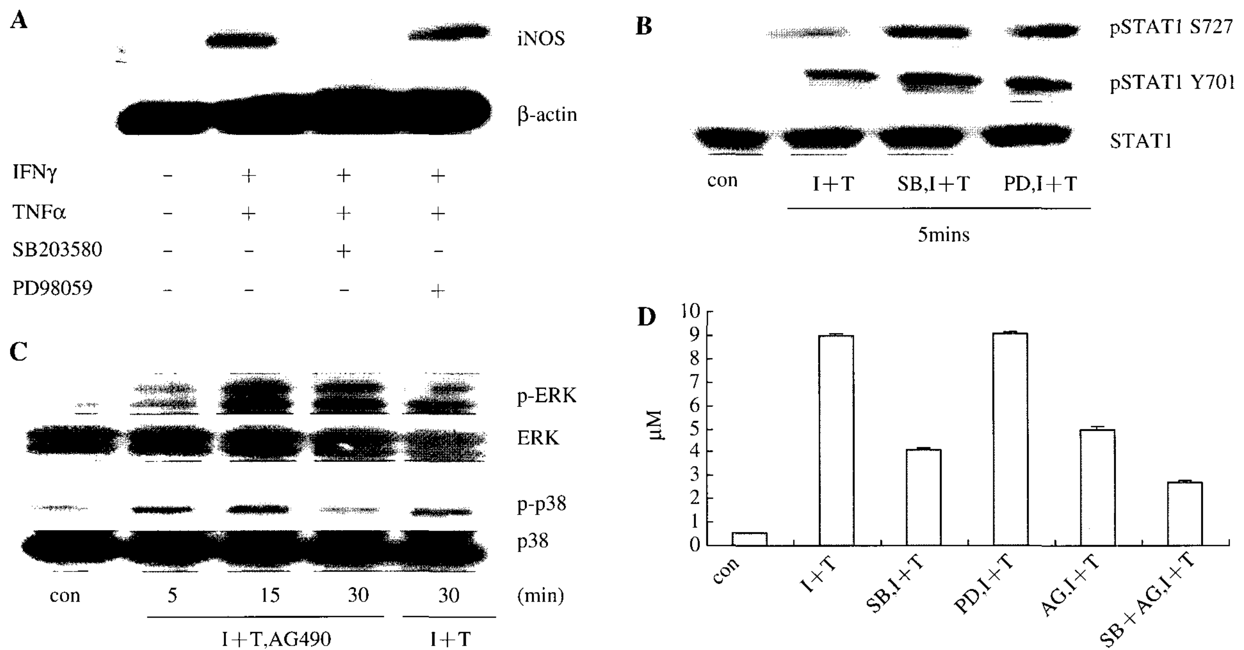

Fig. 3. MAPK and Stat activation in cytokine treated PC12 cells. Cells were treated with 50 ng/mL IFNγ and 50 ng/mLTNFα and cultured for indicated times following treatment with SB203580 (10 μM), PD98059 (10 μM), AG490 (10 μM) for 45 mins. Soluble protein was extracted from treated cells and western blotting was performed with (A) iNOS protein expression (B) Stat1 phosphorylation (C) p38 and ERK activation antibody (I : IFNγ, T : TNFα). (D) Nitirite accumulation was measured with Griess reagents.

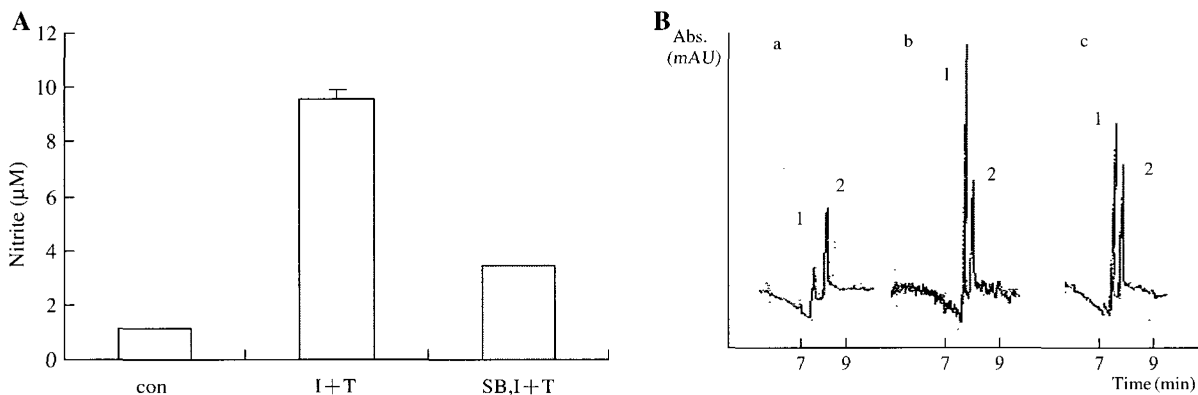

Fig. 4. Nitrite production was detected with the (A) Griess reagent method and the (B) capillary electrophoresis (CE) method. CE electropherogram of nitrite in PC12 cell cultured media was obtained as described in methods and materials. (a) media ONLY (RPMI 1640) (b) with 50 ng/mL IFNγ and 50 ng/mL TNFα (c) treated 10 μM SB203580 prior to 50 ng/mL IFNγ and 50 ng/mL TNFα. Peak 1: Nitrite, Peak 2: internal standard.

or/and kinase inhibition. While ERK was not also involved in NO production as well as in iNOS expression, the inhibition of p38 or Jak/Stat pathway was effectively reduced NO production. Moreover, both inhibition of p38 and Jak/Stat showed additive effect on decrease of NO production. Accordingly, we found the crosstalk between p38 and Jak/Stat pathway in cytokine mediated NO production mechanism of PC12 cells.

Verification of NO Production via Capillary Electrophoresis (CE)

Capillary electrophoresis (CE) provides better specificity and faster ion analysis (NO_2^-), in both aqueous

and biological samples[18]. Therefore, we tried the verification of NO production by CE method in PC12 cells. We obtained the consistent CE data with nitrite assay data using Griess reagent (Fig. 4). CE method was very sensitive and represented the NO production clearly.

Discussion

The short-lived nitric oxide (NO) has emerged as a potent inhibitor as well as inducer of apoptosis. Although NO plays key signaling roles in the nervous systems, excess NO leads to cell death. Recently, cytoprotective role of NO in neuron system has been reviewed[19] and it was also reported that low NO production caused by nNOS and eNOS inhibited apoptosis[20]. On the other hand, several groups reported NO-dependent apoptosis in macrophage, pancreatic islet, neurons, and PC12 cells[21,22]. In addition, Sarker KP *et al.* demonstrated that NO donor-induced apoptosis in human neuroblastoma (NB-1) and PC12 cells[23,24]. In this report, we concentrated in cytokine (IFNγ/TNFα) mediated NO production and its mechanism, which cause PC12 cell death. Firstly, we assessed NO production by IFNγ/TNFα. After 12 hours, nitrite induction was at peaked and cell viability became decrease simultaneously. It is consistent with previous report, which demonstrated that p38 MAPK is involved in NO-induced PC12 cell death[25,26]. It is believed that NO synthase, eNOS and nNOS, produce low levels of NO, which inhibit apoptosis, but iNOS produces a much greater level of NO, which cause apoptosis[27,28]. Therefore, we examined iNOS mRNA and protein level, since cytokine-mediated NO caused cell death. Indeed, nNOS mRNA level was constitutive with low level by cytokine treatment. In Fig. 2, iNOS mRNA level was remarkably increased at 4 hours and prolonged up to 24 hours. Consistent with this result, the increase of iNOS protein expression was also detected from 6 hours post-stimulation with cytokines. Additionally, the results definitely demonstrated that iNOS expression was synergically induced by the combine treatment of these cytokines. Accordingly, IFNγ and TNFα increased NO production caused by iNOS expression, and then PC12 cell death was occurred by NO-induced nitrosative stress.

A great number of studies have shown that activation of p38 MAPK and/or JNK is positively linked to oxidative stress induced apoptosis[29,30]. We investigated the involvement of MAPK in cytokine mediated NO production mechanism. SB203580, p38 MAPK inhibitor, completely inhibited iNOS expression by IFNγ/TNFα (Fig. 3-A), and then NO production was also decreased (Fig. 3-D). Therefore, it is suggested that p38 MAPK is crucial for cytokine mediated NO production mechanism of PC12 cells. On the other hand, we assessed Stat activation and interaction with MAPK, since IFNγ generally activate Jak/Stat signaling pathway[31]. Moreover, it was reported recently that Stat1 activation by IFNγ enhances TNFα-dependent NFκB activation, which leads regulators of apoptosis and cell proliferation[15]. We observed that Stat1 was phosphorylated at both serine and tyrosine residue by IFNγ/TNFα, and it was affected by neither SB203580 nor PD98059 (Fig. 3-B). However, not ERK but p38 MAPK activation was attenuated by AG490, Jak inhibitor, at 30 min after treatment with IFNγ/TNFα (Fig. 3-C). In addition, NO production by IFNγ/TNFα was also reduced by AG490 and additive inhibitory effect was shown by SB203580 and AG490 (Fig. 3-D). In order to verify NO production, we tried capillary electrophoresis, which are developed by our group previously[18] and p38 MAPK involvement in NO production pathway was observed definitely. Therefore, we suggest that CE method is potent tool to detect NO production.

In conclusion, p38 MAPK was found as an essential element in cytokine mediated NO production pathway of PC12 cells. Interestingly, Jak/Stat pathway activated by cytokines interfered with p38 MAPK signaling pathway. While NO production was observed neither by IFNγ nor TNFα, co-stimulation with these cytokines significantly mediated NO production. So that, Jak/Stat would regulate NO production mechanism by attenuation of p38 MAPK activation, it accounts for the synergism of IFNγ and TNFα in No-induced PC12 cell death.

Methods

Materials

Recombinant rat IFN-γ and TNF-α were purchased from R & D Systems (Minneapolis, MN, USA). SB203580, PD98059, and AG490 were purchased from Calbiochem-Novabiochem (San Diego, CA, USA). Anti rabbit polyclonal phospho p38, p38, phospho p42/p44 ERK, p42/p44 ERK, phospho stat1 (Y701), and stat1 antibodies were purchased from New England Biolabs (Beverly, MA, USA). Rabbit polyclonal anti-phospho-STAT1 (S727) was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Mouse monoclonal anti-β-actin antibody and rabbit anti-nitric oxide synthase II polyclonal antibody were purchased from Sigma and Chemicon International, respectively. MTT reagent, 3-(4, 5-dimethyl-2-

thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide and Griess reagent were purchased from Sigma (St. Louis, MO, USA). The ECL-Plus detection system was purchased from Amersham (Piscataway, NJ, USA).

Cell Culture

The PC12 cells were obtained from the American Type Cell Collection (Rockville, MD, USA), and maintained in RPMI1640 medium supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, and 1% penicillin/streptomycin, at 37°C in 5% CO_2. For experiments, the cells were plated onto collagen-coated 60-mm-diameter dishes (2×10^6 cells/mL) or 12-well plates (2×10^5 cells/mL).

Analysis of NO_2^- Accumulation

NO production was assessed by measuring the accumulation of NO_2^- in the culture supernatants, using a colorimetric reaction with Griess reagent [0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 5% H_3PO_4]. Absorbance was measured at 540 nm in an automated plate reader (microplate reader model 550, Bio-Rad, Hercules, CA, USA). NO_2^- concentration was determined from a sodium nitrite standard curve.

Cell Viability

Cell viability was determined by a modified MTT assay method[16]. MTT solution (2 mg/mL in PBS) was added and incubated at 37°C for 3 hours. DMSO was added to the cultures and mixed to dissolve the dark violet formazan crystals. Formazan quantification was carried out using an automatic plate reader at 570 nm (Bio-Rad).

Semi-quantitative RT-PCR

After appropriate treatments and incubation for varying periods, cells were collected and washed with PBS. Total RNA was extracted using the TRIzol (Invitrogen, Inc. Groningen, Netherland)[17]. RNA concentrations were determined spectrophotometrically at the absorbance of 260 nm (VITRO SPEC 2000, Amersham. Buckinghamshire, UK). Intact total RNA (3-5 μg) was reversibly transcribed into first strand cDNA using Superscript II (Invitrogen) at 42°C and which was then amplified using PCR. The reaction mixture for PCR contained 2 μL cDNA template from RT reaction; 200 mM Tris-HCl (pH 8.4), 500 mM KCl, 50 mM $MgCl_2$, 10 mM dNTP mixture, 1.0 μM of each primer, and Taq DNA polymerase (total volume 100 μL). The oligonucleotide primers used in these experiments are as follows; iNOS (GenBank asccession no. D12520) 5′-AGCAT CACCC CTGTG TTCCA CCA-3′, 5′-TGGGG CAGTC TCCAT TGCCA-3′, nNOS (GenBank asccession no. X59949) 5′-GGCAC TGGCA TCGCA CCCTT-3′, 5′-CTTTG GCCTG TCCGG TTCCC-3′, β-actin (GenBank asccession no. V01217) 5′-TTCTA CAATG AGCTG CGTGT GGC-3′, 5′-CTC (A/G)T AGCTC TTCTC CAGGG AGGA-3′. PCR was performed with a DNA thermal cycle (Gene Cycler, Bio-Rad) at 94°C for 30 sec, at 65°C for 30 sec, and at 72°C for 1 min per cycle. The amplified products were visualized by electrophoresis on a 1% agarose gel in the presence of 0.5 μg/mL ethidium bromide. β-Actin primers were used in all cases as the internal control.

Western Blot Analysis

After various treatments, the cells were washed and lysed in lysis buffer containing buffer H (50 mM β-glycerophosphate, 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 1 mM benzaimidine, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 2 μg/mL pepstatin A, pH 7.3)/1% triton X-100. The cell lysates were then centrifuged for 20 minutes at 14,000 rpm at 4°C. Protein concentrations were determined using BCA assays (Pierce, Rockford, IL, USA). Equal amounts of protein were separated on SDS polyacrylamide gel, and electro-transferred onto nitrocellulose membranes (Schleicher & Schuell, Germany). Finally the membrane was probed with indicated antibodies. The Enhanced Chemiluminescence (ECL) detection system was used to visualize the antibody-labeled bands.

Capillary Electrophoresis (CE)

All experiments were conducted using an automated P/ACE 5,000 capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA). The instrument was equipped with a 214 nm wavelength UV detector. Separation was carried out with an untreated fused silica capillary column of 57 cm in length, and a 75 μm I.D. The running buffer consisted of 150 mM Tris-phosphate buffer, and Hexadecyl-trimethylammonium chloride (CTAC) 6 μM buffer, at a pH of 7.0. Sample injections were conducted under gravity for 20 seconds, and a 10 kV applied negative potential. The capillary tube was washed for 5 minutes before each run with 0.5 N NaOH solutions, and then flushed with running buffer solution for 20 minutes. All buffer and sample solutions were filtered through a 0.2 μm filter, and degassed prior to use. The standard curve for nitrite was constructed via the injection of an RPMI1640 media mixture containing known amounts of $NaNO_2$.

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