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Pravastatin Restored the Infarct Size-Limiting Effect of Ischemic Preconditioning Blunted by Hypercholesterolemia in the Rabbit Model of Myocardial Infarction

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OBJECTIVES We tested to find out whether pravastatin restores the infarct size (IS)-limiting effect of ischemic preconditioning (IP) and if it has any effect on the IP-induced activation of adenosine producing enzyme ecto-5′-nucleotidase which plays a key role in the IP-induced cardioprotection.

BACKGROUND The IS-limiting effect of IP is blunted by hypercholesterolemia. Recently, HMG-CoA reductase inhibitors are shown to have direct cytoprotective effects.

METHODS Rabbits were fed with a normal or cholesterol (1%) added diet with or without pravastatin (5 mg/kg/day) treatment. Infarct size was measured after 30 min occlusion and 3 h reperfusion of circumflex coronary artery with or without the IP procedure (5 min occlusion and 10 min reperfusion). Additionally, ecto-5′-nucleotidase activities of ischemic and nonischemic myocardium were measured immediately after IP procedure.

RESULTS This dose of pravastatin did not normalize the increased level of serum cholesterol. The IS-limiting effect of preceding IP (IS reduced from 36.7% to 9.6%, $p < 0.001$) was abolished by hypercholesterolemia (from 46.1% to 31.3%, p = NS) and restored by pravastatin treatment (from 35.2% to 9.4%, $p < 0.001$). Pravastatin treatment did not affect IS or the effect of IP under normocholesterolemia. The activation of ecto-5′-nucleotidase presented as the activity ratio of ischemic to nonischemic myocardium (3.1-fold in normocholesterolemia) was blunted by hypercholesterolemia (1.8-fold, $p < 0.05$) and restored by pravastatin treatment (2.9-fold).

CONCLUSIONS Pravastatin, at the dose serum cholesterol was not normalized, restored the IS-limiting effect of IP and IP-induced ecto-5′-nucleotidase activation, which were both blunted by hypercholesterolemia. The activation of ecto-5′-nucleotidase may be worth further investigation as a possible mechanism for the hypercholesterolemia-induced retardation and pravastatin-mediated restoration of the cardioprotective effect of IP. (J Am Coll Cardiol 1999;34: 2120–5) © 1999 by the American College of Cardiology

Infarct size (IS) is markedly limited when brief periods of ischemia precede the sustained ischemia, a phenomenon known as ischemic preconditioning (IP). This phenomenon can be extended to the clinical settings because clinical evidence suggests the acquisition of ischemic tolerance of the myocardium afforded by antecedent brief periods of ischemia. In the clinical situation, pathophysiology of myocardial ischemia due to coronary arterial stenosis may be modified by many risk factors, e.g., hypercholesterolemia (HC), smoking and hypertension. Recently, we have reported (1) that the IS-limiting effect of IP was blunted by HC in the rabbit model of myocardial infarction (MI). Since IP may be a common phenomenon afforded by preinfarction angina (2) in the patients with acute MI, it is suggested that HC may increase IS in those preconditioned hearts. According to a recent report (3), HMG-CoA reductase inhibitors, which are now commonly used to treat the patients with HC, have direct cytoprotective action to endothelial cells. Therefore, it is of great interest to investigate if pravastatin restores the IS-limiting effect of IP blunted by HC without normalizing serum cholesterol level.

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Abbreviations and Acronyms

ANCOVA = analysis of covariance
ANOVA = analysis of variance
HC = hypercholesterolemia
HCP = hypercholesterolemia with pravastatin treatment
HDL = high density lipoprotein
IP = ischemic preconditioning
IS = infarct size
MI = myocardial infarction
NC = normocholesterolemia
NCP = normocholesterolemia treated with pravastatin
PLSD = protected least significant difference

Furthermore, since ecto-5′-nucleotidase, the enzyme responsible for adenosine production, may mediate (4) IP, and its activation correlates (5) with the IS-limiting effect of IP, we evaluated if HC or pravastatin influences the IP-induced activation of ecto-5′-nucleotidase.

METHODS

Cholesterol feeding and pravastatin treatment. The animal study was approved by the Animal Care and Use Committee of the Animal Research Institute in Osaka University. New Zealand White rabbits (n = 88) eight weeks old weighing about 1.8 kg (Oriental Co., Japan) were randomly assigned to the normocholesterolemia (NC) group (n = 22), the HC group (n = 22), the NC with pravastatin treatment (NCP) group (n = 22) or the HC with pravastatin treatment (HCP) group (n = 22). Hypercholesterolemia was achieved by feeding 1% cholesterol containing diet (RC4, Oriental Co., Japan) for 16 weeks in the HC and HCP groups, and the pravastatin treatment was done by the oral administration of pravastatin (5 mg/kg/day) in the latter eight weeks in NCP and HCP groups. After 16 weeks of feeding, serum levels of total cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides were measured and rabbits were used for the measurement (4) of myocardial IS.

Measurement of myocardial infarct size. Rabbits were anesthetized with intravenous pentobarbital under respiratory control. The heart was exposed with left intercostal thoracotomy and the left circumflex coronary artery was ligated for IP (5 min occlusion and 10 min reperfusion) or MI (30 min occlusion and 3 h reperfusion). The IP procedure was performed in half of the rabbits in each group. The electrocardiogram, systemic blood pressure and heart rate were continuously monitored during the procedures. The oxygen saturation of arterial blood was examined immediately before coronary occlusion and before reperfusion and at 3 h after reperfusion. Cardioversion was not done for ventricular fibrillation. We eliminated the rabbits with continuous ventricular fibrillation from the data analysis.

The heart was excised after religation of the coronary artery and the left atrial injection of Evans Blue dye (Wako, Osaka) to negatively stain the area at risk and then cut into five to six slices and stained with triphenyl tetrazolium chloride to determine the infarct area. The infarct area and the area at risk were determined as the summation of those areas measured on each picture of the slices with the assistance of a computer. The IS was defined as the percentage of infarct area in the area at risk. The weight of the risk region and of the infarct region was also determined.

Measurement of the IP-induced activation of myocardial 5′-nucleotidase. Since we have previously reported (4,5) that the activation of ecto-5′-nucleotidase of myocardium is important for the IS-limiting effect of IP, we measured the 5′-nucleotidase activities of myocardium immediately after IP procedure to clarify the effect of HC and pravastatin on the 5′-nucleotidase and on their activation by IP.

Rabbits (n = 36) were randomly assigned to the NC group (n = 12), the HC group (n = 12) or the HCP group (n = 12). Hypercholesterolemia was achieved by feeding a 0.3% cholesterol containing diet for 10 weeks in the HC and HCP groups, and the pravastatin treatment was done by the oral administration of pravastatin (5 mg/kg/day) in the latter six weeks in the HCP group.

After 10 weeks, serum levels of cholesterol were measured. Immediately after the IP procedure (5 min occlusion and 10 min reperfusion of circumflex coronary artery), myocardium of the ischemic and nonischemic regions was frozen in liquid nitrogen and stored at −80°C or less until use for measuring cytosolic and ecto-5′-nucleotidase activities. The activation of 5′-nucleotidase by IP was evaluated by the activity ratio of ischemic to nonischemic myocardium.

Measurement of 5′-nucleotidase activity. The homogenized myocardium was separated into membrane and cytosolic fractions as described previously (5). We defined 5′-nucleotidase activity in the membrane and cytosolic fraction as ecto- and cytosolic 5′-nucleotidase activity, respectively. The activity of 5′-nucleotidase was assessed by an enzymatic assay technique (6) and reported as units of nmol/min/mg protein.

Statistical analysis. All data were presented as mean ± SD. The changes and differences in the blood pressure, heart rate and the oxygen saturation of arterial blood were analyzed by two-way analysis of variance (ANOVA) repeated measurements with posthoc analysis of Fisher protected least significant difference (PLSD). The differences in the serum levels of total cholesterol, HDL cholesterol and triglyceride, and the activities of 5′-nucleotidase were analyzed by ANOVA with posthoc analysis of Fisher PLSD. The relationship between IS and risk region size was compared among groups with an analysis of covariance (ANCOVA) with the size of the risk region used as the

covariant. The correlation between IS and risk region size was assessed by linear regression analysis using the least square method. A value of $p < 0.05$ was considered significant.

RESULTS

Animals in each group. Excluding some rabbits that died before the completion of 3 h reperfusion, IS was acquired in 7, 9, 7, 7, 9, 9, 9 and 9 rabbits in NC IP(−), NC IP(+), HC IP(−), HC IP(+), HCP IP(−), HCP IP(+), NCP IP(−) and NCP IP(+) groups, respectively. The level of serum total cholesterol (64.9 ± 48.2 mg/dL in the NC group) was significantly, but similarly, elevated by cholesterol feeding in the HC (2049.1 ± 636.7 mg/dL) and the HCP (1847.9 ± 479.2 mg/dL) groups regardless of the pravastatin treatment and was not significantly changed by pravastatin treatment alone in the NCP (18.7 ± 3.6 mg/dL) group. The level of serum triglyceride (45 ± 39 mg/dL in the NC group) was also significantly but similarly elevated in the HC (225.3 ± 148.3 mg/dL) and the HCP (232.4 ± 190.6 mg/dL) groups regardless of the pravastatin treatment and was not significantly changed by pravastatin treatment alone in the NCP (31.8 ± 16 mg/dL) group. The level of serum HDL cholesterol (34.3 ± 18.5 mg/dL) was not changed by cholesterol feeding in the HC (45.6 ± 21 mg/dL) or HCP (45.9 ± 40.0 mg/dL) group but was significantly reduced by pravastatin treatment alone in the NCP group (10.4 ± 2.8 mg/dL).

Effects of IP on myocardial infarct size. The interaction of all factors by two-way ANOVA repeated measurements was not significant for the data of blood pressure, heart rate or the oxygen saturation of arterial blood (97% to 98%). Although no difference was detected among groups, coronary occlusion decreased systolic/diastolic blood pressure from 115 ± 18/74 ± 12 mm Hg to 104 ± 18/66 ± 13 mm Hg and increased heart rate from 275 ± 30 to 281 ± 31 beats/min. Infarct sizes as the percentage of risk region are presented in Figure 1. The relationship between the size of infarct region and the size of risk region is presented in Figure 2. Infarct size was not different among groups without IP, meaning that HC or pravastatin did not change IS. The significant reduction of IS by IP observed in the NC group was not affected in the NCP group; however, it was blunted in the HC group and restored in the HCP group.

Effects of HC and pravastatin on the IP-induced activation of 5′-nucleotidase. The level of serum cholesterol was also similarly higher in the HC (437 ± 310 mg/dL) and HCP (509 ± 171 mg/dL) groups compared with the NC (26 ± 8.5 mg/dL) group in this protocol. Cytosolic and ecto-5′-nucleotidase activities of ischemic and nonischemic myocardium and their activation by IP in the NC, HC and HCP groups are presented in Figure 3. Cytosolic and ecto-5′-nucleotidase activities in the nonischemic region were significantly increased by hypercholesterolemia (HC and HCP groups), and pravastatin had no influence on them. However, the activation of ecto-5′-nucleotidase by IP as shown by the activity ratio of ischemic/nonischemic myocardium was significantly blunted by the HC group and was restored by pravastatin treatment (HCP group).

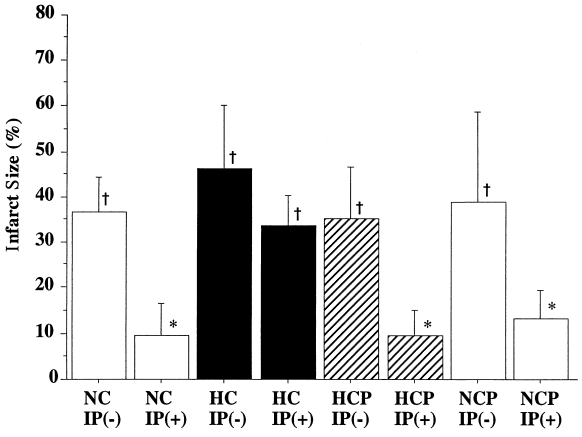

Figure 1. Infarct size as the percentage of risk region. The IS limiting effect of ischemic preconditioning recognized in NC and NCP groups is blunted in the HC group but is restored in the HCP group. Data are presented as means ± SD. HC = hypercholesterolemia group; HCP = hypercholesterolemia with pravastatin treatment group; IP = ischemic preconditioning; IS = infarct size; NC = normocholesterolemia group; NCP = normocholesterolemia with pravastatin treatment group. *Significantly different from NC IP(−) group by ANCOVA (Fig. 2) with size of the risk region used as the covariant. †Significantly different from NC IP(+) group by ANCOVA.

DISCUSSION

We performed two independent experiments and revealed, first, that HC blunts the IS-limiting effect of IP which is restored by pravastatin treatment although HC or pravastatin does not change IS by itself, and second, that HC blunts the activation of ecto-5′-nucleotidase by IP which is restored by pravastatin treatment. Since the activation of ecto-5′-nucleotidase is essential (4) for the IS-limiting effect of IP, it is probable that HC blunts the effect of IP via blunting the activation of ecto-5′-nucleotidase and that pravastatin restores the effect of IP via restoring the activation of ecto-5′-nucleotidase although a causal relationship has not been proved.

Effect of HC and pravastatin on infarct size. We examined the effect of HC and of pravastatin on the IS with and without IP procedure. The results of the study must be interpreted separately for the direct effect on IS and for the effect on IP, and there has been no other study so far on the latter effect. Since the mechanism of IP is not adequately understood, it may be quite difficult to clarify the mechanism by which HC/pravastatin modify the mechanism of IP. However, since there are various accompanying factors that may modify the effect of IP in the patients with

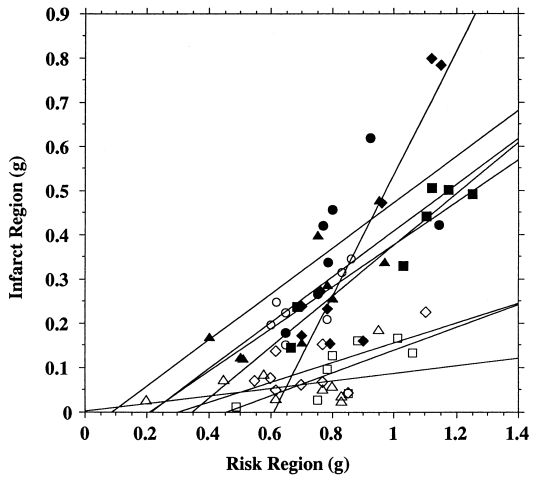

Figure 2. Relationship between the size of infarct region and the size of risk region. The linear regression line of each group is presented. In all groups, IS was positively and linearly related to risk region size. Linear regression equations were as follows: NC IP(+), y = 0.26x − 0.12; NC IP(−), y = 0.58x − 0.20; HC IP(+), y = 0.08x + 0.17; HC IP(−), y = 0.52x − 0.05; HCP IP(+), y = 0.09x; HCP IP(−), y = 0.52x − 0.11; NCP IP(+), y = 0.22x − 0.07; NCP IP(−), y = 1.4x − 0.84. ANCOVA demonstrated that the slope of the regression lines for NC IP(+), HCP IP(+) and NCP IP(+) groups, but not for others, are significantly different from those for the NC IP(−) group, indicating that IS-limiting effect observed in the NC group was abolished in the HC group, restored in the HCP group and not affected in the NCP group. Abbreviations as in Figure 1. **Open circle** = HC IP(+); **closed circle** = HC IP(−); **open triangle** = HCP IP(+); **closed triangle** = HCP IP(−); **open square** = NC IP(+); **closed square** = NC IP(−); **open diamond** = NCP IP(+); **closed diamond** = NCP IP(−).

ischemic heart disease, e.g., HC, hypertension, diabetes mellitus and medical treatments for them, it is very important to investigate the influence of those factors and to take them into consideration when the effect of IP is to be evaluated in clinical trials. We revealed here for the first time that one of the factors HC blunts the IS-limiting effect of IP and was restored by pravastatin treatment without normalizing the serum cholesterol level, meaning that pravastatin exerted its effect, not through the reduction of serum cholesterol levels but through some other unknown mechanism.

Hypercholesterolemia did not change the IS in this study with rabbits although HC was reported to increase IS partly by reducing the collateral flow in dogs (7). Although this difference between dogs and rabbits cannot be explained by the information available so far, this may partly be due to the difference in the amount of collateral flow; collateral circulation is well developed in canine hearts but it is not in rabbit hearts.

Although chronic treatment with the present dose (5 mg/kg/day) of pravastatin in HC rabbits did not significantly change the serum level of total cholesterol in accordance with the results in the previous reports (8–11), the IS-limiting effect of IP was completely restored. Since pravastatin did not decrease IS in NC rabbits, it is likely that pravastatin restored some factors involved in the mechanisms of IP which were disturbed by HC. These factors have never been discussed in the previous reports and further investigations are required to clarify them.

Our hypothesis on the IS-limiting effect of IP and adenosine. The IS-limiting effect of IP would be influenced by the cardiac interstitial adenosine levels following the activation of 5′-nucleotidase. The important determinant of the cardiac adenosine levels in the preconditioned myocardium is the extent of the increases in the activity of 5′-nucleotidase, which can be revealed by the ratio of the activity of 5′-nucleotidase between the preconditioned and nonpreconditioned area of the hearts (bottom panel of Fig. 3) judging from our previous investigations. Since the basal adenosine levels are determined not only by 5′-nucleotidase but also adenosine kinase, adenosine deaminase and the adenosine uptake capability, even if the basal activity of 5′-nucleotidase is high, the interstitial adenosine levels may not be increased. If the basal cardiac adenosine levels are comparable in the HC and the HCP groups and 5′-nucleotidase in the HCP group is further activated beyond the levels of the HC group due to IP procedure, this extent of increases in 5′-nucleotidase may cause the increases in the adenosine levels which may contribute to the limitation of IS. Therefore, the difference in 5′-nucleotidase activity before and after ischemia, but not the basal activity of 5′-nucleotidase itself, may be important for the determination of the IS-limiting effect of IP. Although we need to further investigate whether this hypothesis is correct, we interpreted the present data based on this hypothesis.

Possible mechanism involved in the restoration of the IS-limiting effect of IP blunted by HC. No direct action of HMG-CoA reductase inhibitors to endothelial or myocardial cells has been reported except for one recent report (3) which revealed their direct effect to upregulate endothelial nitric oxide synthase. In this study, we demonstrated that the IP-induced activation (4) of ecto-5′-nucleotidase of myocardium (as shown by the activity ratio of ischemic/nonischemic myocardium) is blunted by HC and is restored by pravastatin treatment possibly through its direct action besides reducing serum cholesterol level since the serum cholesterol level was not reduced in this study. Furthermore, we demonstrated that ecto-5′-nucleotidase activity of the nonischemic myocardium are increased by HC and are not affected by the pravastatin treatment, meaning that the activation of ecto-5′-nucleotidase by IP is blunted by HC regardless of the increased activity of ecto-5′-nucleotidase in the nonischemic region and that pravastatin restored the activation without changing the increased activity of ecto-5′-nucleotidase. Therefore, HC has influence not only on the activity of ecto-5′-nucleotidase itself but also on the mechanism of its activation by IP. Since the activation of ecto-5′-nucleotidase by IP negatively correlates with IS (5),

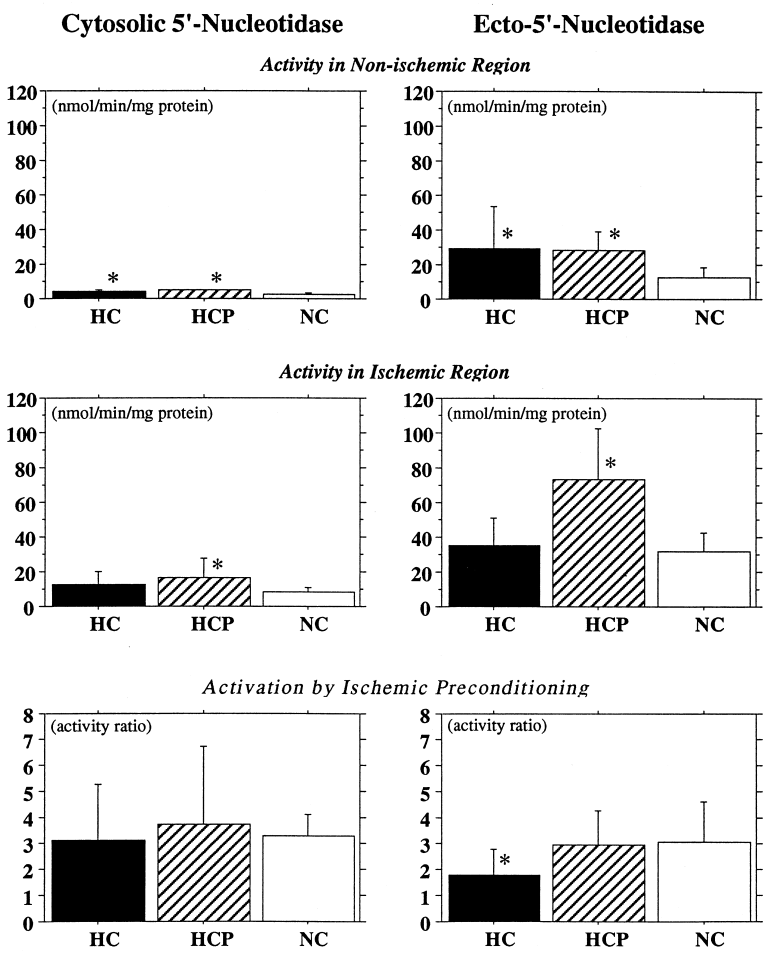

Figure 3. Cytosolic and ecto-5′-nucleotidase activity of myocardium from the ischemic and nonischemic region immediately after the procedure of ischemic preconditioning. Cytosolic and ecto-5′-nucleotidase activities in the nonischemic region were increased by hypercholesterolemia (HC and HCP groups) and pravastatin had no influence on them. However, the activation of ecto-5′-nucleotidase by ischemic preconditioning was blunted by hypercholesterolemia (HC group) and restored by pravastatin treatment (HCP group). *Significantly different from NC group. HC = hypercholesterolemia; HCP = hypercholesterolemia treated with pravastatin, NC = normocholesterolemia.

blunted activation of ecto-5′-nucleotidase in this study may be responsible for the blunted effect of IP in the HC rabbits, and pravastatin may restore the effect of IP by restoring the activation of ecto-5′-nucleotidase. The mechanism of ecto-5′-nucleotidase activation by IP, which is blunted by HC and restored by pravastatin, is left to be investigated. Another possible mechanism left to be investigated is that, since nitric oxide is reported to open the second window of preconditioning (12–14), the depletion of nitric oxide by HC (15) may attenuate cardioprotection, and pravastatin may restore the IS-limiting effect of IP by upregulating (3) the nitric oxide synthase. We do not have evidence to support or negate this idea.

Clinical implications. Recently, increasing numbers of studies have suggested the existence of IP in the clinical settings. Preinfarction angina or prodrome has been reported to be related to good left ventricular function after MI in humans (2), and this may be due to the IS-limiting effect of IP. Repeated reduction of coronary blood flow and transient occlusion of coronary artery by the phenomenon of cyclic flow variation is known to precede the permanent thrombotic occlusion. Therefore, it is quite likely that transient myocardial ischemia including the one without chest pain (silent ischemia) precedes the onset of MI in predominant numbers of patients, and this may induce IP. Considering this evidence, our results suggest that HC increases IS among the preconditioned patients, and this attenuation of cardioprotection may be restored by treatment with pravastatin.

Study limitations. Since there might be species differences, our results with rabbits might not necessarily predict the clinical outcome. Since we used a certain protocol for developing IP, administering pravastatin or feeding cholesterol, the results may be different if some different protocols

(multiple cycles of ischemia for IP, low dose of cholesterol or acute administration of pravastatin) are used, which are left to be tested. Since we revealed the effect of HC and pravastatin on the IS-limiting effect of IP and on the activation of ecto-5′-nucleotidase activity separately, we can not deny the possibility that these two phenomena are coincidental. However, considering the results of the previous studies that IS correlates with the ecto-5′-nucleotidase activity of myocardium and its inhibition can abolish the effect of IP, it may be probable that the changes in the IS are determined by the changes in the ecto-5′-nucleotidase activation. Although we demonstrated the effects of pravastatin, it is left to be determined whether the effects are specific to this particular agent or are common among HMG-CoA reductase inhibitors.

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