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SOLUBILIZATION OF TWO TYPES OF ENDOTHELIN RECEPTORS, ET_A AND ET_B, FROM RAT LUNG WITH RETENTION OF BINDING ACTIVITY

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ABSTRACT

Endothelin (ET) receptors were solubilized from rat lung using digitonin. The binding of ^{125}I-ET-1 and -3 to the solubilized receptors was specific, time-dependent, and saturable. Scatchard-plot analysis of ^{125}I-ET-1 and -3 binding exhibited similar dissociation constants of 20 pM and 14 pM, respectively. However, the concentration of binding sites for the two isopeptides was different: B_{max}=3.4 pmol/mg protein for ^{125}I-ET-1 and 1.0 pmol/mg protein for ^{125}I-ET-3. The order of potency in displacing the binding of ^{125}I-ET-1 to the solubilized receptors was sarafotoxin S6b=ET-1>ET-3. In contrast, for ^{125}I-ET-3, the order of displacement was almost the same among the three peptides. Cross-linking of ^{125}I-ET-1 and -3-labeled solubilized ET receptors with disuccinimidyl tartarate resulted in the identification of one major and one minor band of 48 and 37 kDa, respectively, the latter being presumably a proteolytic degradation product of the major band. These results suggest that digitonin-solubilized ET receptors retain the biochemical characteristics of the membrane-bound receptors and that there are at least two distinct receptor types in the solubilized extracts corresponding to ET_A and ET_B receptor. Moreover, both ET_A and ET_B receptor proteins as well as their mRNAs were abundant in the rat lung.

Endothelin (ET), an extremely potent vasoactive peptide, is classified into a family of three isopeptides named ET-1, -2, and -3 (4, 11, 18). In addition to vasoconstriction, a number of physiological and pharmacological functions induced by ET have been reported, including stimulation of the release of aldosterone (3), atrial natriuretic peptide (2), endothelium-derived relaxing factor (16) and inhibition of renin release (10). These functions are thought to be initiated by endothelin binding to its specific receptors on the surfaces of target tissues (8). Several studies have demonstrated that the effects on the same tissues varied among the three isopeptides, suggesting the existence of multiple receptor types differing in their biochemical properties and which mediate distinct responses to the ligand (3, 4, 7, 13, 14, 17). Recently, the existence of at least two distinct types of ET receptors has been substantiated by cloning the cDNA for two different ET receptor types named ET_A (ET_AR) and ET_B receptor (ET_BR) (1, 6, 12); ET_AR is an ET-1-specific receptor subtype and ET_BR is a non-isopeptide-selective subtype. However, correlation between ET-induced functions and receptor subtypes has not been established. Thus, it is of great interest to examine the relationships among ET isopeptides, the many ET-induced functions, and receptor subtypes. To clarify these issues, detailed biochemical

Abbreviations: CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; DST, disuccinimidyl tartarate

characterization of each type of the receptor is essential. However, only a few investigators have attempted to solubilize and ultimately purify the receptors, both of which are necessary for characterization (8, 9, 14, 15).

In the present study, we solubilized the two ET receptor subtypes, ET_AR and ET_BR, in an active form from the rat lung using digitonin, and compared their binding properties with those of the membrane-bound receptors by Scatchard-plot analysis, affinity labeling, and competition binding. We also found that the lung is an abundant source of both ET_AR and ET_BR proteins as well as their mRNAs.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (200–250 g) were obtained from CLEA (Tokyo, Japan). ET-1, -3, sarafotoxin S6b, leupeptin, and antipain were purchased from the Peptide Institute (Osaka, Japan). ^{125}I-ET-1 and -3 (~2,000 Ci/mmol) were obtained from Amersham International plc (Bucks, U.K.). Digitonin and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS) were from Wako Pure Chemicals (Osaka, Japan) and Dojin Laboratories (Kumamoto, Japan), respectively. Disuccinimidyl tartarate (DST) was from Pierce Chemical.

Homogenizing buffer: 20 mM Tris/HCl, pH 7.4, containing 2 mM or 100 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml leupeptin, and 10 μg/ml antipain; storage buffer: 10 mM Tris/HCl, pH 7.4, containing 0.25 M sucrose and the same protease inhibitors listed above; and binding buffer: 10 mM Tris/HCl, pH 7.4, containing 10 mM $MgCl_2$ and the same protease inhibitors listed above except that EDTA was omitted.

Preparation of Rat Lung Membranes and Solubilization

Rat lung membranes were prepared as described in (7, 17) except for the use of the homogenizing and storage buffers. The membranes were solubilized by incubation at a concentration of 1 mg protein/ml in binding buffer with various concentrations of digitonin or CHAPS at 4°C for 1 h followed by centrifugation at 158,000 *g*. The resulting supernatant was used immediately or stored at −80°C as solubilized extract.

^{125}I-ET Binding Assay with Solubilized Extracts

Solubilized extracts diluted 10-fold with binding buffer (~3 μg protein) were incubated with 50 pM ^{125}I-ET-1 or -3 at 25°C for 2 h in 100 μl binding buffer. Free and bound radioligands were separated by adding 2 ml cold phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) to assay tubes followed by filtration through Whatman GF/F glass fiber filters treated with poly (ethyleneimine) under reduced pressure. Tubes and filters were washed with 2 ml of the same buffer, then the radioactivity retained on filters was counted in a Beckman γ-counter. In equilibrium binding experiments, all procedures were as described above except that the solubilized extracts were reacted with ^{125}I-ET-1 or -3 under various concentrations of radioligands (10 pM-500 pM).

Affinity Labeling

Digitonin-solubilized extracts were diluted 2-fold with binding buffer and incubated with 1 nM ^{125}I-ET-1 or -3 in 50 μl binding buffer at 25°C for 2 h. After dialysis against sodium phosphate, pH 7.4, containing 0.01% digitonin, 10 mM $MgCl_2$, and the same protease inhibitors as binding buffer to remove Tris, which contains amines, the reaction mixtures were cross-linked with 1 mM DST at 25°C for 20 min. The cross-linking reaction was quenched by adding 4 M ammonium acetate (100 mM, final concentration). Samples solubilized in SDS-gel sample buffer were separated by SDS-PAGE according to Laemmli (5) using 1-mm-thick gels (10% acrylamide). After electrophoresis, the gels were stained in Coomassie Brilliant Blue R-250, dried, and autoradiographed at −80°C using Fuji medical X-ray film with an intensifying screen.

RESULTS

Effect of Various Concentrations of CHAPS and Digitonin on the Solubilization of ET Receptors

To determine the optimal CHAPS and digitonin concentrations for solubilization of active ET receptors, rat lung membranes (1 mg/ml) were incubated at 4°C with detergent at various concentrations. After centrifugation, ^{125}I-ET-1 binding

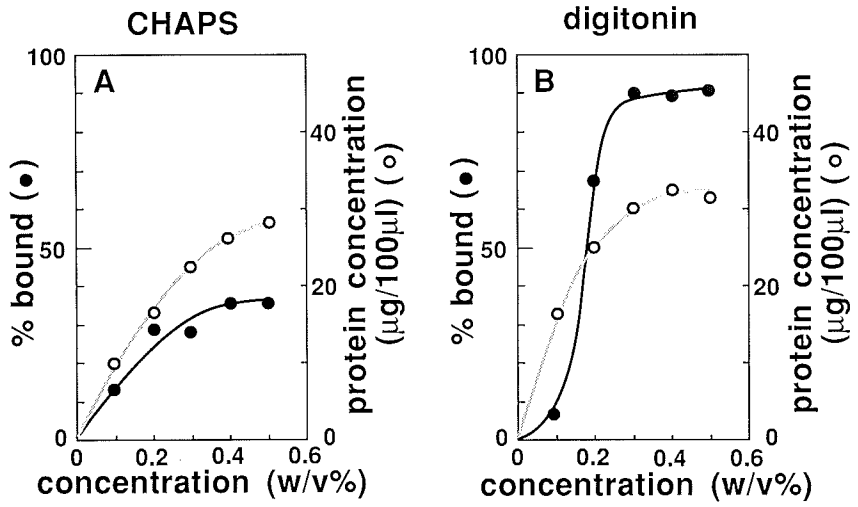

Fig. 1 Solubilization of proteins and ^{125}I-ET-1 binding activity from rat lung membranes using CHAPS or digitonin. Rat lung membranes (1 mg protein/ml) were solubilized with the indicated concentrations of CHAPS (A) or digitonin (B). Each point represents the mean of duplicate determinations.

activity was measured with the soluble fraction which was diluted 10-fold with binding buffer, since ET-1 binding was inhibited at concentrations of CHAPS and digitonin higher than 0.05%. The results illustrated in Fig. 1 show that the appearance of soluble binding activity was virtually proportional to the concentration of solubilized proteins, with saturation at 0.4% CHAPS and 0.3% digitonin, respectively. Although the efficiency of solubilization of membrane proteins was similar with both detergents, the maximum binding activity with digitonin was much higher than that with CHAPS (35% for CHAPS and 90% for digitonin); over 85% of the bound label was displaced by 100 nM unlabeled ET-1. Thus, in subsequent assays, ET receptors were solubilized in 0.3% digitonin.

Time Course of ET Binding to the Solubilized Receptors at Various Temperatures

As illustrated in Fig. 2, the solubilized extracts were incubated with 50 pM ^{125}I-ET-1 or -3 at 4°C, 25°C, or 37°C for the indicated period of time. The rate of association at 37°C was faster than at 4°C and 25°C for both ^{125}I-ET-1 and -3. However, ^{125}I-ET-1 binding at 37°C was a little unstable, since it started to decrease after 1 h. The binding at 25°C also increased rapidly with time, reaching a maximum after 1–2 h of association and remaining

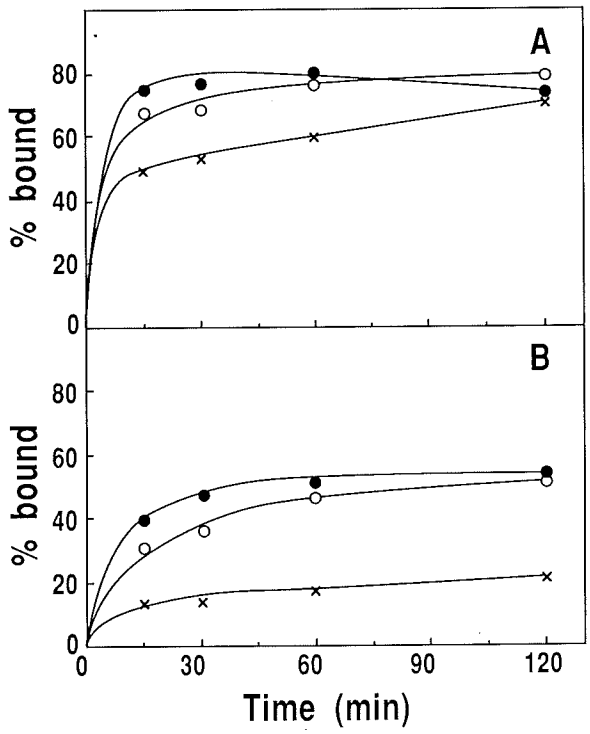

Fig. 2 Time course and temperature dependence of ^{125}I-ET-1 or -3 binding of solubilized ET receptors. Solubilized ET receptors were incubated with ^{125}I-ET-1 (A) or -3 (B) at 4°C (×), 25°C (○) or 37°C (●). At the indicated times, the binding activity of ^{125}I-ET-1 or -3 was measured as described in Materials and Methods. Each point is the average of the duplicate determinations.

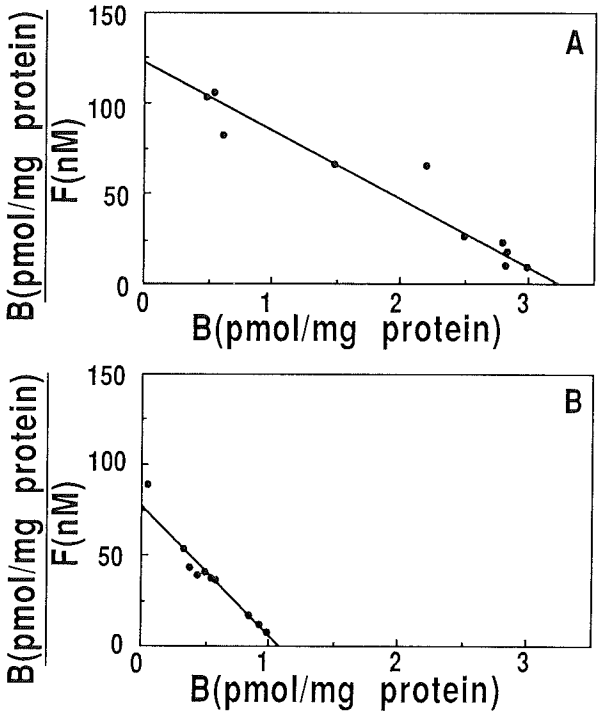

Fig. 3 Scatchard plot of ^{125}I-ET-1 or -3 binding to solubilized ET receptors. The solubilized extracts were incubated with increasing concentrations of ^{125}I-ET-1 or -3 (10–500 pM) in the presence or in the absence of 100 nM unlabeled ET-1 or -3, respectively. The saturation data are plotted according to Scatchard to determine the binding parameters for ^{125}I-ET-1 (A) or -3 (B); the dissociation constant K_d=28 pM, the number of binding sites B_{max}=3.4 pmol/mg protein for ^{125}I-ET-1, and K_d=13 pM, B_{max}=1.1 pmol/mg protein for ^{125}I-ET-3. The data presented is a representative of three different experiments for each radioligand.

stable for at least 4 h, in contrast to the incubation at 37°C. Since the maximal binding at 25°C and 37°C exhibited almost the same values of approximately 80 and 50% for ^{125}I-ET-1 and -3, respectively, the radioligands were reacted with the solubilized extracts at 25°C for 2 h in subsequent experiments.

Scatchard-plot Analysis of ET Binding

As illustrated in Fig. 3, Scatchard-plot analysis of ^{125}I-ET-1 and -3 binding data produced linear plots, which suggested the presence of a single type of binding sites. The binding parameters determined in three different experiments were K_d= 20±10 pM, B_{max}=3.4±0.1 pmol/mg protein for ET-1 and K_d=14±4 pM, B_{max}=1.0±0.2 pmol/mg protein for ET-3. These K_d values were in good agreement with those of membrane-bound ET receptors (32 pM for ET-1 and 27 pM for ET-3) (data not shown), demonstrating that the soluble receptors retained the characteristics of the membrane-bound receptors. Despite the similarity of the K_d values between the two ligands and the linearity of the Scatchard plots, the B_{max} values were different. This indicates the existence of at least two distinct types of the receptors in the soluble extracts. Furthermore, the observation that the concentration of ^{125}I-ET-1 and -3 binding sites was above 1 pmol/mg protein demonstrates that the rat lung is a rich source of both types of ET receptors.

Covalent Cross-linking of ^{125}I-ET-1 and -3 to the Solubilized Receptors with DST

To investigate the molecular mass of solubilized ET receptors, the solubilized extracts were affinity labeled by cross-linking with ^{125}I-ET-1 and -3 using DST. Affinity-labeled proteins were analyzed by SDS-PAGE and subsequent autoradiography. As shown in Fig. 4, one major and one minor band corresponding to 48 and 37 kDa, respectively, appeared when the membranes were prepared in the presence of 100 mM EDTA, a metal protease inhibitor (lanes A and E). The labeling of both bands was specifically inhibited by the presence of 100 nM unlabeled ET-1 and -3 during incubation of the extracts with ^{125}I-ET-1 and -3, respectively, demonstrating that the two bands represent specific ET receptors (lanes B and F). In contrast, when a 2 mM concentration of EDTA was used during membrane preparation, cross-linking resulted in different autoradiographic profiles for both radioligands (lanes C, D, G, and H). That is, the intensity of the 48 kDa band was reduced whereas that of the 37 kDa band was markedly increased (lanes C and G). These findings suggest that the 37 kDa band is a proteolytic degradation product of the 48 kDa protein.

Displacement of ^{125}I-ET-1 and -3 Binding by ET Isopeptides and Sarafotoxin S6b

The specificity of the solubilized receptors for ET isopeptides was further examined by competitive experiments. The solubilized extracts were incubated with 50 pM ^{125}I-ET-1 or -3 and increasing

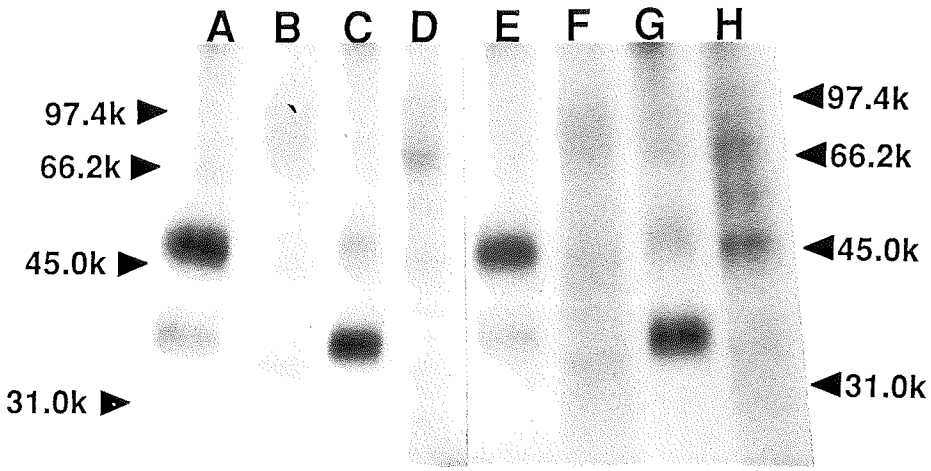

Fig. 4 Affinity-labeling of ET receptors with ^{125}I-ET-1 or -3. Solubilized ET receptors from the membranes prepared in the presence of 100 mM (lanes A, B, E, and F) or 2 mM (lanes C, D, G, and H) EDTA were cross-linked with ^{125}I-ET-1 (lanes A–D) or ^{125}I-ET-3 (lanes E–H), respectively, then separated by SDS-PAGE followed by autoradiography. To detect specific bands, 100 nM unlabeled ET-1 or -3 were added (lanes B, D, F, and H).

concentrations of unlabeled ET-1, -3, and sarafotoxin S6b. As shown in Fig. 5, the order of potency in inhibiting ^{125}I-ET-1 binding was ET-1=sarafotoxin S6b (IC_{50}=200 pM)>ET-3 (IC_{50}=3.2 nM). On the other hand, the potency of these unlabeled peptides in displacing the binding of ^{125}I-ET-3 was sarafotoxin S6b=ET-3 (IC_{50}=190 pM)≥ET-1 (IC_{50}=280 pM). Competition with ^{125}I-ET-1 and -3 using membrane fractions also revealed the same displacement order among these three unlabeled isopeptides (data not shown). These results demonstrate that at least two subtypes of the receptors are present: one has a high affinity for all three peptides while the other preferentially interacts with ET-1, than ET-3. This interpretation is in good agreement with the data from Scatchard-plot analyses.

DISCUSSION

In the present study, we solubilized ET receptors that retained binding activity for ET-1 and -3 from rat lung membranes using the detergent digitonin. CHAPS also solubilized the receptors in an active form but with a considerably lower solubilization efficiency. The solubilized receptors retained the following characteristics of the membrane-bound receptors. Firstly, the K_d values of ^{125}I-ET-1 and -3 binding were similar between the membrane-bound and digitonin-solubilized receptors. Secondly, the specificity of the solubilized receptors for ET isopeptides was almost identical with that of the membrane-bound receptors. Thirdly, the two bands of 48 and 37 kDa were specifically affinity-labeled as observed in membrane fractions (7). These findings showed the importance of characterizing the digitonin-solubilized ET receptors in order to understand the biochemical properties of physiologically active receptors on plasma membranes.

Scatchard-plot analyses and competitive binding studies suggested the existence of two ET receptor subtypes exhibiting different affinities for ET isopeptides, respectively, in the solubilized extracts. Scatchard-plot analyses revealed one class of high affinity binding sites for ^{125}I-ET-1 and -3, respectively, with similar K_d values. However, different B_{max} values (B_{max}=3.4 pmol/mg protein for ET-1, B_{max}=1.0 pmol/mg protein for ET-3) demonstrated the presence of two receptor types. These B_{max} values further indicated that the rat lung is a rich source of both types of the receptors. Competitive experiments also supported the existence of the receptor subtypes. The IC_{50} values of unlabeled ET-1 in blocking ^{125}I-ET-1 and -3 binding were almost the same (IC_{50}=200 pM for ^{125}I-ET-1, IC_{50}=280 pM for ^{125}I-ET-3), indicating that ET-1 binds to both types of the receptors with almost the same affinity (Fig. 5). In contrast, the IC_{50} values of unla-

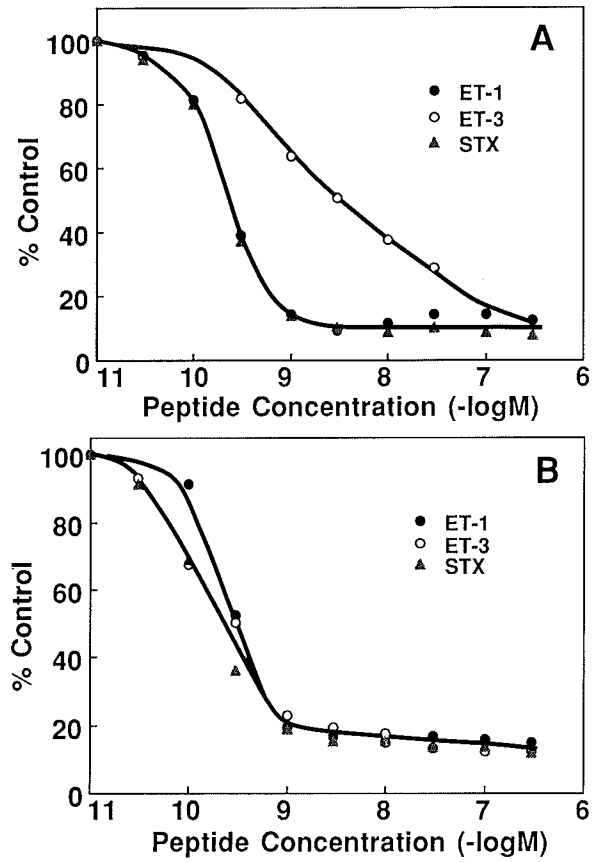

Fig. 5 Competitive displacement of ^{125}I-ET-1 or -3 binding to solubilized ET receptors. The solubilized extracts (100 μl) were incubated with 50 pM ^{125}I-ET-1 (A) or -3 (B) at 25°C in the presence of indicated concentrations of unlabeled ET-1 (●), -3 (○) or sarafotoxin S6b (▲). The data presented are from a representative experiment where each point is the average of duplicate determinations.

beled ET-3 in competing for ^{125}I-ET-1 and -3 binding were considerably different (IC_{50}=3.2 nM for ^{125}I-ET-1, IC_{50}=190 pM for ^{125}I-ET-3), suggesting that ET-3 preferentially interacts with one receptor type (Fig. 5). Together, these data lead to the conclusion that in the digitonin-solubilized extracts there are at least two distinct types of ET receptor; one type preferentially interacts with ET-1 rather than ET-3 whereas the other has virtually identical affinities for ET-1 and -3. Therefore, the former type corresponds to ET_AR and the latter to ET_BR (1, 6, 12).

We have identified an ET-3-specific receptor protein called ET_CR in chick cardiac membranes (17). Recently, Schvartz *et al.* have also suggested the existence of this type in the rat atria (13). However, the results reported here indicate that in the rat lung, ET_CR does not seem to be present, if at all, it is an extremely minor component.

In conclusion, the present study indicates that two different types of ET receptors, ET_AR and ET_BR, on rat lung membranes can be solubilized in an active form using digitonin and that they retain the characteristics of the membrane-bound receptors. Furthermore, the present data clearly demonstrates that the rat lung is an abundant source of ET_AR and ET_BR proteins as well as their mRNAs.

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