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RESEARCH ARTICLE

The Effect of Fetuin-A and Palmitate on the Translocation of Glucose Transporter-4 Through the Activation of Toll-Like Receptor 4 in Skeletal Muscle Cells

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Abstract

Background: Fetuin-A (FetA) is a 63 kD phosphorylated glycoprotein that relates to insulin resistance inhibits autophosphorylation and the activity of insulin receptor tyrosine kinase (IR-TK) at the receptor levels. FetA positively correlates with insulin levels, and homeostasis model of insulin resistance (HOMA-IR). However, the phenomenon is obtained from several studies; a decrease did not always follow the increase of FetA in glucose uptake in the tissue. Aim: To determine the impact of Fetuin-A and Palmitate on TLR4 mediated Glucose Transporter-4 translocation inhibition in normal glucose-tolerance human skeletal muscle cell (h-SkMC). Method: Normal h-SkMC culture were randomly divided into five arms and treated with human- insulin, and deoxy-glucose (GI arm), or human-insulin, deoxy-glucose, and FetuinA (GIF arm), or human-insulin, deoxy-glucose and Palmitate (GIP arm), or human-insulin, deoxyglucose, FetuinA and Palmitate (GIFP arm). The control arm was without any treatment. The effects of insulin, glucose, FetuinA, and Palmitate on the expression of TLR4, Akt, GLUT-4 were evaluated by immunofluorescence microscopy. The expression of TNF-□ and IL-6 were measured by ELISA assay, and glucose uptake was measured by spectrophotometry assay. The results were obtained and subject to comparative test and pathway analysis. Results: The treatment with glucose+insulin (GI), FetA (GIF), Palmitate (GIP), and Palmitate+FetA (GIFP) increased TLR4 expression significantly compared to control arm ($p<0.05$) and only treatment with GI and GIF significantly increased the expression of GLUT-4 compared to control arm ($p<0.05$). All treatment arms and control arms were not significantly different in altering glucose transport. The treatment with Palmitate significantly decreased GLUT-4 expression and independence of TLR4 expression. Conclusion: The treatment with FetA alone or in combination with Palmitate in normal h-SkMC significantly increases the expression of TLR4 but does not alter GLUT-4 translocation and glucose transport. These results provide novel evidence indicating that acute exposure of FetuinA and Palmitate on normal glucose-tolerance h-SkMC does not induce insulin resistance.

Keywords: *FetA, Palmitate, TLR4, GLUT-4, TNFa, IL-6, Glucose transport.*

Introduction

Fetuin-A is a 63 kD phosphorylated glycoprotein synthesized and secreted by liver cells. Current theory states that Fetuin-A is related to insulin resistance because it has been proven that Fetuin-A (FetA) inhibits autophosphorylation and the activity of insulin receptor tyrosine kinase (IR-TK) at the receptor levels. FetA has also been indicated to increase obesity, glucose tolerance impairment, and type 2 diabetes mellitus. FetA positively correlates with insulin levels, and homeostasis model of insulin resistance (HOMA-IR). However, the phenomenon obtained from several studies indicates that the increase of FetA is not always followed by a decrease in glucose uptake in the tissue [1, 2].To date, the FetA theory that causes insulin resistance still leaves problems since the increase of FetA is not always followed by a decrease in glucose uptake in the tissue. Therefore, it is inconsistent and causes insulin resistance. This problem can not be explained yet. FetA draws attention lately because of its role in

glucose metabolism and its being the risk of diabetes mellitus. Its increasing levels are related to insulin resistance. However, a decrease in FetA levels is also associated with the increasing cardiovascular risks [3, 5].In vitro studies, both in experimental animals and in humans, suggest that FetA inhibits insulin autophosphorylation at the level of the insulin receptor. However, the reports from several studies indicate that it causes metabolic effects in the form of glucose uptake disorders and leads to inconsistent results. Several studies have suggested that despite the inhibition of phosphorylation at the level of insulin receptor by FetA, glucose uptake and amino acid transport are not disrupted.

The findings of new therapeutic targets are significant considering the current therapeutic modality for insulin resistance and prevention of the emergence of NOD is still limited. On the other hand, Indonesia currently ranks seventh in the world for the number of patient diabetics [6]. Therefore, there are several unanswered problems from FetA research, both in experimental animals and in humans.

In both in vitro studies and experimental animals, FetA in the phosphorylated form has proven to be a potent inhibitor of insulin receptor autophosphorylation. However, the metabolic impact data generated is inconsistent in showing that FetA causes insulin resistance. There is a positive correlation between the increase of FetA levels with insulin resistance in the state of metabolic syndrome, obesity and type 2 diabetes.

Nevertheless, it is not supported by consistent evidence from both in vitro and in vivo studies, leading to the assumption that FetA does not directly cause inhibition of IR-TK phosphorylation and insulin resistance. The findings of Pal et al. (2012) states that FetA is a protein mediator for free fatty acid (FFA) to bind to Toll Like Receptor 4 (TLR4) in adipocytes which triggers an inflammatory pathway including by increasing TNF-α proinflammatory cytokines, and assumes that FetA in muscle cells does not directly trigger insulin resistance.

However, it requires the presence of FFA in triggering insulin resistance through activation of inflammation by TLR4. The research into the role of FetA as a mediator protein for FFA in causing insulin resistance through the inflammatory pathway will open up new concepts in insulin resistance therapy through the immunometabolism pathway [7].

Method

This research is in vitro laboratory research. This method was chosen because both control and treatment would be more controlled and measured whereas the effect of the treatment would be more reliable. The design of this study was to determine the effects of the exposure of fetA, Palmitate, fetA+Palmitat in the experimental unit with the variables measured after the treatment. TLR4 and PKB/Akt activities, TNF levels, IL-6, GLUT-4 translocation, and glucose uptake by muscle cells in the basal state were used as the controls.

The research samples were the target cells of insulin, which expressed GLUT 4, namely skeletal muscle cells obtained from skeletal muscle cell culture or SkMC. The sample size reached six samples per group. SkMC originates from skeletal muscle obtained from patients without metabolic disorders and diseases undergoing surgery under general anesthesia.

The independent variables of this study were Fetuin and palmitate, whereas the dependent variables were TLR4, PKB/Akt, TNF, IL-6, GLUT-4 GLUT-4 translocation, and glucose uptake. The research was conducted at Dr. Soetomo Regional Hospital (skeletal muscle tissue sampling) and Stem Cell Research and Development Center of Universitas Airlangga for storage, tissue culture, and sample examination. Furthermore, the data were collected and analyzed using Manova, while the pathway analysis used Multiple regression analysis. This study had been through a review. It had been declared "Eligible Ethics" with a Certificate of Ethical Feasibility from the Faculty of Medicine of Universitas Airlangga/Soetomo Teaching Hospital, Surabaya Indonesia.

Results

Examination of TLR4, Akt, GLUT-4 Expressions and TNFα and IL-6 Levels

The following table shows that in the untreated control state, muscle cells express TLR4 even though in low numbers (126 ± 33.94) cells per field of vision with immunofluorescence examination.

Table 1: TLR4 expression in the control groups and treatment groups

Samples	Treatments				
	Controls (K)	D-Glucose + Insulin (GI)	D-Glucose+Insulin + Palmitat (GIP)	D-Glucose+Insulin + Fetuin (GIF)	D-Glucose+Insulin + Palmitat + fetuin (GIFP)
Mean	126.00	802.50	555.83	550.00	655.00
Std. Dev	33.941	31.494	47.793	75.895	130.614
Median	126.00	795.00	545.00	525.00	647.50
Minimum	102	760	500	500	500
Maximum	150	850	625	700	825

Causal Correlation between the Studied Variables

Regression analysis was conducted to find out the causal relationship between research variables and the causes of changes in research variables. Determination of the variables which were involved in the regression analysis using the consideration of the multivariate analysis of dependent variables and the theoretical basis that underlies this study. A positive value indicates that if the independent variables increased by the value of a, the dependent variables had an increase by the value of b. For negative value, on the other hand, if the independent variables had an increase by the value of a, the dependent variables would decrease by the value of b.

Table 2: Regression Analysis (causal correlation) and manova among research variables

No.	Independent variables	Dependent variables		(b)	p
1.	Glucose+Insulin	Akt		0.918	**0.000**
2.	Glucose+Insulin	GLUT-4		0.440	**0.024**
3.	Glucose+Insulin	TLR4		-	**0.000**
4.	Glucose+Insulin	TNF-α		-	**0.012**
5.	Akt	GLUT-4		-	0.804
6.	GLUT-4	Glucose Transport		-	0.558
7.	FetA	TLR4			**0.027**
8.	Palmitate	TLR4		-	**0.040**
9.	Palmitate	GLUT-4		-	**0.001**
10.	TLR4	IL6		-	0.128
11.	TLR4	TNF-α		-	0.078
12.	TNF-α	Akt		-	0.184
13.	IL6	Akt		-	0.475

Note: p <0.05 significant B = standardized regression coefficient

The correlation pattern between variables in Table 2 is explained as follows:

- If the Glucose+Insulin variables increased, Akt would increase, and vice versa, if the Glucose+Insulin variables decreased, the Akt variables would decrease with a standardized coefficient of 0.918.
- If the Glucose+Insulin variables increased, GLUT-4 would increase, and vice versa, if the Glucose+Insulin variables decreased, the GLUT-4 variables would decrease with a standardization coefficient of 0.440.
- If the Glucose+Insulin variables increased, TLR4 would increase, and vice versa, if the Glucose+Insulin variables decreased, the TLR4 variables would decrease.
- If the Glucose+Insulin variables increased, the TNF-α variables would increase, and vice versa, if the Glucose+Insulin variables decreased, the TNF-α variables would decrease.
- If the Akt variables increased, it would cause a change in GLUT-4, but the change was not significant.
- If the GLUT-4 variables increased, it would cause a change in glucose transport, but the change was not significant.
- If the FetA variables increased, TLR4 would increase, and vice versa, if the FetA

variables decreased, the TLR4 variables would decrease.

- If the Palmitate variables increased, TLR4 would increase, and vice versa, if the Palmitate variables decreased, the TLR4 variables would decrease.
- If the Palmitate variables increased, the GLUT-4 variables would decrease, and vice versa, if the Palmitate variables decreased, the GLUT-4 variables would increase.
- If the TLR4 variables increased, it would cause an insignificant change in IL6,.
- If the TLR4 variables increased, it would cause an insignificant change in TNF-α.
- If the TNF-α variables increased, it would cause an insignificant change in Akt.
- If the IL6 variables increased, it would cause an insignificant change in Akt.

Therefore, the significant pathway analysis model is that characterized by thicker lines in the following Figure.

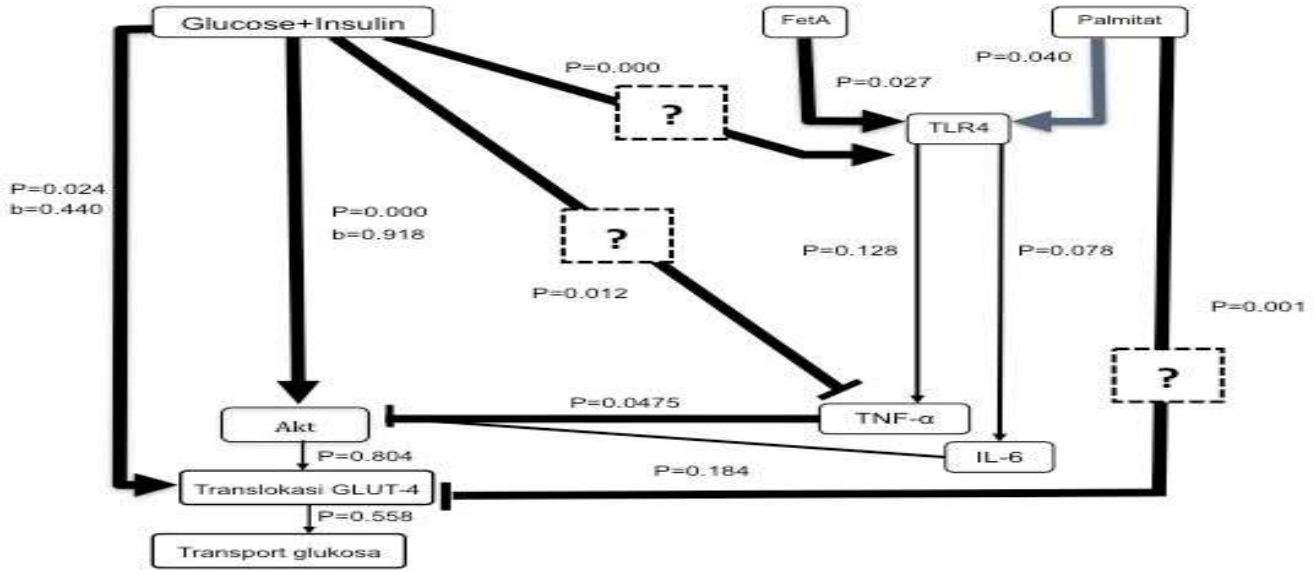

Figure 1: Pathway analysis result model

Discussion

TLR4 Expressions in Normal Skeletal Muscle Cells

In this study, the use of normal skeletal muscle cells after the treatment with glucose+insulin, expression of TLR4 was significantly six times higher than the controls. The treatment with glucose+ insulin+palmitate, and glucose+ insulin+FetA and glucose+insulin +FetA+Palmitate also significantly resulted in higher TLR4 expression than the controls.

The treatment with glucose +insulin also produced TLR4 expression, which was significantly higher than the treatments of glucose+insulin +palmitate, glucose+ insulin +FetA, and glucose+insulin+FetA+palmitate. There were no significant differences between the FeTA treatment and the Palmiate treatment and the combination of both treatments. Previous studies had never reported an increase in TLR4 expression after treatment in normal muscle cells. In general, it was only reported that TLR4 expression increased from adipocytes and skeletal muscle cells with insulin resistance.

An increase in TLR4 expression in the treatments of Glucose+Insulin in normal muscle cells still could not be explained. There might be intermediate pathways or other mediators that needed to be further investigated in normal skeletal muscle cells. Kaur et al. (2012) reported that prolonged hyperglycemia (24 hours) significantly increased the TLR2 and TLR4 expressions in rats' mesangial cells, which was expected to play a role in diabetic nephropathy [8]. Rajamani (2014) also reported that prolonged hyperglycemia (24 hours) significantly increased the TLR4 expression in humans' microvascular retinal culture.

The findings of Kaur (2012) in rat's mesangial cells and Rajamani (2014) in human retinal cells could be used as a comparison of why the increase of TLR4 expression in this study was high after treatment with Glucose+Insulin in normal skeletal muscle cells. However, the mechanism was still not clear [9]. Likewise, why the increase of TLR4 expression was lower in the treatments of Glucose+Insulin+FetA,Glucose+Insulin+Palmitate, and Glucose+Insulin+FetA+Palmitate in normal skeletal muscle cells could not be explained.

For insulin-resistant skeletal muscle cells, it had been suggested that TLR-4 had an important role in causing insulin resistance and inflammation which was triggered by exogenous ligands, which were saturated fatty acid or SFA of diit and lipopolysaccharides (LPS) of the intestine; and the endogenous ligand of the free fatty acids which increased during obesity conditions. Obese individuals and patients with type 2 diabetes had an increase in the TLR4 gene expression and the increase of TLR4 in muscle cells10 significantly.

The findings in this study were also incompatible with previous studies, which indicated that insulin had anti-inflammatory effects. Ghanim et al. (2008) stated that a low dose of insulin infusion (2 units per hour) in patients with type 2 DM for 2 hours significantly decreased the expression of TLR1, 2, 4, 7 dan 9 mRNA in mononuclear cells [11]. De Laat *et al.* (2014) also indicated that hyperinsulinemia in normal (*insulin sensitive*) Wistar rats suppressed TLR4 expression in cardiac myocytes from Wistar rats; however, the expression of cytokines through the TLR4 signalling pathway (IL-6, TNF-a, and SOCS3) and GLUT1,4,8,12 expression of the heart muscle was not affected [11, 12]. Further researches were still needed to determine whether the increase in TLR4 in the treatment of Glucose+Insulin is the effect of hyperglycemia in normal skeletal muscle cells, which causes inflammation.

TNF-α

In our study, the increase of TLR-4 expression was not followed by the increase of the activation of TLR-4 to the downstream signal pathways. This indicated no increase in TNFα expression in the treatments of Glucose+ Insulin+Palmitate, Glucose+ Insulin + FetA, and Glucose + Insulin + FetA + Palmitate.

It could even be seen in Figure 5.8, all treatments indicated lower TNFα expressions than the control groups, although the difference was not significant. On the other hand, all treatments did not show any significant differences. The findings in this research showed no increase in TNFα that was inconsistent with the increase of TLR4 expression might be due to the homeostatic function of normal skeletal muscle cells, which resulted in a different response between acute exposure and chronic exposure. In the condition of skeletal muscle cells where insulin was resistant, FetA exposure and acute Palmitate could affect TLR4 activation and the increase of TNFα.

Further research were still required to answer this question. Our findings are different from the findings of Pal et al. (2012), who reported the increase of TNFα expression in adipocytes from humans whose insulin resistance increased significantly under conditions of free fatty acid induction, only when FetA existed.

Pal et al. also stated that mice with the condition of the FetA gene knockout and TLR4 knockout obtained that the expression of TNF-α was decreased. This research did not support the hypothesis that the FetA with the presence of Palmitate would trigger the activation of TLR4 characterized by TNF-α increase. The research results did not correspond with the findings of Heinrichsdroff and Olefsky (2012) in the in vivo study on rats, which showed that FetuinA should exist on the gene expression of pro-inflammatory triggered by the SFA via TLR4.

It was also different from the findings of Pal et al. (2012) in rat adipocytes, in which the expression of proinflammatory cytokines that induced FFA only occurred when FetuinA and TLR4 existed. Eliminating one of these aspects would eliminate insulin resistance in adipocytes [7, 13].Pal et al. also found out that FetuinA, through the tip of galactoside, directly bound to the residues of Leu100-Gly123 and Thr493-Thr516 on TLR4. The mutations of the TLR4 and the tip of galactoside of FetuinA prevented the free fatty acid from causing insulin resistance in adipocytes.

Pal, et al. also discovered that free fatty acids increased the expression of IL-6 and TNF-α insulin-on resistant human adipocytes only when FetuinA existed.

This research on insulin-sensitive human skeletal muscle cells indicated similarities to the condition of FetA knockout even though it was induced by Palmitate or FetA and Palmitate+FetA. This research's results also differed from those reported by Glass and Olefsky (2012), which suggested that the proinflammatory effects of SFA included the increase of TNFα through the activation of TLR47,10 [7,10].

The study of De Laat, et al. (2014) indicated different results on different treatments in non-insulin resistance Wistar rats, in which the hyperinsulinemia decreased the expression of TLR4. Nevertheless, it was not forwarded on the influence of the activation of TLR4 which was indicated by the absence of change in the levels of TNF-α [12].

In this study, the induction with Glucose+Insulin also increased TNF-α without TLR4. The increase of TNF-α in the state of hyperglycemia+hyperinsulinemia on normal skeletal muscle cells had not been reported previously. For patients with DM and Prediabetes which were set to insulin deficiency, acute hyperglycemia significantly increased the levels of TNF-α. The findings in this research remained unexplainable because there might be an intermediary path which had not been examined and required further research.

IL-6

The research results study on the expression of IL-6 after treatments with glucose+Insulin, Glucose+ Insulin+Palmitat, Glucose+ Insulin+FetA,Glucose+Insulin+FetA+Palmita t looked the same without any significant differences.

The research findings were also different from the those of Pal et al. (2012) who reported the increase of IL-6 expression in adipocytes of humans whose insulin resistances increased significantly under the condition of free fatty acid induction, only when FetA existed.

In this study, the FetA, either independent or in combination with Palmitate, did not increase the expression of IL-6. Although being displayed in Figure 5.9, the treatment of Glucose+Insulin+FetA+Palmitate seemed to give the highest IL-6 expression compared to the other treatments despite not being significant [7].

The results of the IL-6 study were also different from those of De Laat, et al. (2014) with different treatments on non-insulin resistant Wistar rats, which suggested that hyperinsulinemia decreased the expression of TLR4. Still, there was no change in the levels of IL-6. This research findings indicated no increase in IL-6 that was inconsistent with the increase of TLR4 expression. This phenomenon might take place due to homeostasis from normal skeletal muscle cells or because of the presence of insulin which suppressed the activation of TLR412 [12].

Akt

Previous studies did not measure the expression of Akt and phosphorylation of Akt as a result of treatment with the FetA and Palmitateand indicated that the FetA specifically inhibited insulin-induced autophosphorylation and tyrosine kinase activity. GRB2/Sos complex (Growth factor Receptor-bound Protein 2/ son-of-sevenless) that bound to IRS-1 was inhibited by up to 75% by FetA [14]. If the phosphorylation of IRS-1 was inhibited, the line underneath PKB/Akt would also be inhibited. However, there had been no report on the result of Akt expression on FetA exposure.

In this study, the treatments with Glucose+Insulin,Glucose+Insulin+palmitate, Glucose+insulin+FetA,Glucose+Insulin+FetA +Palmitateprovided a higher expression of Akt than the control group significantly. The increasing Akt expression might support the findings of Srinivas, et al. (1996) stating that although FetA inhibited the phosphorylation of IR-TK, it does not cause impaired glucose uptake as a result of insulin resistance.

This study suggests that the Akt expression is not distressed by the FetA treatment, and even significantly higher than the control group. Akt increase was likely mediated by insulin [6]. TNFα also played a role in Akt activation. For insulin-resistant subjects with Pal, et al. had shown that treatment with Palmitan and FetA would cause insulin resistance through the increase of TNF-α.

Plomgaard, et al. indicated that excessive TNFα would interfere with the phosphorylation of Akt substrate 160, which was the very first step of the pathway of the canonical pathway of the insulin signalling [15].

Glut-4

This study suggested that the treatment with Glucose+Insulin, and the treatment with Glucose+Insulin+FetA generated the expression of Glut-4 which was significantly higher than the control group. The treatment with Glucose+Insulin+FetA was also significant to produce the expression of Glut-4 which was higher than the treatment with Glucose + Insulin + Palmitate and the treatment with Glucose+Insulin + FetA + Palmitate with significant differences. These findings assumed that, compared to Palmitate, FetA did not suppress Glut-4 expression.

The new FetA showed the suppression of Glut-4 expression when combined with Palmitate, or the effect of suppressing Glut-4 expression might be the effect of Palmitate, not the effect of FetA+Palmitate combination. Neither Pal, et al. nor the previous researchers of Reyna, et al. examined the expression of Glut-4 in the treatment of FetA and Palmitate. The findings in both treatments of Glucose+Insulin, FetA and Palmitate and the combination of FetA+Palmitate produced higher TLR4 expression, but not followed by the increase of TNFa and IL-6. It was assumed that there was a difference in signalling between healthy muscle cells and insulin-sensitive with insulin-resistant muscle sets.

Insulin-resistant muscle cells had been in a chronic inflammatory state and the increase of TLR4 expression and signalling in insulin-resistant muscle cells were derived from macrophage infiltration or monocytes in muscle cells [7, 16].Meanwhile, for healthy muscle cells and sensitive insulin, the treatment with FetA and Palmitate provided the increase of TLR4 acutely and caused homeostatic reactions so that the TLR4 signalling did not work and caused insulin resistance.

This assumption remained to be proven by further research. This study also indicated that the induction with Palmitate inhibited the translocation of GLUT-4, characterized by a significant reduction of the expression of GLUT-4 in the membrane without passing through the TLR4 pathway. This study also obtained induction with Palmitate which inhibited GLUT-4 translocation characterized by a significant decrease in GLUT-4 expression in the membrane, without passing through the TLR4 pathway. These findings in normal skeletal muscle cells still can not be explained and require further research to understand its mechanism.

D-Glucose

In the previous studies, no one had examined glucose uptake by GLUT4 after the treatments with Glucose+Insulin and the addition of FetA, Palmitate, and FetA+Palmitate. Although there was an increase in the expression of TLR4 for each treatment compared to the controls, and there was a difference in the expression of GLUT4, the glucose uptake by GLUT4 did not experience a significant difference.

Conclusion

In normal skeletal muscle cells, FetA does not increase the expression of TNF-α and IL-6, nor inhibit the expression of Akt, nor cause impaired translocation of GLUT-4, nor cause glucose transport disorders, either in conjunction with the presence of Palmitate and TLR4 or in the absence of Palmitate. Besides, FetA has also not been proven to act as a mediator of Palmitate in the activation of TLR4, which causes GLUT-4 translocation barriers.

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