Regulation of Cytokine-Induced Nitric Oxide Synthesis by Asymmetric Dimethylarginine

Role of Dimethylarginine Dimethylaminohydrolase

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Abstract—In response to vascular insults, inflammatory cytokines stimulate vascular smooth muscle cells (SMCs) to express an inducible isoform of nitric oxide synthase (iNOS). Asymmetric dimethylarginine (ADMA), an endogenous NO synthase inhibitor, is metabolized by dimethylarginine dimethylaminohydrolase (DDAH). To determine whether the ADMA-DDAH system regulates cytokine-induced NO production, cultured rat SMCs were exposed to interleukin-1β (IL-1β). IL-1β (1 to 100 U/mL) dose-dependently stimulated not only iNOS but also DDAH expression and enzyme activity, accompanied by an increase in NO metabolite and by a decrease in ADMA content in culture media. A DDAH inhibitor (4124W, 5 mmol/L) augmented ADMA production (P<0.01) and decreased NO synthesis (P<0.01) in IL-1β–stimulated SMCs. On the other hand, an adenovirus-mediated overexpression of DDAH reduced ADMA and enhanced NO production. Exogenous administration of NO donors (SNAP and SIN-1) dose-dependently increased NO metabolite in the culture media but had no effect on ADMA. Our results indicate two mechanisms of IL-1β–induced NO synthesis: the direct stimulation of the expression of iNOS and the indirect stimulation of iNOS activity by upregulating DDAH and reducing ADMA. The ADMA-DDAH system may be another regulatory mechanism of inflammation-mediated NO production for human vascular diseases. (Circ Res. 2003;92:1146–1152.)

Key Words: nitric oxide • nitric oxide synthase • interleukins • smooth muscle • atherosclerosis

Nitric oxide (NO) plays an important role in the regulation of not only vascular tone but also architecture. There are two types of nitric oxide synthase in vessels, endothelium-derived NO synthase (eNOS) and inducible NO synthase (iNOS); the former is constitutively present in the endothelium, and the latter is induced by the inflammatory stimuli in the vascular wall. 1–3 Dimethyl-L-arginine (ADMA) is an endogenous NOS inhibitor that inhibits activities of both eNOS and iNOS. We and others have demonstrated that increased plasma levels of ADMA are associated with hypertension, 4 hypercholesterolemia, 4 and the clustering of coronary risk factors in vivo in humans. 5 Furthermore, a close association has been found between plasma ADMA level and carotid intima-media thickness 6 or the severity of peripheral arterial occlusive diseases. 6 These lines of evidence suggest that ADMA is involved in the pathophysiology of human vascular diseases. ADMA is metabolized by a key limiting enzyme, dimethylarginine dimethylaminohydrolase (DDAH). 7–9 DDAH is identified in human aortic tissues. 10–13 It has been reported that oxidized low-density lipoprotein upregulates the synthesis of ADMA in cultured human endothelial cells in association with decreased activity of DDAH. 13 Collectively, the ADMA-DDAH system may be involved in the regulation of endogenous NO synthesis. 14–17

Inflammatory response plays a crucial role in human vascular diseases. 18 Monocyte-derived macrophages, recognized at every stage of disease, promote continuous activation of the cytokine growth factor network in lesions. 19 Inflammatory cytokines, such as interleukin-1β (IL-1β), not only stimulate proliferation of vascular smooth muscle cells (SMCs) but also promote free radical formation. 20,21 Although sources of oxidative stress are diverse, several lines of evidence suggest that large production of NO derived from cytokine-induced NO synthase induces vascular injuries. 22–25 In the present study, we hypothesized that the ADMA-DDAH system regulates cytokine-induced NO production. To test this hypothesis, the effects of pharmacological inhibition and adenovirus-mediated overexpression of DDAH on NO production were examined in IL-1β–stimulated SMCs.

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Materials and Methods

Materials

Human recombinant IL-1β was purchased from PeproTech. ADMA, symmetric dimethylarginine (SDMA), 3-morpholino-sydnonimnin (SIN-1), and S-nitroso-N-acetylpenicillamine (SNAP) were purchased from Wako Co. A mouse monoclonal anti-iNOS antibody that recognizes type I isoform (DDAH I) was generated as previously described. A mouse monoclonal anti-iNOS antibody was purchased from Transduction Laboratories (Lexington, KY). S-2-Amino-4-[3-methylguanidino]butanoic acid (4124W) was synthesized by coupling of 2,4-diamino-n-butric acid · 2HCl with N,S-dimethylthiopseudouronium iodide in the same manner described previously. It was crystallized as a hydrochloride, 4124W · HCl · 1/2H2O (found: C, 32.65%; H, 7.56%; N, 25.41%, respectively. Calculated for C6H14N4O2 · HCl · 1/2H2O: C, 32.80%; H, 7.34%; N, 25.50%).

Cultured SMCs

Rat aortic SMCs were isolated by enzymatic digestion from the thoracic aorta of 6-week-old Sprague-Dawley rats (Charles River Japan, Kanagawa, Japan), as previously described. All surgical interventions and anesthesia were conducted in conformity with institutional guidelines and in compliance with international laws and policies (EEC Council Directive 86/609, OJL 358, December 1987; Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985). The cells were cultured in DMEM (Nissui Pharmaceutical Co) containing 10% FBS (Filtron Pty Ltd) together with antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; Life Technologies) at 37°C in a humidified 5% CO2 to air atmosphere, and SMCs from 5 to 10 passages were subjected to the following experiments.

Measurement of Nitrate and Nitrite and ADMA

Measurements of Nitrate and Nitrite and ADMA

Nitrate and nitrite (NOx) were analyzed by high-performance liquid chromatography (HPLC). One hundred microliters of conditioned media were mixed with 200 μL of ethanol by a vortex mixer for 2 minutes. After the mixture was centrifuged (12 000 rpm) at 4°C for 15 minutes, the clear supernatant was injected into the HPLC analyzer. Then nitrate and nitrite were separated by an anion exchange column (Shodex IC-524A, 60×300 mm, Showa Denko), and nitrate was reduced to nitrite in a reduction column packed with copper-plated cadmium fillings (Cd-Cu Reduction Column, Tokyo Kasei Kogyo). The separation and reduction columns were placed in a column oven (L-5025, Hitachi) that was set to 40°C. The mobile phase (NO Carrier Solution A7500, diluted to 50, Tokyo Kasei Kogyo) was delivered by a pump (L-7100, Hitachi) at a rate of 0.6 mL/min. The Griess reagent (NO Regent Solution A7502, B7503:1:1, Tokyo Kasei Kogyo) was delivered by a pump (301, Flom) at a rate of 0.4 mL/min. The reaction product was determined by the absorbance at 540 nm using a flow-through spectrophotometer (SPD-10A, Shimadzu). Data acquisition and integration were performed using an integrator (C-R5A, Shimadzu). Intra-assay and interassay variance was 2.6% and 2.7%, respectively.

ADMA was analyzed by HPLC using the column switching method, which constitutes two separation steps; by a precolumn, the crude fraction containing dimethylarginines was isolated and subsequently ADMA was separated by an analytical column (ODS). Samples were deproteinized by the same manner described above, and the supernatants were incubated for 5 minutes with OPA reagent (2.3 mg/mL OPA in borate buffer, pH 9.7, containing 2.3 mg/mL N-acetylcysteine) before automatic injection (injection volume, 15 μL) into the HPLC column (temperature, 49°C). HPLC was performed on the Hitachi L-6300 system equipped with a F-1080 absorbance detector at 540 nm and emission at 450 nm. Precolumn, in which the mobile phase was 75 mmol/L sodium acetate buffer (pH 6.0) containing 3% acetonitrile at flow rate of 0.8 mL/min, was an anion-exchanged column (Shodex NH2P50, 4.6 mm inner diameter (ID)×50 mm). Analytical column, in which the mobile phase was 75 mmol/L sodium acetate buffer (pH 6.0) containing 2.5% tetrahydrofuran at flow rate of 0.8 mL/min, was an ODS column (L-column ODS, 5 μm, 4.6 mm ID×250 mm, Chemical Inspection and Testing Institute). Intra-assay and interassay variance was 2.8% and 2.4%, respectively, and the detection limit of the assay was 0.02 mmol/mL. NOx and ADMA were adjusted relative to cell numbers, which were counted using a hemocytometer after trypan blue exclusion.

Measurement of DDAH Activity

The DDAH activity was assayed as previously described. Briefly, the cell lysate was incubated with 4 mmol/L ADMA and 0.1 mol/L sodium phosphate buffer (pH 6.5) in a total volume of 0.5 mL for 2 hours at 37°C. The reaction was stopped by the addition of equal volume of 10% trichloroacetic acid, and the supernatant was boiled with diacetyl monoxime (0.8% [wt/vol] in 5% acetic acid) and antipyrine (0.5% [wt/vol] in 50% sulfuric acid). The amounts of l-citrulline formed were determined with the spectrophotometric analysis at 466 nm. As the assay blank, the enzyme preparations heated in a boiling water bath were subjected to the determination of the activity.

Recombinant Adenoviruses

The plasmid, including the entire coding region of rat DDAH cDNA (pDDAH21), was cloned as previously described. To produce AdvCMVDDAH (Adv-DDAH), an adenovirus vector expressing DDAH protein under the control of the CMV promoter, the entire coding region of DDAH was inserted into an E1, E3-deleted, human adenovirus serotype 5 mutant, dl7001, with homologous recombination in 293 cells (American Tissue Culture Collection), as previously described. Adv-LacZ encoding bacterial β-galactosidase was used as a control virus. At the infection of adenovirus vectors, the cells were incubated for 24 hours in DMEM containing 2% FBS and adenovirus vectors at the indicated multiplicity of infection (MOI). Determination of transfection efficiencies by in situ X-Gal staining revealed that at least 80% of cells were positive for β-galactosidase 24 hours after the infection with 100 MOI of Adv-LacZ. There were no significant differences in cell morphology or concentration of lactate dehydrogenase in conditioned media between viral vector–infected cells and mock-infected cells, suggesting a low level of cellular toxicity at the maximal MOI applied in the series of experiments (data not shown).

Western Blotting

Cells were harvested in the lysis buffer containing 50 mmol/L Tris-HCl (pH 6.8), 10% glyceral, 2% sodium dodecyl sulfate (SDS), and 5% mercaptoethanol. Equal amounts of samples normalized by cell number were subjected to SDS-polyacrylamide gel electrophoresis and transferred on PVDF membranes (Immobilon). Membranes were incubated with a mouse monoclonal antibody to iNOS or DDAH I and then with alkaline phosphatase–conjugated secondary antibodies (Oregon Teknika, West Chester, PA) and subjected to colorimetric detection using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate p-tolidine salt (Boehringer Mannheim). To confirm that equal amounts of the proteins were subjected to the Western blotting analysis, the membranes were reprobed with an antibody against β-actin (Sigma, St Louis, Mo). Densitometric analysis was performed with NIH image software, and the relative ratio to the β-actin expression was calculated in each sample.

Statistical Analysis

Experimental groups were compared by ANOVA and, when appropriate, with Scheffe’s test for multiple comparisons. All data were expressed as mean±SD. P<0.05 was accepted as statistically significant.

Results

IL-1β-Induced NO Synthesis Was Associated With a Decrease in ADMA Through Upregulation of DDAH

SMCs were continuously stimulated with IL-1β for as long as 60 hours. IL-1β (100 U/mL) stimulated NO synthesis in...
SMCs. The accumulation of NOx began 24 hours after the stimulation, and it was 9.5 ± 0.9-fold higher than that of the control at 60 hours (*P* < 0.01; Figure 1A). In control cells, ADMA time-dependently accumulated, whereas ADMA accumulation was attenuated in IL-1β–stimulated SMCs (*P* < 0.01; Figure 1B). The expression of DDAH and the enzyme activity were time-dependently upregulated by IL-1β (Figures 1C and 1D, respectively). Because the ADMA production plateaued at 48 to 60 hours, the subsequent experiments were conducted at 60 hours of incubation. Next, we examined the dose-dependent effects of IL-1β. When the cells were stimulated with 1 to 100 U/mL of IL-1β, we observed dose-dependent inductions of iNOS at the protein level and accumulation of NOx in cultured media (Figures 2A and 2B, respectively). Similarly, the expression and the activity of DDAH were upregulated by IL-1β in a dose-dependent manner (Figures 2D and 2E, respectively), in association with dose-dependent reduction of ADMA in the culture media (Figure 2C).

Exogenously Applied ADMA Inhibited NO Synthesis Without Affecting iNOS Expression

To determine whether exogenously applied ADMA inhibits NO synthesis, we added ADMA and its inert isomer, SDMA, into the culture media (1 mmol/L each) with or without IL-1β stimulation. Neither ADMA nor SDMA altered iNOS expression, irrespective of IL-1β stimulation (Figure 3A). However, IL-1β–induced NO synthesis was significantly inhibited by ADMA but not by SDMA (*P* < 0.01; Figure 3B), indicating a reduction of iNOS activity by ADMA.

Effects of Intervention of DDAH on ADMA and NOx

We first examined the effects of 4124W, an inhibitor of DDAH, on ADMA and NOx production in IL-1β–stimulated cells. 4124W did not affect ADMA or NO synthesis in SMCs without IL-1β stimulation. With IL-1β stimulation, 4124W abolished the ADMA reduction (Figure 4A) and attenuated NO synthesis in a dose-dependent manner (Figure 4B). 4124W itself did not alter the expression of iNOS (Figure 4C) or DDAH (data not shown). Second, we examined the effects of upregulation of DDAH on ADMA and NO synthesis in SMCs with or without IL-1β stimulation. The overexpression of DDAH by Adv-DDAH inhibited ADMA production in SMCs, irrespective of IL-1β stimulation (Figure 5A). However, the overexpression of DDAH augmented NOx production in SMCs only with IL-1β stimulation (Figure 5B).
Effects of NO Donors on the ADMA-DDAH System
To examine the effects of NO on the ADMA-DDAH system, chemically synthesized NO donors (SNAP and SIN-1) were added into the culture media without IL-1β stimulation. Although NO donors dose-dependently increased NO to levels similar to those in SMCs stimulated by IL-1β, ADMA levels were not altered (Figure 6).

Discussion
In this study, an inflammatory cytokine, IL-1β, increased NOx by upregulating not only iNOS but also DDAH in vascular SMCs. The upregulated DDAH decreased ADMA, which augmented the activity of iNOS. This is the first demonstration that the ADMA-DDAH system regulates cytokine-induced NO synthesis in vascular SMCs.

DDAH Subtypes in SMCs
We investigated IL-1β-induced DDAH expression at the protein level and activity. Two isoforms of human DDAH (DDAH I and DDAH II) have different tissue distribution.29,30 In the present study, we used a monoclonal antibody against DDAH I, which does not recognize DDAH II. Because DDAH activity was increased in parallel with DDAH I expression when stimulated with IL-1β (Figures 1 and 2), it seems that DDAH I is a major isofrom determining the activity of DDAH in SMCs. DDAH I expression is predominantly found in the tissue expressing neuronal isoform of NOS.29 In this study, we show that DDAH I plays an important role in the cytokine-induced NO synthesis by vascular SMCs as well.

iNOS and DDAH Expression in SMCs
In humans, vascular SMCs play an important role in the development of vascular diseases, and several factors are involved in their activation and disease progression.18–20 Inflammatory cytokine–induced activation of iNOS is a major factor.18–20 It is well accepted that a large amount of NOx produced by iNOS is cytotoxic and atherogenic, rather than cytoprotective, because of formation of highly toxic...
peroxynitrite. Activated SMCs in atherosclerotic lesions produce a large amount of NO by iNOS on exposure to cytokines. Among several inflammatory cytokines, IL-1β is considered one of the most potent stimuli for iNOS induction in SMCs. Accordingly, in this study, we investigated the regulatory mechanisms of NOx production by iNOS in IL-1β–stimulated cultured SMCs. In the control state, without IL-1β stimulation, ADMA accumulated time dependently in the culture media, which may have been attributable to spontaneous degradation of methylated protein, a source of ADMA. Although ADMA competitively inhibits iNOS activity, there was no iNOS expression in the control state (Figure 2A). Thus, despite the increases in the accumulation of ADMA, NOx production was unchanged in the control state.

As reported by many investigators, IL-1β increased iNOS expression at the protein level and NOx production in time- and dose-dependent manners (Figures 1 and 2). At the same time, IL-1β increased DDAH activity and expression at the protein level and inhibited the accumulation of ADMA. Because DDAH is a key enzyme for the metabolism of ADMA, it is likely that the increased DDAH protein decreased the ADMA content. Thus, our results suggest that IL-1β increased NOx production by upregulating iNOS not only at the protein level but also by augmenting iNOS activity via decrease of ADMA, of which decrease is caused by IL-1β–stimulated DDAH. However, ADMA formation increased barely significantly within 24 hours in response to incubation with cytokines in contrast to a strong increase in NOx formation. This observation suggests that changes in NOx production within the first 24 hours are not related to intracellular changes in ADMA. Accordingly, we should limit our observation to this time period. When increasing doses of IL-1β were applied, ADMA and iNOS were changed linearly but NOx was exponentially increased, suggesting that IL-1β–stimulated NOx production is regulated not only by iNOS and ADMA but also other factors. ADMA production is regulated by the protein arginine methyltransferase (PRMT)–induced methylation of arginine residue and the degradation by DDAH, which may explain the relatively small reduction of ADMA compared with the increase in DDAH after IL-1β stimulation. It is possible that IL-1β may also affect PRMTs. This possibility needs to be studied additionally.

Next, we studied whether the inhibition of iNOS activity by ADMA decreases NOx production in our system. As shown in Figure 3, exogenously applied ADMA but not SDMA attenuated IL-1β–induced NOx production without affecting iNOS protein expression. These findings suggest that IL-1β stimulates NO production by a dual pathway, ie, by the transcriptional induction of iNOS protein and by the augmentation of iNOS activity through the DDAH-mediated elimination of ADMA (Figure 7). To additionally test this hypothesis, we manipulated DDAH activity and expression and assessed subsequent NO production.

**DDAH Manipulation: Pharmacological Inhibition**

In the present study, we investigated whether 4124W, an inhibitor of DDAH, inhibits DDAH activity, increases ADMA, and decreases NOx production in vascular SMCs. In the control state, exogenously applied 4124W did not affect ADMA or NOx production (Figure 4), possibly because of the lack of iNOS expression in the absence of IL-1β stimulation. When vascular SMCs were exposed to IL-1β, ADMA was decreased and NOx production was augmented. When 4124W was applied exogenously, it abolished IL-1β–induced
ADMA reduction and attenuated NO production in a dose-dependent manner (Figure 4), indicating that 4124W inhibited IL-1β–induced upregulation of DDAH activity. Although 4124W did not affect iNOS expression induced by IL-1β at the protein level, we could not deny the possibility, based on our findings, that 4124W changed the activity of iNOS and decreased NOx production. Our results suggest that the ADMA-DDAH system regulates the IL-1β–induced NOx production. Consistently with our results, it has been reported that 4124W causes concentration-dependent contraction of precontracted rings of endothelium-denuded rat aorta that have been incubated with the endotoxin to induce the expression of iNOS.\textsuperscript{17} Thus, the ADMA-DDAH system may widely contribute to the inflammation-induced NO synthesis in SMCs.

In this study, relatively high concentrations (1 to 5 mmol/L) of 4124W were required for the inhibition of ADMA metabolism and NO production in cytokine-stimulated SMCs (Figure 4). It has been shown previously that 4124W inhibits methylarginine metabolism and increases intracellular ADMA sufficiently enough to inhibit endogenous NO synthesis.\textsuperscript{17} However, it has been suggested that 4124W is a relatively weak inhibitor of DDAH. In this study, the very high concentration of 4124W was required to suppress NOx in the presence of IL-1β, supporting the weak activity of 4124W. Another reason may be that IL-1β–induced NO production is regulated not only by ADMA-DDAH system but also by a direct effect of IL-1β on iNOS gene.
Next, we examined the effects of transfection of adenovirus encoding DDAH on SMC. In the absence of IL-1β stimulation, the overexpression of DDAH decreased ADMA >30% (Figure 5), suggesting that transfected DDAH is enzymatically active. Despite the decrease of ADMA, the basal NOx level was not affected, because unstimulated SMCs do not express iNOS. When IL-1β was added into the culture medium, ADMA was decreased and NOx was increased, with or without AdvCMVLacZ (Adv-LZ) transfection, as shown repeatedly in this study. When vascular SMCs were transfected by adenovirus encoding DDAH, ADMA was decreased and NOx was increased additionally. This effect of the transfection of Adv-DDAH is not nonspecific, because Adv-LZ transfection did not change ADMA or NOx level compared with controls. Considering the results of the two manipulations of DDAH, ie, inhibition of DDAH by 4124W and overexpression of DDAH, we suggest that the ADMA-DDAH dose regulated NOx production in IL-1β–stimulated SMCs.

Several factors other than DDAH are known to be involved in ADMA metabolism, including (1) arginine methylation by PRMT type I concomitant with protein catabolism; (2) degradation by alanine glyoxalate aminotransferase (AGT-2)36; and (3) renal excretion in vivo.8 Although we did not examine the possible involvement of these factors in the present study, it would be of interest to investigate them in future studies. Nonetheless, several remarks may be made. First, it seems unlikely that AGT-2 played a role in the

Figure 5. Effects of the overexpression of DDAH on IL-1β–induced NO production. Cultured rat SMCs inoculated on 6-well dishes were infected with 100 MOI of Adv-LZ or Adv-DDAH or mock infected (NV). The cells were then stimulated with or without 10 U/mL of IL-1β for 60 hours. ADMA content (A) and NOx content (B) in the conditioned media were measured by HPLC (n=6, respectively).

Figure 6. Effects of NO donors on ADMA production. Rat SMCs inoculated on 6-well dishes were stimulated with NO donors (1 to 100 nmol/L of SNAP or SIN-1). NO production (A) and ADAM content (B) in the conditioned media were measured by HPLC (n=6, respectively).

Figure 7. Hypothetical pathways of mechanisms of NO production by IL-1β. IL-1β produces NOx in vascular smooth muscles by stimulating INOS and DDAH. DDAH decreases ADMA, which subsequently augments INOS activity.
metabolism of ADMA in our study, because AGT-2 has been shown to be localized in nonvascular tissues and to have a low binding affinity for ADMA. Second, it has been suggested that high output of NO plays a role in the posttranslational modification of proteins. Thus, it could be anticipated that enzymatic activity of DDAH is altered by a large amount of NO released from SMCs. To exclude this possibility, NO donors (SNAP and SIN-1) were added into the culture media in the absence of IL-1β. SNAP and SIN-1 markedly increased NOx in the medium to the levels 1.5 to 2 times higher than those by IL-1β stimulation but had no effects on the ADMA level. These results indicate that high output of NO per se does not play a role in the regulation of the ADMA-DDAH system.

Clinical Implications

Our results indicate that in vascular SMCs, IL-1β stimulates both iNOS and DDAH, resulting in the production of NOx, which raises the possibility that not only iNOS but also DDAH plays a role in the pathophysiology of human vascular diseases. In support of this hypothesis, we have recently detected the expression of DDAH in human atherosclerotic lesions, in association with reduced ADMA content and high activity of iNOS (Kató et al, unpublished data). It has been shown that high plasma levels of ADMA are associated with endothelial dysfunction and atherosclerotic diseases. In addition, ADMA release from cultured endothelial cells was augmented in the presence of oxidized LDL. Although serum ADMA levels were elevated in patients with atherosclerosis, the source of ADMA has not been clearly identified, ie, endothelium, vascular smooth muscle, or other cells. Thus, it is possible that elevated serum levels may derive from cells other than vascular smooth muscles. In hypercholesterolemic rabbit model, tissue activities of DDAH in aorta, kidneys, and liver were significantly reduced in all these tissue, whereas its expression was not altered. However, it is not yet clarified which types of cells in the tissue showed decreased DDAH activation. Accordingly, regulations of ADMA and DDAH may be diverse in endothelial cells, vascular smooth muscles, and other types of cells. Although additional in vivo study is necessary to extend our in vitro findings, we do think that the present study does not necessarily conflict with the data provided by previous studies.

In conclusion, IL-1β augmented NO production by upregulating iNOS and DDAH in vascular SMCs. Our data suggest that the ADMA-DDAH system may play an important role in the pathogenesis of human vascular diseases as a potential regulatory mechanism of iNOS activity.

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