Nickel requires hypoxia-inducible factor-1α, not redox signaling, to induce plasminogen activator inhibitor-1

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Inhalation of nickel dust has been associated with an increased incidence of pulmonary fibrosis in humans and rodents (4, 9, 28, 35, 55). A possible mechanism for this effect of nickel was suggested to involve transcriptional induction of the plasminogen activator inhibitor (PAI)-1 gene in human airway cells (5). In these cells, nickel induced PAI-1 mRNA and protein levels, which led to chronic inhibition of fibrinolytic activity (5). The specific mechanisms driving the induction of PAI-1 by nickel are unknown but may involve the ability of nickel to mimic a response to hypoxia. Therefore, the present study investigated the hypothesis that nickel stabilizes the transcription factor hypoxia-inducible factor (HIF)-1α via the NADPH oxidase complex (purported to be an oxygen sensor), reactive oxygen species, or kinases, resulting in transcriptional activation of PAI-1.

HIF-1 is a heterodimeric basic helix-loop-helix-PAS transcription factor that consists of two subunits, HIF-1α and HIF-1β (27). In normal physiology, low cellular oxygen concentrations increase HIF-1α protein levels and transcription of a number of genes. Hypoxia-inducible genes such as vascular endothelial growth factor (20), erythropoietin (34), glucose transporters, and glycolytic enzymes (52) are associated with increased oxygen delivery to tissues or metabolic adaptation to more anaerobic conditions (50). Hypoxia-induced increases in HIF-1α protein are thought to be due mainly to stabilization of the protein, which normally turns over with a 5-min half-life (30, 50).

Metals such as nickel, cobalt chloride, and mersalyl as well as the iron chelator deferoxamine also stabilize HIF-1α protein by unknown mechanisms. This stabilization leads to transcriptional activation of hypoxia-inducible genes (2, 11, 38, 44, 47, 49, 52). There are several theories to explain the ability of nickel to stabilize HIF-1α protein and to activate hypoxia-inducible gene expression. These models are based primarily on experiments done with other stimuli such as hypoxia, cobalt chloride, and deferoxamine. The major models involve the NADPH oxidase complex, a heme oxygen sensor, the mitochondrial electron transport chain, or kinase signaling. Reactive oxygen species are hypothesized to be central signaling molecules in several of the postulated mechanisms for the induction of HIF-1α by hypoxia and cobalt chloride (11, 27, 50, 51). Although several investigators (31, 32, 56) have noted increased production of reactive oxygen intermediates after nickel exposure, it is unclear whether this increase is involved in the stabilization of HIF-1α protein by nickel. Other studies indicate that kinases including diacylglycerol kinase (DAGK) (6), phosphatidylinositol 3-kinase (PI3K) (58), and extracellular signal-regulated kinase (ERK) (37, 45) are required for HIF-dependent transcription in response to hypoxia. The involvement of these kinases in the stabilization of
HIF-1α and transcription of PAI-1 by nickel has not been previously investigated.

Nickel subsulfide is recognized as one of the more toxic forms of nickel because it is durable enough to be retained in the lung yet able to be dissolved inside lung cells (17, 18). Macrophages and epithelial cells ingest particles of nickel subsulfide into endocytic vesicles that fuse with acidic lysosomes. This acidification releases Ni2+, which reacts with cytoplasmic proteins (41) or heterochromatic regions of DNA (42, 53). The bound metal can then redox cycle to produce reactive oxygen species or affect protein conformations (36, 41). Therefore, nickel may activate cell signaling either through direct protein binding or through generation of reactive oxygen species.

In rat hepatocytes, hypoxia-induced PAI-1 expression is mediated by hypoxia-responsive elements in the promoter (43). This study identified both a strong and a weak hypoxia response element (HRE) that binds HIF-1α under conditions of hypoxia (43). Demonstration of active HREs in the promoter of the human gene has not been reported, and the human sequence shows homology only with the weak element in the rat promoter. Thus, although these data are supportive, the role of HIF-1α in nickel induction of the human PAI-1 gene is unresolved. This present study was designed to investigate the potential mechanisms for nickel-induced stabilization of HIF-1α and to determine the involvement of this transcription factor in the transcriptional activation of PAI-1 by nickel. Results of this investigation indicate that increases in HIF-1α protein levels are critical to the ability of nickel to induce PAI-1. Furthermore, the subcellular mechanism driving stimulation of the hypoxia-responsive pathway leading to PAI-1 activation by nickel may involve a kinase such as ERK but does not seem to be affected by changing the redox status of the cell or inhibiting the NADPH oxidase complex or the mitochondrial electron transport chain. These data increase the understanding of mechanisms for nickel-induced PAI-1 transcription, which may be critical for understanding the pathology of pulmonary fibrosis, cancer, and other serious diseases associated with nickel exposure.

METHODS

Cells. Human bronchial epithelial cells (BEAS-2B; American Type Culture Collection, Manassas, VA) were grown to postconfluence in 6- or 12-well plates (Corning Costar, Corning, NY) on a matrix of 0.01 mg/ml of human fibronectin (Collaborative Biomedical Products, Bedford, MA), 0.03 mg/ml of Vitrogen 100 (Collagen Biomaterials, Palo Alto, CA), and 0.01 mg/ml of bovine serum albumin (Sigma, St. Louis, MO). The cultures were maintained in LHC-9 medium (Biofluids, Rockville, MD) at 37°C in an atmosphere of 5% CO2-95% air. The cells were subcultured with 0.1% trypsin-EDTA and plated in tissue culture plates.

Nickel. The respirable-size fraction of nickel used in these experiments was prepared by applying nickel subsulfide (Ni3S2) particles (Aldrich, Milwaukee, WI) to a water column and allowing the larger particles to settle out. Particle size was measured during settling with a particle counter (Couler, Miami, FL). Nickel subsulfide particles <2.5 μm in diameter were decanted, concentrated by centrifugation, and sterilized by baking at 200°C for 18 h. This preparation gives the same quantitative and qualitative responses as a standard preparation of nickel subsulfide obtained from the Nickel Producers Environmental Research Association (Durham, NC; a kind gift from Dr. Andrea Oller).

Treatments. As shown by a clonogenic survival assay, the addition of 2.34 μg Ni/cm² of nickel subsulfide is not toxic to this cell model (5). In the present study, the cells were treated for up to 24 h with 0.58–2.34 μg Ni/cm² (5). Deferoxamine mesylate (260 μM) was applied as a positive control to induce HIF-1α protein levels. Phorbol 12-myristate 13-acetate (PMA; 2–100 nM) was used as a positive control to induce PAI-1 expression. Cells were pretreated with 2–10 mM N-acetyl-l-cysteine (NAC), 2 mM ascorbic acid, 2 mM superoxide dismutase (SOD), 5 mM apocynin (Aldrich), 5 mM rotenone, 10 μM diphenyleneiodonium chloride (DPI), 20 μM SB-203580 (Calbiochem, La Jolla, CA), 10 μM U-0126 (Calbiochem), 1 μM wortmannin (Calbiochem), or 10 μM DAGK inhibitor (DAGK) R-59949 (Calbiochem). Dimethyl sulfoxide (DMSO) was added as a vehicle control for treatment with DPI (0.01 μl/ml) or R-59949 (0.1% vol/vol). All reagents mentioned above were purchased from Sigma unless otherwise noted.

mRNA levels. Total cellular RNA was harvested with TRIZol reagent (GIBCO BRL, Life Technologies, Gaithersburg, MD) according to the manufacturer’s instructions and quantitated by spectrophotometric absorbance at 260 nm. Reverse transcription-polymerase chain reaction (RT-PCR) was performed as previously described by Barchowsky et al. (8) with PAI-1 (forward, 5′-cgctctctcacaatactac-3′ and reverse, 5′-atgctctcaacagatga-3′), β-actin, or HIF-1α (forward, 5′-tcacaccagacaggtacg-3′ and reverse, 5′-cagcaagaagacctagtcg-3′) specific primers that were synthesized in the Molecular Biology Core at Dartmouth University (Hanover, NH) and reagents from Amhion (Austin, TX), Promega (Madison, WI), and Amersham Pharmacia Biotech (Piscataway, NJ). PCR products were either run on agarose gels stained with ethidium bromide or quantified by spectrophotometric absorbance at 260 nm. Densitometry was performed on ethidium bromide stained gels with National Institutes of Health Image software. PAI-1 mRNA expression was normalized to the housekeeping gene β-actin by taking the ratio of the PAI-1 to β-actin band density or PicoGreen fluorescence.

2′,7′-Dichlorofluorescein fluorescence. 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-DCFH-DA) was purchased from Molecular Probes. Ninety-six-well plates of cells were incubated for 30 min to 24 h with antioxidants in 100 μl of LHC-9 medium. CM-DCFH-DA was prepared in LHC-9 medium and added to the cells at a final concentration of 20 μM in 200 μl/well. The cells were incubated with 2.34 μg Ni/cm² for 10 min at 37°C, and the plates were read on a microplate fluorescence reader with excitation at 485 nm and emission at 530 nm. Similar results were obtained when the cells were treated with nickel for 24 h before the addition of CM-DCFH-DA.

Protein levels. The effects of nickel on HIF-1α or β-actin protein levels were determined by Western blotting with a polyclonal antibody to HIF-1α (Transduction Laboratories, Lexington, KY) or a monoclonal antibody to β-actin (Sigma). Immunoblotting was essentially as described by Barchowsky et al. (7). Briefly, at the end of exposure periods, all cells were placed on ice and rinsed with ice-cold stop buffer (10 mmol/l of Tris-HCl, pH 7.4, 10 mmol/l of EDTA, 5 mmol/l of EGTA, 100 mmol/l of NaF, 200 mmol/l of sucrose, 100 μmol/l of...
sodium orthovanadate, 5 mM sodium pyrophosphate, 4 µg/ml of leupeptin, 4 µg/ml of soybean trypsin inhibitor, 1 mmol/l of benzamidine, 20 µmol/l of calpain inhibitor-1, 100 µU/ml of aprotinin, and 100 µmol/l of phenylmethylsulfonyl fluoride). The stop buffer was replaced with a minimal volume of 2× SDS-PAGE buffer. The proteins were separated by PAGE and transferred to polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, MA), and the membranes were blotted with 5.0% milk in 0.01 mol/l of Tris, pH 8.0, 0.15 mol/l of NaCl, and 0.05% Tween 20. Primary antibodies were blocked by antioxidants. Confluent cells were pretreated with 10 mM NAC or 2 mM ascorbic acid before the addition of 20 µM CM-DCFH-DA and nickel subsulfide. The results in Fig. 2 support previous reports from other cell types (31, 48) that nickel increases

**RESULTS**

Time course and dose response of nickel subsulfide-induced HIF-1α in BEAS-2B cells. Nickel disrupts the fibrinolytic cascade by inducing transcription of PAI-1 (5). To investigate the hypothesis that nickel increases the levels of HIF-1α to induce transcription of the PAI-1 gene, we first examined the time course for nickel induction of HIF-1α protein levels in our cell model. Exposure of postconfluent BEAS-2B cells to 2.34 µg Ni/cm² for up to 24 h increased HIF-1α protein levels beginning at 4 h, with maximal increases at 24 h. The Western analysis shown in Fig. 1A demonstrated that doses as low as 0.058 µg Ni/cm² could increase HIF-1α protein at the 8-h time point. In contrast, HIF-1α mRNA levels were not induced and, in fact, were significantly decreased after 15 h of nickel treatment (Fig. 1, B and C). Thus increased protein stabilization appears to be the primary explanation for the dose- and time-dependent induction of HIF-1α protein levels induced by nickel.

**Antioxidants inhibit increases in intracellular oxidations induced by nickel.** CM-DCFH-DA fluorescence was used to confirm that nickel subsulfide induced intracellular reactive oxygen species (31, 32, 56) in the BEAS-2B cells. The cells were left untreated or were pretreated with 10 mM NAC or 2 mM ascorbic acid before the addition of 20 µM CM-DCFH-DA and nickel subsulfide. The results in Fig. 2 support previous reports from other cell types (31, 48) that nickel increases
CM-DHCFH-DA fluorescence and that these increases can be blocked by pretreatment with antioxidants such as NAC and ascorbic acid.

**Antioxidants, NADPH oxidase, and mitochondrial inhibitors do not block nickel-induced HIF-1α protein.** Reactive oxygen species, the mitochondrial electron transport chain, and the NADPH oxidase complex are potentially involved in mechanisms that stabilize HIF-1α protein in response to nickel, cobalt chloride, and hypoxia (11, 50, 51). Therefore, the ability of NADPH oxidase inhibitors, mitochondrial electron transport chain inhibitors, or antioxidants to inhibit nickel-induced HIF-1α protein levels was determined. In Fig. 3A, confluent cells left untreated or pretreated with NAC, apocynin, or rotenone were exposed to nickel subsulfide for 24 h. The HIF-1α immunoblot in Fig. 3A shows that neither the antioxidant NAC nor the mitochondrial inhibitor rotenone blocked nickel-induced HIF-1α protein. Although the apocynin treatment decreased the intensity of the nickel-induced HIF-1α band, the concomitant decrease in basal HIF-1α levels suggested that this may not be a specific block. Figure 3B shows that increasing the dose of the antioxidant NAC to 10 mM still did not inhibit the induction of HIF-1α protein by nickel. In fact, NAC seemed to have a stimulatory effect on HIF-1α expression. The HIF-1α immunoblot in Fig. 3C confirms that pretreatment with the NADPH oxidase inhibitors apocynin and DPI did not block HIF-1α responsiveness to nickel subsulfide. There was no effect of adding 0.01 μl/ml of DMSO, the vehicle for DPI treatment.

**Antioxidants and NADPH oxidase inhibitors do not block induction of PAI-1 mRNA by nickel.** To investigate whether reactive oxygen species and the NADPH oxidase complex mediate nickel induction of PAI-1 mRNA, cells pretreated with NAC, ascorbic acid, SOD, or apocynin were exposed to nickel subsulfide or deferoxamine (a positive control for activating HIF-1α) for 24 h. As seen in Fig. 4A, antioxidants do not block nickel-induced PAI-1 mRNA levels. The NADPH oxidase inhibitor apocynin alone seemed to stimulate nickel-induced PAI-1 mRNA levels; however, apocynin pretreatment did not block nickel-induced PAI-1 (Fig. 4B). Deferoxamine, which is known to promote HIF-1α responses, induced a significant increase in PAI-1 mRNA levels.

**HIF-1α antisense oligonucleotide blocks nickel-induced HIF-1α protein.** HIF-1α antisense oligonucleotide was used to investigate the hypothesis that HIF-1α is necessary for PAI-1 transcriptional activation by nickel. To verify the effectiveness of the HIF-1α antisense oligonucleotide, it was added for up to 56 h followed by a 6-h treatment with nickel subsulfide. HIF-1α sense oligonucleotide and PMA were added for 24 h as a control. The immunoblot in Fig. 5 shows that the HIF-1α antisense oligonucleotide effectively inhibited nickel-induced HIF-1α protein levels at all time points tested. The HIF-1α sense oligonucleotide slightly elevated HIF-1α protein levels in these cells.

**HIF-1α antisense but not sense oligonucleotide blocks nickel-induced PAI-1 mRNA.** To determine whether inhibiting HIF-1α nickel responsiveness with an anti-
sense oligonucleotide would inhibit induction of the endogenous PAI-1 gene, cells were pretreated with HIF-1α antisense or sense oligonucleotide followed by 6 h with nickel subsulfide. Only the antisense oligonucleotide blocked the ability of nickel but not of PMA to stimulate PAI-1 mRNA levels (Fig. 6, A and B). In contrast, treatment with the HIF-1α sense oligonucleotide had no effect on the induction of PAI-1 by nickel (Fig. 6, C and D).

**Upstream kinases may be involved in the stabilization of HIF-1α by nickel.** Reports in the literature (6, 14, 58) suggest that phosphorylation of HIF-1α by several upstream kinases may be involved in the transactivation of HIF-driven genes. Cells were pretreated with kinase inhibitors followed by 24 h of treatment with nickel subsulfide to determine whether these kinases were involved in the stabilization of HIF-1α by nickel. The ERK inhibitor U-0126 partially inhibited nickel-stimulated HIF-1α protein levels, whereas the DAGKI had a less dramatic inhibitory effect (Fig. 7). In contrast, the p38 inhibitor SB-20358 and the PI3K inhibitor wortmannin did not decrease nickel-induced HIF-1α protein levels.

**Kinases may be involved in the induction of PAI-1 by nickel.** PAI-1 mRNA levels were compared in cells that were pretreated with and without kinase inhibitors to determine whether kinases were involved in the transcriptional activation of PAI-1 by nickel. U-0126 caused the most significant inhibition of nickel-induced PAI-1 mRNA. SB-20358, wortmannin, and the DAGKI all had modest effects on nickel-stimulated PAI-1 mRNA levels.

**DISCUSSION**

The purpose of this study was to test the hypothesis that the induction of PAI-1 mRNA by nickel was mediated by the transcription factor HIF-1α. The mechanisms for stimulating increases in HIF-1α protein levels have been controversial and may be stimulus specific. The actions of nickel are further complicated because it has the paradoxical actions of promoting increased cellular redox cycling and mimicking hypoxia. The data presented in Fig. 2 confirm that nickel increases intracellular oxidation, but neither these oxidants, the mitochondrial electron transport chain, nor the NADPH oxidase complex mediate nickel-induced HIF-1α protein levels or expression of PAI-1. Eliminating HIF-1α from the cells, however, prevents the nickel-induced transcription of PAI-1 mRNA (Fig. 6). Pretreatment with kinase inhibitors, particularly U-0126, inhibits both the stabilization of HIF-1α by nickel as well as the ability of nickel to induce PAI-1 mRNA.

Reactive oxygen species are hypothesized to be central signaling molecules in several different postulated mechanisms for the induction of HIF-1α by hypoxia...
HIF-1α

Fig. 7. Kinases may be involved in the induction of PAI-1 by nickel. Cells were pretreated for 90 min with 1 μM wortmannin (Wort) or for 30 min with 20 μM SB-203580 (SB), 10 μM U-0126, or 10 μM diacylglycerol kinase inhibitor R-59949 (DAGKI) followed by 24 h of exposure to 2.34 μg Ni/cm². DMSO (0.1% vol/vol) was added as the vehicle control, and 260 μM DFO was added as a positive control for HIF-1α induction. Cells were harvested in 2× SDS buffer, and Western blots were performed with an antibody to HIF-1α. Data are representative of at least 3 replicates from separate cultures.

and cobalt chloride (11, 27, 50, 51). The generation of free radicals by nickel (31, 32, 56) has been cited as a mechanism for the stimulation of other transcription factors such as nuclear factor-κB and genes including intercellular adhesion molecule-1 (24) and interleukin-1 (15). If changes in reactive oxygen levels were critical for nickel-induced stabilization of HIF-1α protein, one would expect that the addition of antioxidants would prevent nickel from increasing HIF-1α protein levels. In contrast, the data in Fig. 3 indicate that pretreatment with a variety of antioxidants including NAC, which increases intracellular glutathione levels (19), or ascorbic acid, which scavenges reactive oxygen species (46), were completely ineffective in inhibiting nickel-induced HIF-1α protein levels. As shown in Fig. 2, these concentrations of antioxidants blocked the increases in intracellular oxidants by nickel. These results indicate that changes in reactive oxygen species levels are not necessary for nickel signaling in the HIF-1α-PAI-1 pathway. This finding supports a study by Salnikow et al. (49) showing that induction of another hypoxia-responsive gene, Cap43, by nickel was also not mediated by reactive oxygen species. Furthermore, the fact that HIF-1α is stabilized in the presence of the enhanced oxidative state after nickel exposure argues against the hypothesis that a decrease in oxidants is required to activate HIF-1α (Fig. 1).

The mitochondrial electron transport chain is also postulated to be involved in the hypoxic signaling cascade. Even though the mitochondrial electron transport chain complex I inhibitor rotenone does block the induction of HIF-1α protein in response to hypoxia, it does not affect HIF-1α expression induced by cobalt chloride or deferoxamine (3, 13). In the present study, pretreatment with rotenone failed to inhibit the HIF-1α response to nickel (Fig. 3A), indicating that mitochondrial electron transport is probably not involved in the induction of HIF-1α by this metal.

Other models of hypoxia-stimulated signal transduction indicate the involvement of a NADPH oxidase complex. The oxidants produced by this complex act as chemical messengers, mediating increases in HIF-1α protein stabilization (1, 23). If the NADPH oxidase complex were critical to the induction of HIF-1α by nickel, one would expect that NADPH oxidase inhibitors would block this induction. The data in Fig. 3C do not support this hypothesis. Treatment with apocynin, which inhibits NADPH oxidase by blocking the SH3 domain on the p47phox subunit, preventing it from interacting with the complex (33, 54, 57), failed to block nickel-induced HIF-1α protein. Likewise, treatment with DPI, an inhibitor of electron transfer through the gp91 subunit of the NADPH oxidase complex (40), also did not inhibit the induction of HIF-1α by nickel. Thus...
it is unlikely that this complex or a change in the oxidants generated by the complex is necessary for the nickel induction of HIF-1α (Fig. 3C).

Another possible mechanism for the action of nickel and the inability of NAC to protect against increases in HIF-1α protein levels might involve a heme oxygen sensor (11). According to this model, a lack of oxygen bound to the heme center stimulates a signaling cascade, which ultimately stabilizes the HIF-1α protein (22, 25, 26, 29). Nickel is unique among metals in its ability to interact with iron-sulfur centers. Metals like cobalt and nickel or iron chelators like deferoxamine might substitute for the iron in the center of the heme or prevent oxygen binding, triggering the hypoxia response pathway (11, 44, 50). It is also possible that nickel reacts directly with oxidized sulfur in the center to cause activation. NAC would not be expected to protect against this type of reaction.

Nickel may stimulate proximal events in signaling cascades, such as the insulin-like growth factor receptor, following a pathway similar to that activated by mersalyl, an organic mercury compound (50). Reports in the literature (10, 37, 45) indicate that the transactivation of HIF by hypoxia may require phosphorylation by ERK. Involvement of ERK in nickel signaling was supported by the data in Figs. 7 and 8 showing that treatment with U-0126 partially blocked the stabilization of HIF-1α and the induction of PAI-1 mRNA by nickel. ERK also phosphorylates other transcription factor binding proteins including c-Jun, which binds to the PAI-1 promoter.

The involvement of PI3K and signaling through DAGK to increase phosphatidic acid (6) have also been reported to mediate stabilization of HIF-1α (Fig. 3C). These data indicate that ERK and possibly DAGK are involved in the upstream signaling pathways leading to the stabilization of HIF-1α and the induction of PAI-1 mRNA by nickel.

The hypothesis that increased levels of the transcription factor HIF-1α could be mediating nickel-induced transcription of the PAI-1 gene was supported by the data in Fig. 1 indicating that HIF-1α protein levels and PAI-1 mRNA levels are increased after a similar time course of nickel exposure. The increases start at 4 h, with the highest HIF-1α and PAI levels detectable at 24 h (5). HIF-1α antisense oligonucleotide dramatically inhibited the induction of PAI-1 mRNA levels by nickel (Fig. 6). These data implicate HIF-1α as a critical transcription factor involved in the nickel induction of PAI-1 gene transcription. Data in Fig. 4B further support the importance of HIF-1 to activation of the PAI-1 promoter because treatment with another agent that increases HIF-1α levels, deferoxamine, also induced a significant increase in PAI-1 mRNA levels. These results are consistent with a report (43) that the rat PAI-1 promoter contains an active HIF-1α-responsive element that was critical for the induction of PAI-1 by hypoxia. Although HREs have not been previously reported in the human PAI-1 promoter, sequences matching the consensus site for this binding element are located in the human PAI-1 promoter region. Transiently transfected plasmids expressing the full-length PAI-1 promoter linked to luciferase were significantly induced by nickel (data not shown). Further studies are needed to demonstrate which potential HREs or other transcription factor binding sites are necessary and sufficient for the induction by nickel.

In conclusion, the results of this study demonstrate that nickel transcriptionally activates PAI-1 by a mechanism that involves increases in HIF-1α protein mediated by ERK but does not require the NADPH oxidase complex, the mitochondrial electron transport chain, or increases in reactive oxygen species. Further investigation is needed to identify the rate-limiting upstream signaling pathways involved in nickel-induced stabilization of HIF-1α and the enhanced transcriptional activation of profibrotic genes such as PAI-1. These investigations will provide a better understanding of the pathological mechanisms of pulmonary fibrosis, cancer, and other diseases associated with exposure to nickel.

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