Uncoupling protein 2, in Vivo Distribution, Induction upon Oxidative Stress, and Evidence for Translational Regulation*

Received for publication, August 2, 2000, and in revised form, November 26, 2000
Published, JBC Papers in Press, November 29, 2000, DOI 10.1074/jbc.M006938200

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Uncoupling protein 2 (UCP2) belongs to the mitochondrial anion carrier family and partially uncouples respiration from ATP synthesis when expressed in recombinant yeast mitochondria. We generated a highly sensitive polyclonal antibody against human UCP2. Its reactivity toward mitochondrial proteins was compared between wild type and ucp2(−/−) mice, leading to non-ambiguous identification of UCP2. We detected UCP2 in spleen, lung, stomach, and white adipose tissue. No UCP2 was detected in heart, skeletal muscle, liver, and brown adipose tissue. The level of UCP2 in spleen mitochondria is less than 1% of the level of UCP1 in brown adipose tissue mitochondria. Starvation and LPS treatments increase UCP2 level up to 12 times in lung and stomach, which supports the hypothesis that UCP2 responds to oxidative stress situations. Stimulation of the UCP2 expression occurs without any change in UCP2 mRNA levels. This is explained by translational regulation of the UCP2 mRNA. We have shown that an upstream open reading frame located in exon two of the ucp2 gene strongly inhibits the expression of the protein. This further level of regulation of the ucp2 gene provides a mechanism by which expression can be strongly and rapidly induced under stress conditions.

The common characteristic of these proteins is to uncouple the respiratory chain from ATP synthesis by dissipating the proton electrochemical gradient when overexpressed in yeast mitochondria (for reviews see Refs. 9 and 10). They may also transport anions, like the other mitochondrial carriers, but these substrates are currently unknown. Over the last few years, several physiological roles have been proposed for UCP2, based on the expression of its mRNA upon various physiological conditions (for review see Ref. 11). Genetic studies suggested the novel UCPs might be linked to hyperinsulinemia (4) or to the resting metabolic rate (12) and consequently to the control of body weight. It was also proposed that UCP2 contributes to the inflammatory response and regulates the production of reactive oxygen species from mitochondria (13, 14). Recently, it has been shown that ucp3(−/−) mice exhibited no consistent phenotypic abnormality, despite a reduced proton leak in muscle mitochondria and a higher level of intracellular ROS in muscle. Moreover, double knockout ucp1-ucp3 phenotype was indistinguishable from the single ucp1(−/−) phenotype (15, 16).

One of the main problems encountered in the physiological studies of the new UCPs is the absence of reliable immunological tools to detect the proteins in vivo. More than 100 publications have described variation of UCP2 mRNA, whereas only seven analyses were performed at the protein level (for reviews see Refs. 9 and 10). In the present study, we have addressed this problem by generating antibodies against the complete human UCP2 sequence, rigorously evaluated their specificity and sensitivity, and compared them with commercial antibodies. Mitochondria were prepared either from ucp2(+/+) or ucp2(−/−) mice that were jointiy created by the Ricquier and Collins laboratories (17). UCP2 protein was immunodetected without ambiguity in spleen, lung, stomach, and gonadal VAT (gWAT) but not in other tissues. Although UCP2 protein was found in vivo at a low level in spleen, lung, stomach, and VAT mitochondria, its expression was strongly increased upon fasting and LPS treatment in lung and stomach. Analysis of UCP2 mRNA and protein levels in transfected COS cells showed that the translation of UCP2 transcript is strongly inhibited by an upstream ORF in the exon two of the gene.

MATERIALS AND METHODS

Chemicals, Media, and Antibodies—LPS, benzamidine, aprotinin, pepstatin, leupeptin, bestatin, and phenylmethylsulfonyl fluoride, CAPS, Tween 20, bicinchoninic acid kit, rabbit, sheep, and goat horse-radish peroxidase-conjugated antibodies were purchased from Sigma. TPCK and mouse UCP2-IP antibody were obtained from Calbiochem. Dulbecco’s modified Eagle’s medium, fetal calf serum, and LipofectAMINE were purchased from Life Technologies, Inc. NHS-activated Sepharose 4 fast flow and ECL detection kit were purchased from Amersham Pharmacia Biotech. Human UCP2-NP antibody was ob-
translated from Research Diagnostics Inc., and mUCP2-A and hUCP2-A antibodies were from Alpha Diagnostic International (San Antonio, TX), and anti-cytocrome c antibody from Santa Cruz Biotechnology (Santa Cruz, CA).

**Animals and Treatments**—All mice were 7 to 10 weeks old. UCP2 (−/−) mice were generated on an inbred 129 and C57BL/6J background (17). C57BL6 mice were purchased from Elevage Janvier (Orleans, France) and were submitted to 24 h of starvation with free access to water or were injected intraperitoneally with 100 mg/ml TPCK, and 0.1 mM phenylmethylsulfonyl fluoride. Minced tissue or COS cells were carefully disrupted in a pcDNA3 vector (Invitrogen, Gronioen, Netherlands) after digestion by NotI. The pUC2-ORF1 was digested by restriction with the excision

**Endonuclease HindIII. Mutations of the upstream initiator methionine positions 123, 159, and 183 were achieved with the Gene Editor kit (Promega, Charbonnieres, France) in combination with 2 of the rat

**Preparation of RNA and Northern Blot Analysis**—Total RNAs were extracted from frozen tissue as described previously (21) or from transfected cells with a RNaseq kit (Qiagen, Courtaboeuf, France). Northern analysis of 20 ng of total tissue RNA or 5 ng of total cell culture RNA was carried out as described (22) using an α-32P-labeled and the complete mouse UCP2 cDNA as probe (GenBank™ accession number U96135). Quantification of UCP2 signal was determined with a Packard instant imager (Packard Instrument Co., Meriden, CT), and the signal was normalized after hybridization of the membrane with an 18 S rRNA probe.

**Purification of UCP Proteins and Production of Anti-UCP2 Antibodies**—Fragments of mouse UCP2 and the full-length rat UCP1, human UCP2, and mouse UCP3 were produced as inclusion bodies in the E. coli C41 (DE3) bacterial strain and purified as described previously (23). Proteins were refolded according to Qiagen’s protocol and purified in the presence of Fc12 detergent (Anatrace, Maumee, OH) using nick-nitriolactric acid resin (Qiagen, Courtaboeuf, France) and the BioLogic Duo-flow high pressure liquid chromatography system (Bio-Rad). Purified hUCP2 protein was cross-linked to NHS-activated Sepharose 4 fast flow. Three hundred micrograms of purified peptides or proteins were injected in 5 different rabbits, twice within 15 days and 1 month later. Animals were bled 14 days after the last boost. Blood was left 1 h at room temperature and then overnight at 4 °C and centrifuged at 3,000 × g. After inactivation of the complement at 55 °C for 20 min, serum was precipitated with ammonium sulfate according to Ref. 24 and purified by affinity chromatography on 2-ml hUCP2-NHS column as described before (25).

**Cell Culture and Transient Transfections**—The simian kidney epithelial cell line, COS-7 cells, was routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were transiently transfected using LipofectAMINE Plus Reagent according to the manufacturer’s instructions and harvested 24 h after transfection for UCP2 mRNA and protein analysis.

**Isolation of Mitochondria**—All steps were carried out at 4 °C. Fresh tissues were minced in TES buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 250 mM sucrose) supplemented with the following protease inhibitors: 1 mM benzamidine, 4 mM mg/ml aprotinin, 1 mg/ml pepstatin, 2 mg/ml leupeptin, 5 mg/ml benzonamide, 4 μg/ml aprotinin, 1 μg/ml pepstatin, 2 μg/ml leupeptin, 5 μg/ml benzanide, 4 μg/ml aprotinin, 1 μg/ml pepstatin, 2 μg/ml leupeptin, 5 μg/ml benzonamide, 4 μg/ml aprotinin, 1 μg/ml pepstatin, 2 μg/ml leupeptin, 5 μg/ml benzo...
chondrial pellet was resuspended in 1 ml of TES buffer. Mitochondria were submitted to another round of 10 min of centrifugation at 750 and 10,000 × g, respectively. Mitochondrial protein content was assayed by the bicinchoninic acid method according to the manufacturer’s protocol.

RESULTS

Generation of Highly Sensitive UCP2 Antibodies—Fragments of the mouse UCP2 and the full-length rat UCP1, human UCP2, and mouse UCP3 were produced as inclusion bodies in the E. coli C41(DE3) bacterial strain (23). Full-length human UCP2 and mUCP2 (amino acid residues 95–206) proteins were purified on nickel columns in the presence of Fc12 detergent and injected into rabbits. Three antisera were obtained, two anti-hUCP2 sera and one anti-mUCP2 (95-206) serum. Table I summarizes the data obtained with all UCP2 antibodies tested in this study. The rUCP1-375-5 serum, an antibody that we raised against the purified rat UCP1 (26), displayed high sensitivity toward UCP1. This antibody could detect only 80 ng of UCP2 inclusion bodies. The mUCP2-IP (Calbiochem), hUCP32-A and mUCP22-A (Alpha Diagnostic) antibodies had a low titer and poor reactivity toward UCP2. Among the antipeptide antibodies, the hUCP2-NP antibody (Research Diagnostic Int.) was the most sensitive toward UCP2 and was 50 times more selective for UCP2 than either UCP1 or UCP3. However, mUCP2-2/3 and both antibodies we raised against the full-length UCP2, hUCP2-605 and 606, displayed higher sensitivity toward UCP2 than all the other antibodies (Table I).

In Vivo Distribution of UCP2—Since the hUCP2-605 serum we obtained was the most sensitive antibody and equipotent toward human or mouse UCP2, it was selected to investigate UCP2 distribution in mouse tissues. Western blot analysis revealed the presence of UCP2 protein in spleen, stomach, intestine, lung, and gWAT. Mitochondria were prepared either from ucp2(+/+ or ucp2(−/−) mice tissues, and 30 μg of mitochondrial proteins were loaded onto an SDS-12.5% PAGE gel. Western blot analysis was performed using the hUCP2-605 antibody at 0.1 μg/ml (10 min of exposure of the membrane). To confirm that the equivalent amount of proteins were loaded, the same membrane was probed with an anti-cytochrome c antibody. A, positive control: 5 ng of mouse UCP2 inclusion bodies; immunodetection of UCP2 in spleen, stomach, intestine, lung, and gWAT. B, immunoreactivity of the hUCP2–605 antibody in muscle, heart, liver, brain, and BAT mitochondria.

UCP2 Protein Is Increased in Lung and Stomach upon Fast ing and LPS Treatment—It has been previously reported that fasting and LPS treatment increased the level of UCP2 mRNA in skeletal muscle and liver mitochondria, respectively (29–
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**FIG. 2. Tissue blot analysis with other anti-UCPs antibodies.** Mitochondria were isolated from tissues of ucp2+/+ mice or ucp2−/− mice and analyzed for their UCP2 content by Western blot. Gels were loaded with 30 μg of mitochondrial proteins except for B where 3 μg of BAT mitochondria were analyzed. Membranes were probed with different antibodies. A, hUCP2-NP (Calbiochem, 1 μg/ml, 10 min of exposure), the most sensitive anti-UCP2 peptide antibody; B, rUCP1-375-5 (0.1 μg/ml, 60 min of exposure), our anti-UCP1 antibody (26) that was reported to detect UCP2 by immunocytochemistry analysis (40, 43); C, hUCP32-A (Alpha Diagnostic Int., 1 μg/ml, 10 min of exposure), an anti-UCP3 antibody that recognizes UCP2 as easily as UCP3 (see Table I).

**FIG. 3. Quantitative comparison between UCP1 level in BAT and the level of UCP2 in spleen mitochondria.** Increasing amount of spleen and BAT mitochondrial proteins were loaded on SDS-12.5% PAGE. The membrane was probed with hUCP2-605 antibody at 0.1 μg/ml to detect UCP2 in spleen and UCP1 in BAT mitochondria (15 min of exposure).

**Table II**

Expression of UCP2 in mouse tissues

| Mouse tissues (mitochondrial fraction) | Relative amount of UCP2 protein | Relative amount of UCP2 mRNA |
|--------------------------------------|---------------------------------|-----------------------------|
| Spleen (control)                     | 1 ± 0.05 n = 11                 | 1 ± 0.01 n = 3              |
| Spleen (LPS)                         | 0.9 ± 0.17 n = 6 NSb            | 1.1 ± 0.03 n = 3 NSb        |
| Spleen (LPS)                         | 0.24 ± 0.01 n = 14              | 1.2 ± 0.01 n = 3            |
| Lung                                 | 1.5 ± 0.25 n = 11 p < 0.001     | 1.3 ± 0.05 n = 3 NSb        |
| Lung (LPS)                           | 2.9 ± 0.45 n = 5 p < 0.001      | 1.3 ± 0.52 n = 2 NSb        |
| Lung (LPS + cycloheximide)           | 0.25 ± 0.04 n = 4 p < 0.001     | ND                          |
| Stomach                              | 0.1 ± 0.004 n = 14              | 1.1 ± 0.05 n = 4            |
| Stomach (cycloheximide)              | 0.58 ± 0.08 n = 5 p < 0.001     | 1.2 ± 0.07 n = 3 NSb        |

**a** Spleen was chosen as reference for both UCP2 mRNA and protein levels. Results are expressed as mean ± S.E.; n, number of independent experiments. p value was calculated versus the control condition using Student’s t test.

**b** NS, not significant.

**c** ND, not determined.

32). Therefore, C57BL/6 mice were either treated with LPS (injected intraperitoneally with 100 μg of LPS or PBS buffer) or fasted for 24 h, and several organs were analyzed for their UCP2 protein content. Following 24 h of fasting, UCP2 remained undetectable in muscle and constant in spleen mitochondria, but a 6-fold increase of UCP2 protein was observed in stomach and lung mitochondria (Fig. 4, A and B). Two LPS-treated mice and two PBS-treated mice were killed 6, 8, 10, 12, 14 (3 mice), 18, and 24 h after injection. Western blot analysis showed that UCP2 protein appears 10 h after LPS injection only in lung mitochondria and reaches its maximal level 2 h later. UCP2 protein returned to its basal expression level 24 h after injection (Fig. 5, A and B). No change in UCP2 protein was observed in the control mice treated with PBS (Fig. 5B) and in stomach, liver, duodenum, kidney, heart, muscle, and spleen of LPS-treated mice. Quantitative analysis of LPS stimulation revealed that UCP2 protein increased 12-fold in lung mitochondria 14 h after injection (p < 0.001, Table II). To assess whether the apparent increase of UCP2 protein in lung mitochondria could simply reflect an increase of UCP2 protein stability, 280 μg of cycloheximide, an inhibitor of protein translation, was also injected intraperitoneally in four mice 9.5 h after LPS injection. Two and one-half hours later all mice were killed and analyzed for their UCP2 content in lung mitochondria. Cycloheximide treatment completely abolished UCP2 stimulation observed 12 h after LPS injection (98.2% inhibition p < 0.001, Table II). This experiment showed that LPS treatment stimulates the de novo synthesis of UCP2 protein in lung.

UCP2 mRNA Levels Do Not Reflect UCP2 Protein Content in Mitochondria—To investigate whether the fluctuations in the protein content correlated with gene expression, total RNAs were prepared, and UCP2 mRNA level was estimated by Northern blot analysis. Although UCP2 protein level was 4 and 10 times lower in lung and stomach, respectively, than in spleen mitochondria, UCP2 mRNA levels in those tissues were very similar (Table II). The same discrepancy between UCP2 protein and mRNA levels was observed in stimulated conditions. The levels of UCP2 mRNA in lung and stomach marginally increased upon fasting (Fig. 4, C and D, and Table II).
UCP2 mRNA levels also remained surprisingly unchanged over the LPS time course experiment (Fig. 5C). Fourteen hours after LPS injection, UCP2 mRNA levels in spleen and lung were comparable, although UCP2 protein content in lung had increased 12 times to reach three times the basal level of UCP2 in spleen (Table II). Finally, despite the strong variations of UCP2 protein that were observed in basal or stimulated conditions, the level of UCP2 mRNA did not correlate with the amount of UCP2 protein.

An Open Reading Frame in the Exon 2 of ucp2 Gene Inhibits the Translation of the Protein—The striking difference between the abundance of UCP2 mRNA and the tiny amount of UCP2 protein detected in mitochondria as well as the discrepancy between UCP2 mRNA and protein levels prompted us to investigate whether expression was regulated at the translational level. We have shown that ucp2 gene contained a short open reading frame (ORF1) in exon 2 which potentially encodes a putative peptide of 36 amino acids (33) (Fig. 6A). Although the translation of UCP2 protein was not affected by the ORF1 in reticulocyte lysate (33), we examined whether its presence could influence the expression of UCP2 in transiently transfected COS cells. COS cells were transfected with the pcDNA3 vector, recombinant UCP2 mRNA had a higher apparent molecular weight than mouse UCP2 mRNA (Fig. 6B). Although UCP2 mRNA level was 56 times higher in cells transfected with pUCP2 construct than in mouse spleen, the amount of UCP2 protein was only 3.5 times higher in COS cell mitochondria than in spleen mitochondria (Fig. 6B and Table III). Thus, the coupling between transcription and translation of ucp2 gene is more efficient in mouse spleen than in transfected COS cells. However, transfection of COS cells with pUCP2-ORF1 or pUCP2-ATG increased the production of UCP2 protein 50 and 17 times, respectively, in COS cell mitochondria, without major changes in UCP2 mRNA levels (Fig. 6, B and C). In the absence of ORF1, UCP2 protein content in COS cell mitochondria reached up to 176 times the level of UCP2 in spleen mitochondria, although the UCP2 mRNA level had increased only 28 times (Table III). These results demonstrated that the ATGs present in ORF1 strongly inhibits the translation of UCP2 mRNA and supports the lack of correlation between UCP2 mRNA levels and protein content observed in mouse tissues.

UCP3, BMCP1, and Other Mitochondrial Carriers Contain Upstream ORF—The 5′-untranslated region of all known mammalian mitochondrial carriers was analyzed for the presence of upstream AUG or ORF. Among the 33 human and...
rodst.cDNAsequencesavailable,9ofthemcontainedatleast
to the gene inhibits the translation of UCP2. A, amino acid sequence of oneupstream AUG or ORF. We found that ucp3 and bmcp1 also contained 1 and 3 ORFs, respectively (Table IV). Statistical parameters developed by Kochetovet al. (34) were used to predict translational efficiency of these mitochondrial carriers. Messenger RNA that are weakly translated generally possess a messenger RNA that are weakly translated generally possess a 5'-UTR (more than 50 nucleotides), a G + C content in their 5'-UTR around 60%, and a context of complementary nucleotides given by the A/U ratio between 0.75 and 1.25. In addition to their upstream ORF, the five mitochondrial carriers that contained upstream ORFs fulfilled at least two out of the three conditions described above (Table IV). These results indicate that ucp3 and bmcp1 are probably subject to translational regulation and, consequently, are poorly expressed in vivo.

**DISCUSSION**

**Immunodetection of UCP2**—Several attempts were made to identify in vivo UCP2 protein. Peptide antibodies (35–37), antibodies against the full-length UCP2 (38), or anti-UCP1 antibodies (14, 39, 40) were employed to detect the protein either by Western blot or immunocytochemistry analysis (41–44). The identification of UCP1, UCP2, or UCP3 was established by correlation with the tissue distribution of the mRNA. It was also advocated that competition with synthetic peptides and the apparent molecular weight confirmed the identification of the bands as UCP1, UCP2, or UCP3 (38, 45). These arguments are not sufficient to identify the UCPs in vivo since most of the anti-UCP antibodies cross-react with other proteins. This is especially true in liver mitochondria since four antibodies tested in this study detected nonspecific bands (Figs. 1 and 2). We also tested the N-terminal UCP2 antibody (Santa Cruz Biotechnology) that has been used by Diehl's group (35, 37). One strong nonspecific band was detected in liver mitochondria around 32 kDa (data not show). To reconcile biochemical data previously observed in liver and our immunological data, one could speculate that there is another member of the UCP family, specifically expressed in liver mitochondria, that might have a similar function than UCP2. Our hUCP2-605 antibody also shows a moderate specificity. Therefore, mitochondria from tissues of ucp2(−/−) mice were essential to identify the protein in vivo. By doing so, we demonstrated that UCP2 protein is undoubtedly expressed in spleen, lung, stomach, and gWAT mitochondria. Regarding the other tissues, UCP2 protein might be present at very low levels, expressed only in some subset of cells or simply not expressed. For instance, although UCP2 mRNA has a neuronal localization by in situ hybridization, its distribution is not uniform in the brain, and the mRNA is especially abundant in the suprachiasmatic nucleus and other nuclei (46).

**Translational Regulation of UCP2 Expression**—Several lines of evidence support the possibility that UCP2 mRNA does not correlate with the variation of the protein. First, UCP2 protein is 10 times less expressed in stomach mitochondria than in spleen even if the relative amount of UCP2 mRNA in both organs is similar (Table II). Second, the increase of UCP2 protein in lung and stomach upon fasting or LPS treatment is
not accompanied by an increase of UCP2 mRNA. Third, inactivation of ORF1 in ucp2 gene increased 50 times the expression of UCP2 protein in transfected COS cells without any major changes at the mRNA level. Cotransfection of COS cells with a vector encoding ORF1 peptide and pUCP2-ATG did not repress the expression of the UCP2 protein, and disruption of the first or the second ATG of the upstream ORF also stimulated the expression of UCP2 (data not shown). Thus, the ucp2 gene is down-regulated in cis at translational level by an upstream ORF. These results do not exclude transcriptional regulation of the ucp2 gene. UCP2 mRNA is highly expressed in spleen, lung, stomach, and WAT but is weakly expressed in the other tissues. Since UCP2 protein was found only in tissues where the its mRNA was highly abundant, it is possible that the tissue specificity of UCP2 might be to protect the organism from oxidative stresses provoked by infection, allergy, or pollution.

**Physiological Aspects**—It is admitted that UCP1 is a slow proton transporter highly expressed in brown adipose tissue mitochondria. In cold-adapted rat, UCP1 protein reaches up to 5% of mitochondrial proteins (48), and its thermogenic contribution relies on its abundance and its activation by free fatty acids. After LPS stimulation, UCP2 protein increases in lung but reaches only 0.02% of the mitochondrial proteins, suggesting that either both proteins do not work the same way or they do not have the same function. On one hand, since UCP2 and UCP3 are weakly inhibited by purine nucleotides (49–51), it is possible that UCP2 is constantly activated in vivo whereas UCP1 is always inhibited by nucleotides in BAT mitochondria. On the other hand, one could also propose that the function of the new UCPs is not to trigger thermogenesis by a strong mitochondrial uncoupling. Negre-Salvayre et al. (14), Lee et al. (13), and Yang et al. (37) have proposed that UCP2 regulates the production of reactive oxygen species by uncoupling the respiratory chain, as suggested by Skulachev (52). In fact, studies on ucp3(−/−) mice (16) and ucp2(−/−) mice (17) showed that both proteins do indeed regulate the production of ROS since higher levels of ROS were observed in muscle and macrophage, respectively. The finding that UCP2 is increased in lung and stomach upon LPS treatment and starvation also support this hypothesis. The kinetics of induction of UCP2 protein after LPS injection is consistent with a primary immune response leading to an oxidative burst in lung. Activation of macrophage receptors by LPS stimulate the production of proinflammatory cytokines such as TNFα (53) which activates the NF-κB pathway. The cellular content of reduced glutathione decreases, and consequently, the level of intracellular ROS increases (for review see Ref. 54). Since starvation has also been shown to provoke a marked decrease of reduced glutathione level in lung (54) and in stomach (55), one could propose that UCP2 does not have any function in basal conditions but is up-regulated when the level of intracellular reactive oxygen species is too high. In this context, translational regulation of UCP2 might ensure a rapid cellular response, based on the large pool of UCP2 mRNA available. Finally, our data point out the lung and the stomach as two major organs where UCP2 plays an important role. Since both organs are constantly exposed to toxic compounds and pathogens, the natural function of UCP2 might be to protect the organism from oxidative stresses provoked by infection, allergy, or pollution.

**Acknowledgments**—We are grateful to Hiroki Onuma for work in characterizing the ucp2(−/−) mice; L. P. Kozak for the gift of pHis17 expression vector; D. Sanchis for the cloning of mouse UCP3 cDNA; M. Marsolo and A. Pigent for technical assistance; and K. Marheineke for critical reading of the manuscript.

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