Inhibitory Effects of Alpha-zearalenol on Angiotensin II-Induced Integrin β_3 mRNA via Suppression of Nuclear Factor-κB[1]

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Objective To investigate the effect of α-zearalenol on angiotensin II-induced β_3 integrin mRNA expression in human umbilical vein endothelial cells (HUVECs). **Methods** The mRNA level in integrin β_3 was determined by reverse transcription–polymerase chain reaction. Endothelial NF-κB activity was determined by the luciferase activity assay of plasmid NF-κB-LUC. **Results** The angiotensin II- induced β_3 integrin mRNA expression was inhibited by α-zearalenol and 17β-estradiol (10 nmol/L -1 μmol/L), but not influenced by ICI 182, 780, a pure competitive antagonist for estrogen receptor or a nitric oxide inhibitor N^{ω}-Nitro-L-arginine methyl ester hydrochloride. Alpha-zearalenol and 17β-estradiol suppressed the angiotensin II-induced activation of NF-κB in endothelial cells. **Conclusion** Alpha-zearalenol inhibits angiotensin II-induced integrin β_3 mRNA expression by suppressing NF-κB activation in endothelial cells.

Key words: Alpha-zearalenol; Integrin β_3; Endothelial cell; NF-κB; 17β-estradiol

INTRODUCTION

Estrogens may act at several steps in the atherosclerosis process to prevent cardiovascular diseases[1]. However, the concerns have been raised about the effects of conventional estrogen on the progression of breast and uterine neoplasms and the tendency to promote thrombosis[2]. Even a randomized, blinded, placebo-controlled clinical trial for secondary prevention of coronary heart disease in postmenopausal women fails to confirm its anti-atherosclerotic action[3]. Recently, phytoestrogens, plant-derived partial estrogen receptor agonists, including isoflavones, lignans and coumestans, have been shown to be anti-atherosclerotic for their lipid-lowering effects and to have ability to prevent low-density lipoprotein oxidation. Most importantly, they exert their anti-atherosclerotic functions without increasing the risk of breast and uterine caner and with less thrombotic tendency[2]. Zearalenone, a kind of mycotoxin, is metabolized into α-zearalenol and β-zearalenol in the presence of NADPH and UDPglucuronosyltransferase. Alpha-zearalenol shows greater ability to bind to estrogen receptor than both zearalenone and β-zearalenol[4] and is classified as a new kind of phytoestrogens. Alpha-zearalenol improves endothelial-dependent relaxation in ovariectomized rats[5] and prevents progression of atherosclerosis in ovariectomized cholesterol-fed rabbits without promoting the hyperplasia of uterus[6].

Conventional and plant-derived estrogens are anti-atherosclerotic by at least three mechanisms. Release of nitric oxide (NO) and activation of endothelial nitric oxide synthase (eNOS) gene are believed to play the most important roles in their protecting endothelium[3,7]. In addition, estrogen regulates gene expression via its receptor[8]. These genes include eNOS. Once estrogen binds to its receptor, the ligand-receptor compound is formed and acts as a transcription factor to directly interact with classical or non-classical estrogen-responsive elements[9] or to affect the activity of other transcription factors[10].

Estradiol has been reported to hinder generation of endothelium-derived superoxide anion and activation of nuclear factor -κB (NF-κB)[11]. Oxidative-stress is involved in the onset and development of endothelial dysfunction. NF-κB is a pivotal oxidative-stress related transcription factor[12]. Inflammatory cytokines, oxidized lipids, and oxidative stress,

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Biographical note of the first author: Su-Min LI, female, born in 1972, majoring in mechanism by which integrin β_3 gene is regulated by atherogenic factors during the atherosclerosis process.

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factors or events present in human atheroma, can activate NF-κB *in vitro*. Several genes up-regulated in endothelial cells during atherogenesis, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion mecule-1 (ICAM-1)[13], interleukin-1, tumor necrosis factor-α, *c-myc*[14] and integrin β_3[15], contain functional NF-κB elements in their promoter/ enhancer regions. The NF-κB/Rel family of proteins consists of homo- or heterodimers[16-17]. Subunits include NF-κB1 (p50), NF-κB2 (p52, p49, p50B), p65 (RelA), RelB, and c-Rel. All are expressed ubiquitously except for RelB and c-Rel, which are largely restricted to lymphoid and hematopoietic cells, respectively. In cultured endothelial cells, p50/p65 is the predominant NF-κB species[18]. In quiescent cells, NF-κB is localized in the cytoplasm, where it is retained through its association with an inhibitor[16-17]. Inhibitors of NF-κB (IκBs) include IκBα, IκBβ, IκBε, bcl-3, p105 (precursor of p50) and p100 (precursor of p52) and IκBγ. Different IκBs bind preferentially to different NF-κB dimers. Diverse stimuli can activate NF-κB through phosphorylation and activation of IκB kinase complex[16-17]. Activated IκB kinases phosphorylate IκBs leading to their polyubiquiti- nation and degradation. NF-κB dimers are then liberated and transported to the nuclei to promote transactivation of target genes[19].

The adhesion molecule integrin $\alpha_v\beta_3$ on endothelial cells plays an important role in atherosclerosis by stimulating platelet/endothelium interactions and white blood cell adhesion to and then transmigration across the endothelial membrane[20]. We have previously found that integrin $\alpha_v\beta_3$ could be induced by tumor necrosis factor α, high glucose, high proinsulin[21] and angiotensin II[22]. The increased integrin $\alpha_v\beta_3$ promotes platelet adhesion to endothelial cells[21]. However, it has not been determined that α-zearalenol affects $\alpha_v\beta_3$ integrin expression induced by atherosclerotic risk factors in endothelial cells. The number of $\alpha_v\beta_3$ on the surface of cells (except the megakaryocyte/platelet lineage) is decided by the transcription level of β_3 gene. Integrin $\alpha_v\beta_3$ is one of the β_3 integrins family numbers: $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$. Alpha$_{IIb}\beta_3$ expression is restricted to the megakaryocyte/platelet lineage, whereas $\alpha_v\beta_3$ is expressed in endothelial cells, smooth muscle cells, osteoclasts, monocyte-derived macrophages, as well as some malignant cell lines besides in megakaryocytes and platelets. In addition, α_v is associated with β_3, β_1, β_5, β_6, and β_8[23]. Moreover, it has been shown that β_3 gene expression is controlled at transcription level[24]. As a reasonable deduction, the mRNA level of β_3 gene approximately represents the level of $\alpha_v\beta_3$ on the surface of most cell types except for megakaryocytes and platelets.

This study was focused on the mechanism of phytoestrogen α-zearalenol affecting the angiotensin II-induced integrin β_3 mRNA expression in human endothelial cells. As a comparison, the effect of the conventional estrogen 17β-estradiol was observed at the same time.

MATERIALS AND METHODS

Materials

Complete phenol red-free medium200 was purchased from Cascade Ltd. Collagease I, HEPES, trypsin and TRIzol reagent were obtained from Life Technologies. Angiotensin II, 17β-estradiol, N^{ω}- Nitro-L-arginine methyl ester hydrochloride (L-NAME) were from Sigma. Alpha-zearalenol was provided by Professor Shunling Dai (Peking Union Medical College, Beijing, China). ICI 182, 780 was from AstraZeneca. Transfection reagent FuGene 6 was purchased from Roche. Plasmid pcDNA3 was purchased from Invitrogen. Plasmid NF-κB-LUC (containing three repeats of the NF-κB binding sites upstream of a minimal thymidine kinase promoter and a luciferase gene in pGL2 vector) was a generous gift from Dr. Hiroyasu Nakano (Juntendo University, Japan). Random primers, moloney murine leukemia virus reverse transcriptase, plasmid pSV-β- galactosidase vector, luciferase assay system and Luminescent β-galactosidase assay system were obtained from Promega.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were prepared as described previously[25]. The umbilical cords from female fetus were collected immediately after delivery and stored in sterile containers at 4℃. The veins were cannulated, washed with D'-Hanks balanced salt solution, and filled with collagenase (37℃, type I, 0.2 mg/mL). After incubation in a water bath (37℃, 10 min), the contents of vein were collected. Cells were centrifuged (400×g, 10 min) and the pellet was resuspended in the complete phenol red-free medium containing 2% (vol/vol) fetal bovine serum, 10 ng/mL human epidermal growth factor, 3 ng/mL basic fibroblast growth factor, and 10 mg/mL heparin and 1 mg/mL hydrocortisone. Cells were maintained at 37℃ in a humidified 5% CO_2 atmosphere. Endothelial cells were identified by their characteristic monolayer cobblestone appearance and positive staining for factor VIII-related antigen (von Willebrand factor, vWF)[26]. Cells from passages 2 to 4 were used in the study.

Drug Treatment

Cells were incubated with vehicle, α-zearalenol or 17β-estradiol for 12 h followed by co-incubation with angiotensin II for the indicated time[27]. The inhibitors, when involved, were added into the medium 90 min before their corresponding drugs. In the transfection experiment, reagents were added one day after the transfection.

Reverse Transcription-Polymerase Chain Reaction

For reverse transcription-polymerase chain reaction (RT-PCR), total RNA from HUVECs cultured in 6-well plates was isolated using TRIzol reagent following the manufacturer's procedures. Reverse transcription of 1-2 μg total RNA was performed according to the manufacturer's protocol of moloney murine leukemia virus reverse transcriptase. Two μL of cDNA products was amplified in a 25 μL PCR system containing 2.5 U LA Taq- DNA polymerase and 1 μmol/L human β_3 integrin primers. As an internal control for RT-PCR, 0.5 μmol/L 1human β-actin primers was also included in each sample at the same time. The sequences of the forward and reverse primers for the β_3 integrin were 5′ GCA ACC GTT ACT GCC GTG ACG AGA TT 3′ and 5′ CCC GGT ACG TGA TAT TGG TGA AGG TAG ACG 3′ respectively. The sequences of the forward and reverse primers for β-actin were 5′ GAT TCC TAT GTG GGC GAC GA 3′ and 5′ CCA TCT CTT GCT CGA AGT CC 3′ respectively. The parameters for PCR were at: 94℃ for 3 min and 28 cycles of denaturation at 94℃ for 30 s, annealing at 59℃ for 60 s, extension at 72℃ for 60 s and at 72℃ for 5 min. PCR product sizes for the β_3 integrin and β-actin were 392 bp and 532 bp according to their mRNA sequences[28-29]. The agarose gel photographic and densitometric records were derived from stained and UV trans-illuminated gels. The densitometric values of β-actin bands were used to standardize the results.

Transient Transfection

As a measure of NF-kB activity, luciferase reporter plasmid containing NF-κB binding sites (NF-kB-LUC) was used[30]. HUVECs were grown to 60% to 80% confluence and plated in 6-well dishes at the density of 4×10^5 cells/mL on the previous day. On the transfection day, cells were incubated for 3-4 h with a total DNA -FuGene6 mixture (1: 2, μg: μL) in 1 mL of Medium 200 without cell growth supplement. Psv-β-gal plasmid was simultaneously transfected to account for the variation in transfection efficiency. Afterwards, the cells were maintained in complete medium until the next day when they were exposed to drugs. Finally, the cells were washed twice with phosphate-buffered saline (PBS), resuspended in 80 μL of 1 × lysis buffer and lysed by repeated freezing and thawing. Cell extract was used for the measurement of lusiferase activity and β-galactosidase activity respectively using the luciferase assay system and luminescent β-galactosidase enzyme assay system.

Statistical Analysis

Values were expressed as $\bar{x} \pm s$, and n represented the number of experiments. One–way analysis of variance (ANOVA) followed by least-significant-difference test was used. $P \leqslant 0.05$ was considered statistically significant.

RESULTS

Alpha-Zearalenol and 17β-Estradiol Inhibited Angiotensin II-Stimulated Integrin β_3 mRNA Expression in HUVECs

We determined the effects of α-zearalenol and 17β-estradiol on the angiotensin II-stimulated integrin β_3 mRNA expression in HUVECs using RT-PCR. One μmol/L, 0.1 μmol/L and 10 nmol/L α-zearalenol and 17β-estradiol inhibited the stimulation of angiotensin II respectively (Fig. 1). Alpha-zearalenol was significantly more potent than 17β-estradiol at the dose of 0.1 μmol/L (Fig. 1C).

Nitric Oxide Inhibition and Estrogen Receptor Blockade Did Not Reverse the Effects of Alpha-Zearalenol and 17β-Estradiol

NO inhibitory L-NAME effects on α-zearalenol and 17β-estradiol were estimated using RT-PCR. L-NAME failed to reverse the inhibition on a-zearalenol and 17β-estradiol (Fig. 2A).

To elucidate whether their inhibitory effects were through estrogen receptor stimulation, ICI182, 780, a pure competitive antagonist for estrogen receptor, was used. We hypothesized that ICI182, 780 would reverse the inhibitory effects estrogen receptor in a dose-dependent manner. Surprisingly, ICI182, 780 failed to reverse its inhibitory effects (Fig. 2B). ICI182, 780 alone had little effect on the β_3 integrin mRNA expression (data not shown).

Alpha-Zearalenol and 17β-Estradiol Suppressed Activity Of NF-κB Induced by Angiotensin II in HUVECs

Angiotensin II markedly increased the activity of NF-κB. Treatment with either α-zearalenol or 17β-estradiol blocked such increase. Meanwhile, ICI182,

780 had no effects on the action of α-zearalenol and 17β-estradiol. Moreover, inhibition was significantly more potent by α-zearalenol than by 17β-estradiol (Fig. 3).

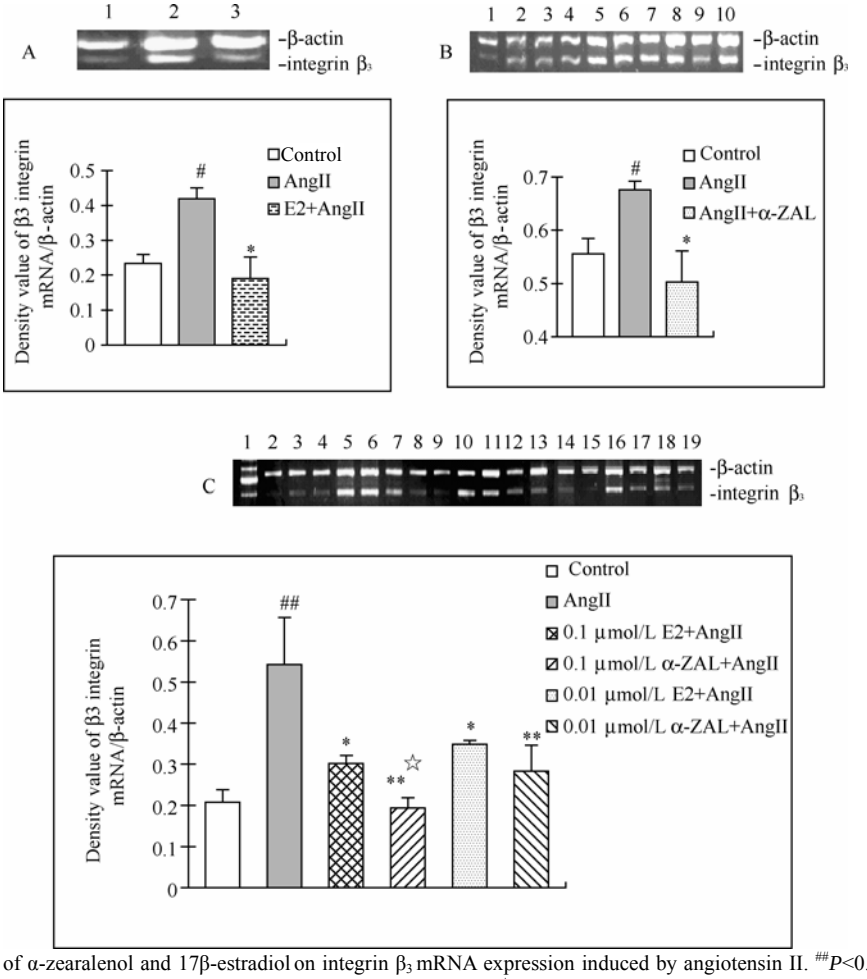

FIG. 1. Effects of α-zearalenol and 17β-estradiol on integrin β_3 mRNA expression induced by angiotensin II. $^{\#\#}P<0.01$, $^{\#}P<0.05$, AngII vs. control. $^{**}P<0.01$, $^{*}P<0.05$, E_2 or α-ZAL vs. AngII. $^{\star}P<0.05$, α-ZAL+AngII vs. E_2+AngII. A. Effect of 1 μmol/L 17β-estradiol. Lane 1: control; lane 2: AngII; lane 3: E_2+AngII. B. Effect of 1 μmol/L α-zearalenol. Lane 1: 100 bp DNA ladder, lanes 2-4: control; lanes 5-7: AngII; lanes 8-10: α-ZAL +AngII. C. Effects of 0.1 μmol/L, 10 nmol/L 17β-estradiol and α-zearalenol. Lane 1:100 bp DNA ladder; lanes 2-4: control; lanes 5-7: AngII; lanes 8-9: 0.1 μmol/L E_2 +AngII; lanes 11-13: 10 nmol/L E_2+AngII; lanes 14-16: 0.1 μmol/L α-ZAL+AngII; lanes 17-19: 10 nmol/L α-ZAL+AngII.

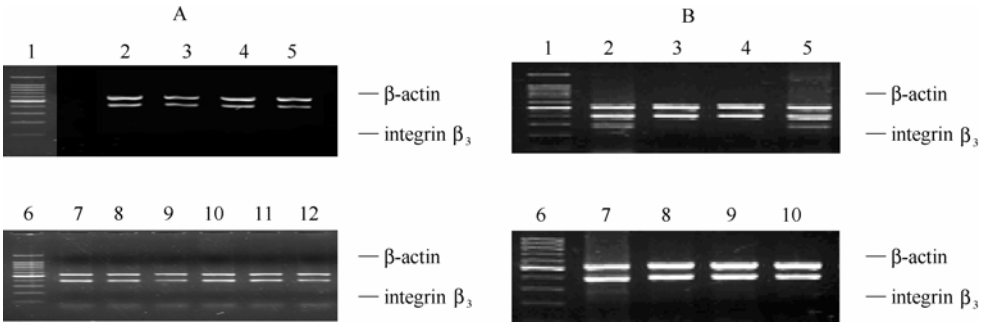

FIG. 2. Effects of L-NAME (A) and ICI (B) on α-zearalenol and 17β-estradiol. A. Effects of L-NAME. Lanes 1, 6: 100 bp DNA ladder; lane 2: E_2+AngII; lane 3: E_2+AngII+L-NAME; lane 4: α-ZAL+AngII; lane 5: α-ZAL+AngII+L- NAME; lane 7: blank control; lane 8: E_2 alone; lane 9: α-ZAL alone; lane 10: L-NAME alone; lane 11: E_2+L-NAME; lane 12: α-ZAL+L-NAME. B: Effects of ICI. Lanes 1, 6: 100 bp DNA ladder; lane 2: E_2+AngII; lane 3: E_2+AngII+1 μmol/L ICI; lane 4: E_2+AngII+ 0.1 μmol/L ICI; lane 5: E_2+AngII+10 nmol/L ICI; lane 7: α-ZAL +AngII; lane 8: α-ZAL +AngII+1 μmol/L ICI; lane 9: α-ZAL +AngII+0.1 μmol/L ICI; lane 10: α-ZAL +AngII+10 nmol/L ICI.

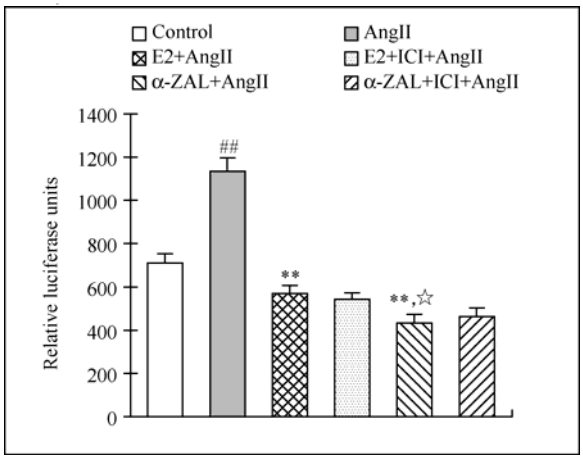

FIG. 3. Effects of α-zearalenol and 17β-estradiol on angiotensin II-induced activity of NF-κB. $^{\#\#}P<0.01$ AngII vs. control (−AngII). $^{**}P<0.01$ E_2+AngII, α-ZAL +AngII vs. AngII. $^{☆}P<0.05$, α-ZAL +AngII vs. E_2+AngII.

DISCUSSION

The present study demonstrated that α-zearalenol and 17β-estradiol could inhibit the angiotensin II-stimulated integrin β_3 mRNA expression via the suppression of NF-κB activity in HUVECs. One μmol/L and 0.1 μmol/L of α-zearalenol were more potent than the same concentrations of 17β-estradiol (Fig. 3, Fig. 1C). Similar to our findings, 17β-estradiol is reported to inhibit expression of VCAM-1 and ICAM-1 induced by interleukin-1 in HUVECs[31].

Conventional and plant-derived estrogens increase production and release of NO, thus relieving the dysfunction of endothelium. NO derived from endothelial cells has a number of anti-atherosclerotic actions, such as inhibiting the expression of E-selectin, ICAM-1, and VCAM-1[32]. That is one of the reasons why estrogens can protect endothelium. However, NO is unlikely to increase integrin β_3 expression. Our results indicate that NO inhibitor L-NAME fails to abolish the inhibition elicited by α-zearalenol and 17β-estradiol on the angiotensin II stimulation (Fig. 2A). Some authors reported that NO increases integrin $\alpha_v\beta_3$ expression in HUVECs[33].

Estrogen-receptor can work as a transcription factor in regulating some gene expression[8]. These genes include eNOS gene. ICI 182, 780, a pure estrogen receptor antagonist, could not abolish the inhibition, indicating that their actions are possibly transduced via the pathways excluding estrogen receptors, although a theoretical estrogen-responsive element can be found within the β_3 integrin promotor region. The results are consistent with the experimental data that L-NAME cannot reverse the inhibitory effects of both estrogens because activation of estrogen receptors can also regulate eNOS gene. Unlike integrin β_3, ICAM-1 and VCAM-1 expressions induced by interleukin-1 are inhibited by 17β-estradiol through the interactions between estrogen receptors and estrogen-responsive elements because the pure estrogen antagonist ICI 164 384 efficiently abrogates the inhibition[29].

It has been shown that estrogen may act as an anti-oxidant via estrogen receptor independent mechanisms in vascular smooth muscle cells[34]. Production of oxidative stress is one of the characteristics of atherosclerosis. Angiotensin II can significantly increase the generation of reactive oxide species[35] and activate NF-κB and up-regulate the expression of VCAM-1, ICAM-1 and E-selectin[26]. Thus it is reasonable to speculate that the ability of angiotensin II to suppress NF-κB activation may be critical for phytoestrogen's anti-atherosclerotic actions. In our study, NF-κB activation was inhibited by both estrogens in HUVECs. Also our previous study demonstrated angiotensin II could induce integrin β_3 expression by the same pathway[15].

It can be concluded from our studies that α-zearalenol and 17β-estradiol inhibit the angiotensin II-induced integrin β_3 mRNA expression via suppression of NF-κB in endothelial cells. But their mechanisms associated with suppression of NF-κB are not fully understood. Some phytoestrogens, such as daidzein, genistein and resveratrol are able to increase intracellular total GSH level by enhancing expression of the rate-limiting enzyme for GSH synthesis, γ-glutamylcysteine synthetase and thereby decrease the level of intracellular reactive oxygen species to prevent activation of NF-κB[36-38]. 17β-estradiol can be converted into several metabolites by cytochrome *P*-450 enzymes, among which 2-hydroxyestradiol and 2-methoxyestradiol are potent antioxidants and have minimal affinity for estrogen receptors[37]. But the effect of the metabolites may be mild on endothelial cells because cytochrome P-450 enzymes are produced mostly in hepatocytes. A common mechanism between them is possibly due to the reducible hydroxyl groups in their molecular structures[38]. The present work provides evidence that the antioxidant property of α-zearalenol has significance for cardiovascular disease although a further investigation is necessary to uncover the anti-oxidative mechanism and to evaluate the anti-oxidative activity of α-zearalenol.

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