Down-regulation of Hypoxia-inducible Factor-2 in PC12 Cells by Nerve Growth Factor Stimulation*

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Cellular responses to low oxygen tension are mediated, at least in part, by the activation of the hypoxia-inducible factors (HIFs). In the presence of oxygen, specific HIF residues become hydroxylated by the action of a recently described group of dioxygenases. These post-translational modifications target HIF for proteosomal degradation and prevent its transcriptional activity. Despite these detailed studies, little is known about the regulation of HIF by stimuli other than hypoxia. Here we report that, in rat pheochromocytoma PC12 cells, nerve growth factor (NGF) stimulation results in a decrease of both basal and hypoxia-induced levels of HIF-2α protein. NGF treatment did not increase HIF-hydroxylase gene expression or activity, and the reduction of the HIF-2α protein level upon stimulation was observed even in the presence of HIF-hydroxylase inhibitors such as deferoxamine or dimethyloxalurate. Thus, in contrast to the response to hypoxia, the effect of NGF on HIF-2α protein levels is not mediated by the HIF hydroxilases. Quantitative real time (RT)-PCR showed that NGF stimulation results in a decrease of the HIF-2α mRNA level similar to that found at the protein level. Interestingly, NGF effect was specific for HIF-2α mRNA because it did not affect HIF-1α mRNA levels. NGF treatment reduced HIF-2α mRNA levels even in the presence of actinomycin D, suggesting an effect on mRNA stability. Finally, the effect of NGF on HIF2α correlates with reduction of both basal and hypoxia-induced vascular endothelial growth factor mRNA levels. Reporter assays suggest that the reduced expression of hypoxia-inducible genes upon NGF treatment is related, at least in part, to the reduction of HIF-2α protein. Hence, in PC12 cells the level of HIF-2α protein and its effect on gene expression can be down-regulated by stimuli other than oxygen.

Most of the changes on gene expression induced by hypoxia are mediated by a group of basic helix-loop-helix-Per/ARNT/Sim (bHLH-PAS) transcription factors, called hypoxia-inducible factors (HIF). These transcription factors are heterodimers of a constitutively expressed β subunit (HIF-β also known as the aryl hydrocarbon receptor translocator (ARNT)) and an oxygen-regulated α subunit (HIF-α). There are three types of α subunits, HIF-1α, HIF-2α (also known as endothelial PAS domain (EPAS)), and HIF-3α, encoded by different genes. Most of the data about regulation and function of these proteins have been obtained through the study of HIF-1α and, to a lesser extent, HIF-2α; however, it is currently accepted that both proteins behave similarly in response to changes in oxygen concentration. The half-life and transcriptional activity of HIFα subunits are controlled by oxygen availability, and recently, the core mechanism responsible for the oxygen sensing has been elucidated. In the presence of oxygen HIFα proteins undergo hydroxylation at two specific proline residues (1, 2) and one asparagine residue (3). These post-translational modifications modulate the interaction between HIF and other proteins, namely VHL (1, 2) and p300 (3). Specifically, proline hydroxylation is required for the binding to VHL, the substrate recognition subunit of an E3 ubiquitin ligase complex, which targets HIF for proteosomal degradation. On the other hand, asparagine hydroxylation interferes with the interaction of p300 with the C-terminal transactivation domain of HIF. None of these modifications occur in the absence of oxygen, thus under hypoxic conditions HIF half-life is dramatically increased and at the same time its transcriptional activity is enhanced by p300 binding.

The enzymes responsible for these hydroxylations have been recently identified as 2-oxoglutarate-dependent dioxygenases (4–7). Because these types of enzymes require molecular oxygen for their catalytic reaction, it is admitted that they act as the oxygen sensors that directly control HIF stability/activity in response to oxygen variations. To date, three different HIF proline hydroxylases have been described (4, 5): EGL-nine homolog 1 (EGLN1), also known as HIF proline hydroxylase 2 (PHD2), EGLN2/HPH3/PHD1, and EGLN3/HPH1/PHD3 (hereafter referred to in the EGLN nomenclature). Although this model explains how HIF is regulated by oxygen, it does not rule out that other mechanisms might contribute or modulate this response. In this sense, at least one of the three identified HIF proline hydroxylases is inducible by...

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1 The abbreviations used are: HIF, hypoxia-inducible factor; HRE, hypoxia response element; DMOG, deoxymethylglutarate; VHL, von Hippel-Lindau; NGF, nerve growth factor; RT, real time; Q, quantitative; bFGF, basic fibroblast growth factor; EGLN, EGLN-nine homolog; ERK, extracellular regulated kinase; EGF, epidermal growth factor; VEG, vascular endothelial growth factor; ARNT, aryl hydrocarbon receptor translocator; EPAS, endothelial Per/ARNT/Sim; E3, ubiquitin-protein isopeptide ligase; GST, glutathione S-transferase.
growth factor stimulation in certain systems (8) and another post-translational modification of HIF, acetylation, has recently been shown to modulate the affinity of the VHL-HIF interaction (9). Moreover, this model does not explain some of the reported observations such as the effect of CO on HIF stabilization (10) nor the role of radical oxygen species (11) and phosphatidic acid (12). Moreover, several works (13–19) describe the induction of HIF, under normal oxygen tension, in response to a variety of stimuli, including growth signals, hormones, and cell to cell contacts. Hence, it seems clear that HIF regulation is complex and that factors other than oxygen might contribute to the activity of these transcription factors. The effect of soluble extracellular signals is of particular interest. Several groups have demonstrated that signals such as insulin-like growth factor-1 (13, 14), insulin (13, 14), interleukin-1 (15), tumor necrosis factor (16), angiotensin (17), or EGF/herguline (18, 19), lead to the stabilization of HIF protein. However, the mechanism by which this occurs is controversial, and although some authors point to an increased HIF mRNA translation (18), other groups argue against this possibility (20). On the other hand, we have previously shown that HIF stabilization is not a general consequence of growth factor signaling because stimulation of different cell types such as HepG2, HeLa, or PC12 with insulin-like growth factor-1 or EGF among other signals does not induce HIF (21). Moreover, it has been described (22, 23) that nerve growth factor (NGF) stimulation of PC12 cells results in a decreased induction of HIF activity by hypoxia concomitant with a reduced or delayed induction of HIF-responsive genes such as tyroxine hydroxylase (TH). Although, no explanation for this latter observation is provided, it suggests that growth factor stimulation does not always result in HIF activation.

In this work we decided to further study the regulation of HIF protein stability by growth factors in PC12 cells. The rat pheochromocytoma PC12 cells have been widely used to study oxygen-induced responses because they present certain characteristics that make them particularly suited for the study of hypoxia. Unlike others, these cells are excitable and rapidly respond to decreases in oxygen tension with membrane depolarization. In addition, these cells respond even to relatively small changes in oxygen tension. Finally, pheochromocytomas arise from the neuroendocrine medullary cells of the adrenal gland, which is involved in the systemic response to oxygen (24).

Here we show that the level of basal (normoxic) as well as hypoxia-induced HIF-2α in PC12 cells is diminished by the presence of growth signals. A mechanism that might explain this effect is presented and the implications are discussed.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The rat pheochromocytoma PC12 cells were maintained in RPMI 1640 medium with GlutaMAX-I (Invitrogen) supplemented with 10% horse serum (Invitrogen) and 5% fetal bovine serum. Cells were maintained in RPMI 1640 medium with GlutaMAX-I (Invitrogen). For analysis purposes the amplicon for each of the analyzed genes was cloned, and known amounts of the cloned product were used to generate a standard curve. The number of copies of the interest gene in each sample was extrapolated from the corresponding standard curve by the indicated software. For each sample, duplicate determinations were done on each figure.

Quantitative RT-PCR—Immediately after treatments cells were harvested into 1 ml of Ultraspec reagent (Biotex, Houston, TX). Total RNA was extracted, quantified, and integrity was tested by gel electrophoresis. 1 μg of total RNA from each sample was retrotranscribed to cDNA using the Transcriptor reverse transcription kit (Roche Applied Science). 1–3 μl of the cDNA product were used as template for amplification reactions carried out with the LC Fast Start DNA master SYBR Green I kit (Roche Applied Science) following the manufacturer’s instructions. PCR amplifications were carried out in a Light Cycler System (Roche Applied Science) and data analyzed with LightCycler software 3 version 3.5.28 (Idaho Technology Inc.). For analysis purposes the amplicon for each of the analyzed genes was cloned, and known amounts of the cloned product were used to generate a standard curve. The number of copies of the interest gene in each sample was extrapolated from the corresponding standard curve by the indicated software. For each sample, duplicate determinations were done on each figure.

Regulation of HIF-2α by NGF

| Gene     | Primer Sequences (5′–3′) |
|----------|-------------------------|
| HIF-2α   | CGAGGGACATCTCGACTTGACAGTTGGAGACGACTCG-3′ and HIF5631R (5′-AGCTCTTCGAGAGACGACTCG-3′) |
were made, and the gene copy number was normalized by the amount of β-actin and/or glyceraldehyde-3-phosphate dehydrogenase on the same sample. The primer pairs used in this study (Table I) were chosen so they hybridize to different exons to avoid amplification of potential genomic DNA contaminant. The primer selection was done with the aid of the LightCycler Probe Design Software, version 1.0 (Idaho Technology Inc.).

RESULTS

NGF Stimulation Reduces HIF-2α Protein Level—In a previous work (21) we found that in PC12 cells, NGF stimulation does not increase HIF-2α protein levels under normoxic conditions as reporter for other cell types upon growth factor stimulation (13–19). Moreover, we noticed that NGF treatment actually reduced the basal level of HIF-2α found in normoxic PC12 cells. To further investigate this effect we stimulated rat phaeochromocytoma PC12 cells with NGF for different periods of time, and the basal (normoxic) level of HIF-2α protein was determined by western-blot. As shown in Fig. 1A, NGF treatment results in a small, but reproducible, transient increase in HIF-2α protein level at all time points tested (Fig. 1). Therefore, NGF treatment results in a reduction of HIF-2α protein regardless of the oxygen tension.

NGF Effect on HIF-2α Protein Is Not Mediated by HIF Proline Hydroxylases—One possible mechanism by which NGF could reduce HIF protein level is the induction of HIF proline hydroxylase activity. Interestingly, sm-20, the rat homologue of EGLN3, is induced by mitogenic stimulation in some cell types (4, 26). Thus, we next investigated whether NGF modified the expression of the EGLN genes. To this end PC12 cells were cultured with NGF for 7–12 h, and the EGLN mRNA expression was analyzed by quantitative RT-PCR (Q-RT-PCR). As a control for the induction of EGLN mRNAs, PC12 cells were exposed to 1% oxygen for the same period of time (Fig. 2A). As previously described (4, 26), EGLN1 and -3, but not EGLN2 mRNAs, were up-regulated by hypoxia. In contrast, NGF treatment had no effect on EGLN1, EGLN2 or EGLN3 mRNA levels (Fig. 2A). The basal levels of the three EGLN mRNAs were very different (Fig. 2B), EGLN1 being the most abundant, whereas EGLN3 was the one with the lowest expression. In agreement with our results, a recent report (26) demonstrated striking differences in the basal amount of the EGLN1, -2, and -3 mRNAs in U2OS cells.

Still, it could be possible that NGF effect on HIF-2α protein level was due to an increase on EGLN activity without affecting

### Table I

| Target gene | Sequence (5’ to 3’) | Amplicon |
|-------------|---------------------|----------|
| HIF2α       | Forward GTCACACAGAACTTTGTCG | 249 87.3 |
|             | Reverse CAAAGATGCTGTGATCGG | 193 81.3 |
| HIF1α       | Forward GTTTACATAAGAACAGACACACCT | 237 87.5 |
|             | Reverse TTCTGGAAGAAGGGAAGG | 327 84.7 |
| EGLN1       | Forward TGGACGACTGATACCG | 330 87.6 |
|             | Reverse TGGCTGACACGCTGGGGG | 193 86.3 |
| EGLN2       | Forward CCAATCGAGGCACCTCTG | 365 87.5 |
|             | Reverse GTGGAAGTGGATCGGCG | 277 88.7 |
| VEGF        | Forward TGGCAGAAGAGATATCTG | 365 87.5 |
|             | Reverse GTCAGGTCGATCCAG | 277 88.7 |
| β-Actin     | Forward CAGAGGAGAGAAGG | 365 87.5 |
|             | Reverse GCTCAGAGGAGAAGG | 277 88.7 |
| GAPDH       | Forward CAGAAGACTGTGGATGGG | 237 88.7 |
|             | Reverse GTTCACACCTCTTTCG | 237 88.7 |
their gene expression level. To explore this possibility, we decided to indirectly assay EGLN activity on lysates from PC12 cells challenged with NGF or hypoxia. Because, VHL binds to HIF only when the relevant prolines had been hydroxylated by the EGLNs, the binding of [35S]VHL to a HIF-derived peptide (VHL capture assay) is considered as an indirect measurement of EGLN activity (1, 2, 4, 5, 27, 28). Recombinant GST-HIF comprising amino acids 521–542 (including Pro-531 residue, which is homologous to Pro-564 from HIF-1α/HIF-2α protein) was exposed to lysates from control, NGF, or hypoxia-treated PC12 cells under conditions that allow EGLN activity (the assay was done at normoxic conditions, see “Experimental Procedures”). Recombinant GST-HIF fusion protein from bacteria does not bind [35S]VHL (Fig. 2C, lane 1) unless it has been exposed to cell lysate (Fig. 2C, lanes 2–8). Exposure of GST-HIF to lysate from control cells results in [35S]VHL captured by the intensity of the GST-HIF band. The experiment was repeated once with the same result. D, PC12 cells were grown in 24-well plates for 48 h and then treated with 150 ng/ml NGF or left untreated for 3 h. Then cells were left at normoxic or transferred to hypoxia (1% oxygen) for additional 4 h. Where indicated deferoxamine (DFX, 380 μM) or DMOG (1 mM) were added to culture media after the first 3 h incubation with NGF and right before incubation at normoxia/hypoxia for 4 additional hours. The experiment shown is representative of three independent experiments.
controls (Fig. 2C, lanes 5 and 6), in agreement with the lack of effect of this treatment on EGLN mRNA level.

All these results suggested that NGF effect on HIF-2α protein was not mediated by the induction of EGLN activity. To rule out the involvement of this activity we decided to study the effect of NGF on HIF-2α protein in the presence of inhibitors of EGLN activity. HIF proline dioxygenases depend on Fe^{2+} for its catalytic activity and require 2-oxoglutarate as a co-substrate, thus the iron chelator deferoxamine and the 2-oxoglutarate mimetic DMOG inhibit their activity (1, 2). PC12 cells were stimulated by various combinations of NGF and hypoxia in the presence or absence of these drugs. As shown in Fig. 2D, DMOG, deferoxamine or hypoxia treatment resulted in HIF-2α accumulation; however, NGF treatment was able to reduce the level of HIF-2α in all the cases even when hypoxia and EGLN inhibitors were combined. Altogether these results suggest that the reduction of HIF-2α protein induced by NGF was independent of EGLN activity.

**NGF Treatment Reduces the Level of HIF-2α mRNA**—The experiments described above suggest that the reduction of HIF-2α induced by NGF treatment was not mediated by changes in EGLN activity. Therefore we studied whether NGF was affecting HIF-2α at the gene expression level. As shown in Fig. 3A, the level of HIF-2α mRNA in NGF-treated cells was reduced as compared with control cells. This reduction of HIF-2α mRNA, 48.6% (glyceraldehyde-3-phosphate dehydrogenase correction, n = 6) or 36.2% (β-actin correction, n = 7) of the control value is roughly the same reduction observed at the protein level (Fig. 1). Importantly, NGF treatment affected HIF-2 α mRNA but had no effect on HIF-1 α mRNA (Fig. 3A). We were unable to prove that NGF had no effect on HIF-1 α protein because it was not detected on PC12 cell lysates by any of the antibodies we tested.2 The abundance of HIF-1 α mRNA in PC12 cells was about 70 times lower than HIF-2α mRNA, suggesting a possible explanation for the impossibility of detecting HIF-1α by western-blot. The effect of NGF treatment on HIF-2α mRNA level was also observed under hypoxia (Fig. 3B).

Several mechanisms can be responsible for the observed reduction of HIF-2α mRNA levels including reduced gene transcription and/or mRNA stability. As shown in Fig. 3C, NGF treatment resulted in a reduction of HIF-2α mRNA even in the presence of actinomycin D, at doses sufficient to inhibit c-fos induction (Fig. 3C, inset). Thus, NGF reduces HIF-2α mRNA, at least in part, by decreasing its stability, although effects at the transcriptional level can not be ruled out.

Finally, we investigated whether the effect of NGF on HIF-2 α mRNA was evoked by other stimuli. Both EGF and bFGF challenge resulted in a reduction of HIF-2α mRNA (Fig. 3D). However, the effect of EGF and bFGF was less pronounced than that of NGF, despite all of them inducing intracellular signaling with similar potency as judged by Erk (Fig. 3D) or Akt2 activation. In agreement with the modest effect of EGF and bFGF on HIF-2α mRNA their effect was not always observed at the protein level,2 in contrast NGF reduction of HIF-2 α protein was highly reproducible (Fig. 1C).

**NGF Stimulation Reduced Both Basal and Hypoxia-induced Vascular Endothelial Growth Factor (VEGF) mRNA Levels**—The data presented above shows that the level of HIF-2α protein in PC12 is decreased upon NGF challenge, probably by down-modulation of its mRNA. We next asked what was the effect of NGF stimulation on HIF target genes. To address this question, PC12 cells were NGF or mock stimulated for 3–4 h and then transferred to 1% O_{2} atmosphere, 5% O_{2} atmosphere,
under the control of the wild type or an HRE-mutant form of the human erythropoietin promoter (25). After transfection cells were mock or NGF processed for luciferase activity determination. We assigned the value of 1 (solid line) to the normalized VEGF level on control (normoxic, untreated) cells. The level of VEGF, normalized by the amount of /H9251 mRNA was determined by Q-RT-PCR as indicated under "Experimental Procedures." We assigned the value of 1 (solid line) to the normalized VEGF level on control (normoxic, untreated) cells. The level of VEGF, normalized by the amount of β-actin, in the rest of samples is represented as fold over control. The results obtained in four independent experiments and their average values are shown. B, the average reduction (n = 4) of VEGF due to NGF treatment is represented as the inhibition over untreated samples at each oxygen concentration (21, 5, or 1% oxygen). C, PC12 cells were grown in 10 cm plates for 24–48 h then transfected with a reporter plasmid encoding for the firefly luciferase gene under the control of the wild type or an HRE-mutant form of the human erythropoietin promoter (25). After transfection cells were mock or NGF (150–200 ng/ml) stimulated for 4–5 h and then transferred to hypoxia (5% oxygen) or left at normoxic conditions for 3 additional hours. Cells were processed for luciferase activity determination. We assigned the value of 1 (solid line) to the luciferase activity (normalized by renilla activity) on control (normoxic, untreated) cells. The activity of luciferase, normalized by renilla activity, in the rest of samples is represented as fold over control. The results obtained in three independent experiments and their average values are shown.

oxygen tensions (Fig. 4B), resulting in an important inhibition of VEGF expression at 21 and 5% oxygen (around 60% below untreated controls), but a modest effect at 1% O2 (about 20% the control values). Our interpretation is that because at low oxygen tension the amount of HIF-2α protein is much higher than at 5 or 21% oxygen, even a reduction of fifty percent of this amount is sufficient to induce VEGF. In contrast, when HIF-2α becomes limiting, as occurs at higher oxygen tensions, a reduction in the total amount of protein has a more profound impact on the expression of HIF target genes.

It could be argued that the observed effect of NGF on HIF-dependent gene expression is independent of HIF and through a response element other than HRE. To investigate this possibility we transiently transfected PC12 cells with a reporter plasmid construct expressing the firefly luciferase gene under the control of the wild type erythropoietin gene promoter or an HRE-mutated version of this promoter. After transfection cells were stimulated with NGF for four hours and then transferred to 5% oxygen or left at normoxia for an additional four hours. As shown in Fig. 4C, NGF stimulation results in a reduction of basal as well as hypoxia-induced luciferase expression, as found for the endogenous VEGF gene. This result is in agreement with a previous report showing that NGF treatment dramatically affected luciferase expression driven by a promoter containing three tandem copies of the HRE element (23). Although some effect was still observed, the reduction of luciferase expression by NGF was less pronounced when the reporter gene was under the control of a promoter lacking functional HRE elements. Hence, the inhibition of HIF-target gene expression observed after stimulation by NGF is attributable, at least in part, to the reduction of HIF acting through HRE.

DISCUSSION

Here we have shown that, in PC12 cells, NGF stimulation results in a decrease of HIF-2α mRNA and protein. This reduction is independent of the amount of oxygen so it can be observed at normoxia, hypoxia, and during reoxygenation. This is the first report showing a reduction of HIF protein and mRNA in response to extracellular signals and suggests that HIF activity can be regulated, at least in some circumstances, at the mRNA level in addition to the protein level. We found that NGF treatment does not increase EGLN activity and that it reduces HIF-2α protein even in the presence of EGLN inhibitors. On the other hand, we observed a good correlation between reduction of HIF-2α protein and mRNA, being the magnitude of these reductions equivalent. It has been recently described that acetylation of HIF by ARD1 increases the affinity of VHL for hydroxylated HIF and as a consequence favors HIF degradation (9). Because NGF reduces HIF-2α protein level even in the absence of HIF hydroxylation (Fig. 2D), it is unlikely that it is due to an increase in ARD1 activity. ARD1 activity is modulated, at least in part, by regulation of its mRNA (9). We found that NGF challenge did not affect ARD1 mRNA level, 2 supporting the lack of a role for this enzyme in the reduction of HIF-2α by NGF. Altogether our results strongly suggest that the reduction of HIF-2α by NGF is mediated by a reduction of HIF-2α mRNA, rather than augmented targeting for VHL-mediated degradation. We also found that the induction of EGLN1 and EGLN3 mRNAs by hypoxia correlate with an increased EGLN activity. This enhancement of EGLN activity induced by hypoxia is likely to be responsible for the decrease of HIF’s protein found after long periods of hypoxia2 and explains why the rate of HIF-1α degradation depends on the duration of the hypoxic stress (29).

The decline of HIF-2α protein after NGF stimulation has functional effects on HIF-dependent genes, being particularly evident at moderate levels of hypoxia. Importantly, reporter experiments indicate that the negative effect of NGF on HIF-dependent genes is mediated, at least in part, through HRE in the promoter of these genes. These results are in agreement with a previous report showing that NGF stimulation results in a reduction of hypoxia-responsive genes in PC12 cells (22, 23) and reduced HIF activation upon hypoxia (22, 23).

The reported effect of NGF on HIF-2α seems to be quite specific because it is observed in PC12 cells but not in other cell types, it affects HIF-2α but not HIF-1α, and it is observed upon NGF stimulation with other growth factors such as EGF and bFGF less potent. Although we found that in PC12 cells, NGF stimulation reduces HIF2α levels, previous reports showed that, in other cell types, growth factor stimulation induces an increase in HIF protein amount (13–17) or has no effect (21). Altogether these
studies suggest that, despite HIF being regulated primarily by oxygen availability, other signals received by the cell modulate this response. Alternatively, these findings might indicate tissue-specific roles for HIF proteins in response to stimuli other than hypoxia.

Given that NGF induces differentiation of PC12 cells to neuronal phenotype, it could be concluded that differentiation alters the response to oxygen. Alternatively, a reduction of HIF-2α may be necessary for PC12 differentiation induced by NGF. In fact, overexpression of VHL protein results in neural differentiation, whereas VHL mRNA antisense oligonucleotides inhibit differentiation of central nervous system progenitor cells (30). In agreement with a negative role of HIF activity on neuronal differentiation, hypoxia decreases the expression of several neuronal markers and causes de-differentiation of neuronal cells (31).

Because EGF, bFGF, and particularly NGF are potent factors for PC12 cells, one further implication of our results is that HIF-2α activity might be involved in the control of PC12 cell apoptosis. The role of HIF on apoptosis is controversial; although most authors agree that HIF activation has a pro-apoptotic role through the induction of genes required to the adaptation to hypoxia, several works propose a pro-apoptotic role for this protein (32-34). In fact, B-Nip and caspase-9, two pro-apoptotic genes, have been shown to be under the control of HIF (35, 36). Taking as valid that, at least under some circumstances, HIF activity promotes apoptosis, NGF-mediated inhibition of HIF-2α could explain its potent anti-apoptotic effects. Further work is required to address all these issues.

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