The presence and role of hormone-sensitive lipase in heart muscle

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Hormone-sensitive lipase (HSL) catalyses the initial, rate-limiting, reaction in adipose-tissue lipolysis. Hormone-stimulated lipolytic activity has also been observed in the heart, where endogenous triacylglycerol is the major energy store. However, the identity of the intracellular lipase responsible has yet to be established. We have partially purified a neutral lipase from bovine heart muscle and compared its properties with those of HSL from bovine adipose tissue. The heart lipase has the same subunit M_r as HSL, is immunoprecipitated by antiserum raised against purified HSL and is phosphorylated by cyclic AMP-dependent protein kinase, apparently at the same site as HSL (as judged by h.p.l.c. of tryptic phosphopeptides). Phosphorylation of the heart lipase was found to result in increased enzyme activity, demonstrating the lipase's potential to respond to hormonal stimuli. The heart lipase was shown to be present in myocytes by its immunoprecipitation from homogenates of rat myocytes by anti-HSL antiserum. These findings are consistent with the conclusion that HSL is responsible for intracellular lipolysis in heart.

INTRODUCTION

Hormone-sensitive lipase (HSL) catalyses the rate-limiting step in adipose-tissue lipolysis and is under acute neural and hormonal control (Yeaman *et al.*, 1987). Lipolytic agents such as noradrenaline (released from sympathetic nerve endings), adrenaline and corticotropin lead to an increase in the intracellular concentration of cyclic AMP, resulting in phosphorylation and activation of HSL by cyclic AMP-dependent protein kinase (Nilsson *et al.*, 1980). Conversely insulin, the major anti-lipolytic hormone, decreases phosphorylation (and activity) of HSL, in part by lowering the intracellular concentration of cyclic AMP (Londos *et al.*, 1985). In addition to its role in lipolysis, HSL is implicated in steroidogenesis, having been shown to be responsible for the cholesterol ester hydrolase activity of adrenal cortex and corpus luteum (Cook *et al.*, 1982, 1983).

Intracellular lipolytic activity has also been observed in heart muscle, where endogenous triacylglycerol is the major energy store. The release of fatty acids from this store, to be oxidized within cardiac muscle cells, is under acute hormonal control, in response to the energy requirements of the heart (reviewed in Severson, 1979). The nature of the cardiac lipase is controversial, with conflicting views that either an intracellular form of lipoprotein lipase (LPL) (Oscai, 1979; Palmer *et al.*, 1981) or a distinct neutral lipase (Goldberg & Khoo, 1985; Ramirez *et al.*, 1985) is responsible for lipolysis.

After perfusion of isolated rat hearts with heparin to remove extracellular LPL activity, a residual lipase activity with several properties identical with those of LPL remained; it was concluded that this lipase was either an intracellular storage pool for endothelial-bound LPL, or its soluble precursor (Hülsmann *et al.*, 1982).

Hormone-sensitivity of heparin-non-releasable LPL was demonstrated by increased lipolytic activity at pH 8.1 in homogenates of heparin-perfused hearts previously treated with glucagon (Oscai, 1979) or adrenaline (Palmer *et al.*, 1981). Consequently the lipase was termed 'type L-HSL', as it was hormone-sensitive and showed the properties of LPL (Palmer & Kane, 1983).

However, the presence of heparin-non-releasable LPL activity in heart homogenates does not necessarily indicate that it is intracellular (Jansen *et al.*, 1980), and, indeed, the role of this enzyme has been disputed, with claims that a distinct neutral lipase is responsible for intracellular cardiac lipolytic activity (Goldberg & Khoo, 1985; Ramirez *et al.*, 1985). LPL apparently constitutes 65–70 % of the total lipase activity in heart homogenates, and it has been argued that this masks the properties of the neutral lipase (Goldberg & Khoo, 1985). The presence of an intracellular neutral lipase in heart, with properties distinct from those of LPL, was first demonstrated by Ramirez *et al.* (1985); it was subsequently shown to be activated by cyclic AMP-dependent protein kinase (Goldberg & Khoo, 1985), suggesting that the increased lipolytic rate in the heart after hormonal stimulation results from cyclic AMP-dependent protein kinase activation of this distinct neutral lipase. The classical neutral hormone-stimulated lipase is HSL, and evidence has been presented for the presence of HSL in homogenates of whole rat hearts by Western blotting using antibody specific for rat adipose-tissue HSL (Holm *et al.*, 1987) and Northern blotting with a cDNA probe for HSL (Holm *et al.*, 1988). In the present paper we report several further pieces of evidence for the presence of intracellular HSL in heart muscle, and directly demonstrate that HSL is present in isolated myocytes, indicating that HSL is responsible for hormonally responsive cardiac lipolysis.

Abbreviations used: HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; DFP, di-isopropyl fluorophosphate; PAGE, polyacrylamide-gel electrophoresis; MOME, 1(3)-mono[³H]oleoyl-2-*O*-oleoylglycerol.

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EXPERIMENTAL

Materials

[γ - 32 P]ATP and [3 H]DFP were from Amersham International. Hexokinase was from Boehringer, Pansorbin from Calbiochem and trypsin [tosyl-phenylalanylchloromethane ('TPCK')-treated] were from Worthington. Collagenase (type II) and hyaluronidase (type I-S) were from Sigma. Sources of substrates, proteinase inhibitors and the non-ionic detergent $C_{13}E_{12}$ were as described in Cordle *et al.* (1986).

Enzyme preparations

Bovine heart was obtained from a local abattoir, transported on ice and processed within 45 min of slaughter. The heart was trimmed of all visible fat before homogenization, and the neutral lipase was prepared by essentially the same procedure as that used for purification of HSL from bovine perirenal adipose tissue (Cordle *et al.*, 1986). All steps were carried out at 4 °C. Briefly, the purification method involves isoelectric precipitation at pH 5.0, followed by partial solubilization in Triton-N101 and ion-exchange chromatography on DEAE-cellulose DE-52. After detergent exchange into $C_{13}E_{12}$ on hydroxyapatite, the enzyme was further purified by chromatography on phenyl-Sepharose and heparin-Sepharose.

The catalytic subunit of cyclic AMP-dependent protein kinase was purified to homogeneity from bovine adipose tissue (Strålfors & Belfrage, 1982).

Enzyme activity

Lipase activity was routinely assayed against cholesterol [3 H]oleate, a convenient substrate for the enzyme (Belfrage *et al.*, 1977), or tri[3 H]oleoylglycerol in ethanolic suspension (Yeaman *et al.*, 1980). Activity against 1(3)-mono[3 H]oleoyl-2-*O*-oleoylglycerol (MOME) was assayed as described by Fredrikson *et al.* (1981). A unit of enzyme catalyses the release of 1 μ mol of fatty acid/min. Protein was determined by the method of Bradford (1976).

SDS/polyacrylamide-gel electrophoresis (SDS/PAGE)

This was performed by the method of Laemmli & Favre (1973), with 4% acrylamide stacking gel and 8% running gel. After staining with Coomassie Brilliant Blue R, gels were either dried and autoradiographed for 1–3 days, or treated with sodium salicylate (Chamberlain, 1979), dried and fluorographed for 1 week. Fuji RX X-ray film was used for both autoradiography and fluorography; the latter was performed at –70 °C by using Du Pont Cronex intensifying screens. Densitometric scanning was carried out with a Bio-Rad densitometer (model 1650) linked to a Spectra-Physics SP4920 integrator.

Phosphorylation of the purified lipase preparations

Before phosphorylation, the lipase preparations were transferred into buffer A [5 mM-imidazole/HCl (pH 7.0), 1 mM-dithiothreitol, 30% (w/v) glycerol, 50 mM-NaCl, 5 μ g of leupeptin/ml and 1 μ g of pepstatin/ml], by repeated 10-fold dilution of the enzyme into this buffer, followed by concentration to the original volume with Amicon Centricon 30 centrifugal micro-concentrators.

Phosphorylation of the lipase preparations was performed in the presence of 0.15 mM- $[\gamma$ - 32 P]ATP

(1000–5000 d.p.m./pmol), 10 mM-MgCl₂ and 3 μ M catalytic subunit of cyclic AMP-dependent protein kinase (Garton *et al.*, 1988).

Antiserum against the M_r -84000 subunit of bovine adipose-tissue HSL was raised in a New Zealand White rabbit as described previously (De Marcucci & Lindsay, 1985; Cordle *et al.*, 1986). An immunoglobulin fraction was prepared by the addition of (NH₄)₂SO₄ (final concn. 60% satn.) to the antiserum. After standing for 30 min, the immunoglobulins were collected by centrifugation (5 min at 10000 *g*), resuspended in 50 mM-K₂HPO₄/KH₂PO₄ buffer, pH 7.0, and then dialysed extensively against this buffer. Immunoprecipitates were prepared by incubating the phosphorylated lipase preparation (15 μ l, containing approx. 0.25 μ g of lipase protein) with 50 μ l of immunoglobulin (2.5 mg/ml), by the procedure detailed in Jones & Yeaman (1986). Controls were incubated with normal rabbit serum, then treated in an identical manner.

Tryptic digestion of the M_r -84000 phosphoprotein and separation of the phosphopeptides by reverse-phase h.p.l.c. was carried out as described by Garton *et al.* (1988). The phosphopeptides were also analysed by high-voltage electrophoresis at pH 1.9 and autoradiography.

Phosphorylation was also performed in the presence of non-radioactive ATP, under the same conditions as outlined above, and activation of the lipase was assayed against 0.125 mM-tri[3 H]oleoylglycerol in 25 mM-Tris/HCl (pH 8.3) containing 5 mM-NaCl as described by Fredrikson *et al.* (1981).

Inhibition by DFP

DFP was dissolved in propylene glycol, and 0.05 vol. was added to the lipase preparations to give the final concentrations indicated. After incubation at room temperature for 30 min, the lipase was assayed against cholesterol oleate and triacylglycerol. A control incubation was performed with 0.05 vol. of propylene glycol alone, which had a negligible effect on the enzyme activity measured against either substrate.

Lipase preparations, transferred to buffer A as described above, were incubated in 70 μ M- $[\gamma$ - 32 P]DFP (8×10^3 d.p.m./pmol) for 30 min at room temperature. The reaction was terminated by the addition of an equal volume of Laemmli sample buffer [0.3 M-Tris/HCl (pH 6.8), containing 25% (v/v) glycerol, 5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.00025% (w/v) Bromophenol Blue] and, after boiling for 5 min, the samples were subjected to SDS/PAGE followed by fluorography.

Preparation of myocyte homogenates

Myocytes were prepared by the 'chunk method' of Isenberg & Klockner (1982), from hearts taken from 150–200 g male Porton Wistar rats which had been fasted for 24 h; from three rat hearts, a total of 2.1×10^7 myocytes were isolated. Myocytes were separated from any contaminating adipocytes by centrifugation at 20 *g* for 2 min; myocytes are recovered in the pellet, and adipocytes float to the surface.

The cell pellet was resuspended in 5 mM-imidazole/HCl (pH 7.0), containing 0.1 mM-benzamidine, 1 mM-dithiothreitol, 50% glycerol, 0.2% (w/v) $C_{13}E_{12}$, 50 mM-NaCl, 5 μ g of leupeptin/ml and 1 μ g of pepstatin/ml, and the myocytes were disrupted by sonication for 4 $\times$ 30 s on ice by using an exponential microtip coupled

to a Dawe type 7533A ultrasonic generator. Cell debris was removed by centrifugation at 10000 *g* for 5 min, and the soluble fraction was stored at -70°C until use.

Phosphorylation and immunoprecipitation of the myocyte homogenate

Homogenate (250 μl , containing approx. 1.25 mg of protein) was incubated for 5 min at 30°C in the presence of 0.1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (5000–10000 d.p.m./pmol), 1 mM- MgCl_2 and 3 μM catalytic subunit of cyclic AMP-dependent protein kinase. Phosphorylations were terminated by the addition of EDTA (final concn. 20 mM), immunoprecipitates were prepared from the phosphorylated extract by using the anti-HSL antiserum as described above, and then analysed by SDS/PAGE followed by autoradiography.

RESULTS

By using the procedure for the purification of HSL from bovine adipose tissue, a neutral lipase was purified approx. 500-fold from bovine heart muscle (Table 1). The final material has a specific activity of 2.8 units/mg against MOME (a diacylglycerol analogue) and is also active against triacylglycerol (sp. activity 46 munits/mg) and cholesterol oleate (sp. activity 68 munits/mg). A similar activity ratio against these substrates has been found with HSL from adipose tissue (Fredrikson *et al.*, 1981).

LPL was removed from the neutral heart lipase preparation by the final heparin–Sephadex purification step (results not shown), the major peak of neutral lipase being eluted from the column at approx. 120 mM-NaCl, whereas LPL was eluted at approx. 1 M-NaCl (Olivecrona *et al.*, 1971). Previous work has shown that HSL consists of a single polypeptide of M_r 84000 (Fredrikson *et al.*, 1981; Cordle *et al.*, 1986). A M_r -84000 polypeptide with many of the properties of HSL was identified in the heart lipase preparation by several criteria. DFP, a serine hydrolase inhibitor which covalently binds to the active-site serine of HSL (Fredrikson *et al.*, 1981; Cook *et al.*, 1982), was found to inhibit the triacylglycerol and cholesterol ester hydrolase activities of the heart lipase, with the activities being lost over the same concentration range of DFP as the activities of adipose tissue HSL (Fig. 1a). When the heart lipase was incubated with $[\text{H}^3]\text{DFP}$, a single ^3H -labelled polypeptide of M_r 84000

was identified (Fig. 1b), indicating that the triacylglycerol and cholesterol ester hydrolase activities are both catalysed by this polypeptide.

Further evidence that the polypeptide of M_r 84000 is HSL was obtained by incubating the heart lipase preparation with the catalytic subunit of cyclic AMP-dependent protein kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. After such treatment, the M_r -84000 polypeptide became phosphorylated (Fig. 2), with a stoichiometry of 0.3–0.4 mol of phosphate/mol of protein; phosphorylation was estimated from the Čerenkov radiation of the excised M_r -84000 polypeptide and the specific radioactivity of the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and the amount of lipase polypeptide was estimated from protein determination (Bradford, 1976), by assuming that the specific activity of the enzyme is the same as that of adipose-tissue HSL (Cordle *et al.*, 1986). Antiserum raised against purified HSL from bovine adipose tissue selectively precipitated the M_r -84000 phosphopeptide from heart lipase preparations (Fig. 2). Immunoprecipitation of the phosphoprotein was blocked in a competitive manner by the presence of increasing amounts of non-radioactive adipose-tissue HSL (results not shown).

After incubation with the catalytic subunit of cyclic AMP-dependent protein kinase and MgATP , the heart lipase became activated against the triacylglycerol substrate. Activation of the heart triacylglycerol hydrolase activity was found to correlate with phosphorylation of the M_r -84000 polypeptide (Fig. 3).

The site on adipose-tissue HSL which is phosphorylated by cyclic AMP-dependent protein kinase has been characterized and sequenced (Garton *et al.*, 1988). Upon reverse-phase h.p.l.c. of the phosphopeptides produced by tryptic digestion of the phosphorylated heart lipase, two major peaks of radioactivity were recovered, corresponding in elution position to phosphopeptides T1 and T2 of adipose-tissue HSL phosphorylated and digested under the same conditions (Fig. 4). The T1/T2 ratio differs for phosphopeptides derived from heart and adipose-tissue HSL; however, phosphopeptides T1 and T2 of adipose-tissue HSL have been shown by amino acid sequencing to be different forms of the same phosphopeptide, and the T1/T2 ratio can vary greatly between different adipose-tissue HSL phosphopeptide preparations (Garton *et al.*, 1988). When the tryptic phosphopeptides were analysed by high-voltage electrophoresis and autoradiography, the

Table 1. Purification of the neutral lipase from bovine heart muscle

Starting material was 1 kg of bovine heart muscle. Protein was determined by the method of Bradford (1976).

Fraction	Protein (mg)	Substrate . . .		Cholesterol oleate		MOME	
		Activity (munits)	Sp. activity (munits/mg)	Activity (munits)	Sp. activity (munits/mg)	Activity (munits)	Sp. activity (munits/mg)
13000 <i>g</i> supernatant	25600	3000	0.12	148800	5.8		
pH 5.0 precipitate	4635	814	0.18	35072	7.6		
DE-52	266	271	1.02	11970	45		
Hydroxyapatite	264	243	0.92	9672	37		
Phenyl-Sephadex	11.2	115	10.3	4494	401		
Heparin-Sephadex	0.725	49	68	2034	2806

Fig. 1. (a) Inhibition of the neutral heart lipase and adipose-tissue HSL by DFP, and (b) [^3H]DFP labelling of the neutral heart lipase and adipose-tissue HSL

(a) Adipose-tissue HSL ($\circ$) and partially purified heart lipase ($\bullet$, $\square$) were incubated with the indicated concentrations of DFP (added in 0.05 vol. of propylene glycol) for 15 min at room temperature and then assayed against cholesterol [^3H]oleate ($\bullet$, $\circ$) or tri [^3H]oleoylglycerol ($\square$). Activities are expressed as a

Fig. 2. Phosphorylation and immunoprecipitation of the neutral heart lipase and adipose-tissue HSL

The enzyme preparations were incubated for 30 min at 30 °C with the catalytic subunit of cyclic AMP-dependent protein kinase (3 μM), 1 mM- MgCl_2 and 0.15 mM- $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ [$(5\text{--}15) \times 10^6$ c.p.m./nmol]. Phosphorylations were terminated by the addition of an equal volume of Laemmli sample buffer and subjected to SDS/PAGE and autoradiography. Lanes: 1, phosphorylated adipose-tissue HSL; 3, phosphorylated heart lipase. Immunoprecipitates were prepared from the phosphorylated enzyme preparations (containing approx. 0.25 μg of lipase protein), as described in Jones & Yeaman (1986), by using an Ig fraction prepared from anti-HSL antiserum, and analysed by SDS/PAGE and autoradiography. Lanes: 2, adipose-tissue HSL + 50 μl of anti-HSL Ig (2.5 mg/ml); 4, heart lipase + 25 μl of anti-HSL Ig; 5, heart lipase + 50 μl of anti-HSL Ig.

same electrophoretic pattern was observed for phosphopeptides derived from both heart lipase and adipose-tissue HSL (results not shown).

When purifying the heart lipase, although all visible traces of fat were removed from the heart before homogenization, it was not possible to discount completely the presence of intracardiac adipocytes and the possibility that the purified HSL was derived from these cells. Therefore isolated myocytes were prepared by collagenase digestion of rat heart and separated from any contaminating adipocytes by centrifugation. The specific immunoprecipitation of a M_r -84000 phosphoprotein from phosphorylated homogenates of these myocytes by anti-HSL antiserum was then demonstrated (Fig. 5).

percentage of that observed in the presence of propylene glycol (0.05 vol.) alone. (b) Adipose-tissue HSL and partially purified heart lipase were incubated with [^3H]DFP (70 μM , 3.5 mCi/mmol), for 30 min at room temperature. The reactions were terminated by the addition of an equal volume of Laemmli sample buffer, and the samples were subjected to SDS/PAGE and fluorography. Lanes: 1, mixture of the two ^3H -labelled lipases; 2, ^3H -labelled heart lipase; 3, ^3H -labelled adipose-tissue HSL. '84K' indicates the M_r -84000 polypeptide.

Fig. 3. Activation (●) and phosphorylation (○) of the neutral heart lipase

The heart lipase preparation was incubated at 30 °C with the catalytic subunit of cyclic AMP-dependent protein kinase (3 μ M), 1 mM-MgCl₂, and either 0.15 mM-ATP (●) or 0.15 mM-[γ -³²P]ATP (○). At the indicated times, samples were removed and phosphorylation was terminated by either 10-fold dilution into 10 mM-EDTA containing 0.1 mg of bovine serum albumin/ml and 1 mM-dithiothreitol (●), or by addition of an equal volume of Laemmli sample buffer (○). Activation was determined by assay against tri[³H]oleoylglycerol, and phosphorylation by scanning densitometry of an autoradiograph of the *M_r*-84000 polypeptide on an SDS/polyacrylamide gel. Maximum activation observed was approx. 30% over control experiments performed in the absence of MgATP. All values are expressed as a percentage of the maximum value observed.

DISCUSSION

We report here the partial purification of a neutral lipase from bovine heart muscle; comparison of its properties with those of HSL from adipose tissue leads to the conclusion that this heart lipase and HSL are the same enzyme. In particular, incubation of adipose-tissue and heart preparations in the presence of [³H]DFP resulted in the specific radiolabelling of a *M_r*-84000 polypeptide in both cases. Similarly, incubation with the catalytic subunit of cyclic AMP-dependent protein kinase and [γ -³²P]ATP led to the phosphorylation of a

Fig. 4. Separation of tryptic phosphopeptides by reverse-phase h.p.l.c.

The neutral heart lipase and adipose-tissue HSL were [³²P]phosphorylated as described in the legend to Fig. 2. After SDS/PAGE, the *M_r*-84000 phosphoprotein was excised from the gel, extracted and digested with trypsin. The tryptic phosphopeptides generated were chromatographed on a Vydac C₁₈ h.p.l.c. column. The peptides were applied to the column in 0.1% (v/v) trifluoroacetic acid and eluted with a linear gradient of 0–35% acetonitrile (---) containing 0.1% trifluoroacetic acid, with an increase in acetonitrile concentration of 0.5%/min. —, ³²P radioactivity.

M_r-84000 polypeptide in the heart preparation which can be immunoprecipitated by antiserum raised against purified adipose-tissue HSL. Tryptic digestion of the *M_r*-84000 phosphoproteins from adipose tissue and heart gave the same pattern of phosphopeptides when subjected to reverse-phase h.p.l.c. and high-voltage electrophoresis. All this evidence indicates that the *M_r*-84000 polypeptide found in heart lipase preparations is identical with adipose-tissue HSL.

It is important to stress that all traces of external fat were removed from the heart before homogenization, to minimize possible contamination by adipose-tissue enzymes. Furthermore, to exclude the possibility that the heart lipase activity was derived exclusively from HSL present in intramuscular adipocytes or other cell types, it was demonstrated that the HSL phosphoprotein was present in isolated myocytes; this was achieved by the specific immunoprecipitation of the *M_r*-84000 HSL phosphoprotein from phosphorylated rat myocyte homogenates by anti-HSL antiserum. The finding that HSL can be detected in homogenates of isolated myocytes is significant, since it has been reported that only 6% of

Fig. 5. Phosphorylation and immunoprecipitation of the rat myocyte homogenate

The adipose-tissue HSL and myocyte homogenate were [32 P]phosphorylated as described in the Experimental section, and immunoprecipitates were prepared from the phosphorylated extracts as described in the legend to Fig. 2. Lanes: 1, phosphorylated adipose-tissue HSL (0.25 μ g) + 50 μ l of anti-HSL Ig (2.5 mg/ml); 2, phosphorylated myocyte homogenate (75 μ g of protein); 3, phosphorylated myocyte homogenate (1.25 mg) + 50 μ l of anti-HSL Ig.

the neutral cholesterol ester hydrolase activity measured in whole rat ventricular homogenates is of myocytic origin and that the predominant activity is derived from other cell types, including possibly cardioadipocytes (Stam *et al.*, 1987). It should be stated that our present data cannot quantify the amount of HSL originating from each cell type, and the absolute contribution of intramyocytic HSL to the total lipase activity observed in heart homogenates remains unclear.

In the heart, as previously mentioned, endogenous triacylglycerol is the major energy store, and non-esterified fatty acids are the major source of fuel, being utilized in preference to any other oxidative substrate. The lipolytic rate must therefore be controlled in response to the metabolic demands of the heart, and is under acute hormonal control. This regulation must be exerted via the activity of the enzyme which catalyses the rate-limiting step of lipolysis, namely the hydrolysis of triacylglycerol to yield diacylglycerol and non-esterified fatty acid. HSL has all the required properties to fulfil this role. The presence of HSL in the heart does not exclude the possible presence of other intracellular triacylglycerol hydrolases. However, since no other identified intracellular lipase is known to be hormone-sensitive, any additional intracellular lipases are unlikely to play a role in the hormonal control of lipolysis in the heart, which is essential for cardiac function.

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