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Tissue and developmental variations in the heat shock response of sea lampreys (*Petromyzon marinus*): effects of an increase in acclimation temperature

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Abstract

Induction of the heat shock response (HSR) was investigated in liver, kidney and intestine tissues of *Petromyzon marinus* larvae (ammocoetes) acclimated to control temperatures of either 9 or 13°C. Labelling with [³⁵S]methionine detected the enhanced synthesis of heat shock proteins (HSPs) with molecular weights of 70 kDa (HSP70) and 90 kDa (HSP90) after an abrupt elevation of water temperature. In ammocoetes maintained at 9°C, a clear induction of HSP synthesis was detected in all tissues following an increase of 20°C, and a slight induction was detected in kidney and intestine after a 15°C increase. Induction of HSP synthesis in ammocoetes acclimated to 13°C was detected after a temperature increase of 12°C in liver and kidney, and an increase of 16°C in intestine. Thus, it appears that the threshold induction temperature decreased from 29 to 25°C in ammocoete liver following an increase in acclimation temperature from 9 to 13°C. This result would be unique to lampreys, since increases in acclimation temperatures usually result in increased threshold induction temperatures in aquatic poikilotherms. The types of HSPs synthesized by liver, kidney and intestine did not change over the lamprey life cycle, suggesting that this aspect of the lamprey HSR is not regulated post-embryonically. © 1999 Elsevier Science Inc. All rights reserved.

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1. Introduction

All organisms can protect themselves from the detrimental effects of increased temperature and other stressors by eliciting a response typically referred to as the heat shock response (HSR). This is a universal response characterized by the rapid synthesis of a set of highly conserved proteins known as heat shock proteins (HSPs) [23]. HSPs are commonly classified on the basis of molecular weight and members of the 70 kDa (HSP70) and 90 kDa (HSP90) families have been extensively studied. Constitutive HSPs are members of these

families that can accumulate to high levels in unstressed cells and are only mildly stress-inducible [29]. Conversely, other HSPs are inducible and their expression is enhanced greatly following stress.

HSP70 and HSP90 belong to the family of molecular chaperones that prevent protein aggregation and facilitate proper protein folding by binding to nascent or denatured proteins [8]. Experiments using amino acid analogues and substances that are known to denature proteins revealed that the HSR is induced as a result of the accumulation of aberrant or misfolded proteins (reviewed in [8]). Both nascent and denatured proteins act as 'substrates' for HSPs, and HSR induction occurs when 'substrate' levels are greater than the levels of constitutive HSPs available to bind to them [3]. In addition, HSPs may be involved in developmental pro-

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cesses. Constitutive HSP levels and the ability to induce the HSR are regulated developmentally in a variety of systems including plants, fungi, *Drosophila*, sea urchin, *Xenopus*, and mammals [10,11].

Many aspects of the HSR of aquatic poikilotherms are regulated in a tissue-specific manner including the spectrum of HSPs synthesized, the minimum temperature that induces HSP synthesis (i.e. the threshold induction temperature), the level or strength of induction, and constitutive HSP levels [33]. Recently, many studies have examined the effects of seasonal variations in temperature on the threshold induction temperatures and constitutive HSP levels of aquatic poikilotherms [4,7,14]. Many events during the lamprey life cycle are influenced by seasonal changes in water temperature including embryogenesis [26,31], larval somatic growth [15,36], metamorphosis [16,17], and spawning [1,9].

Previously, the HSR was characterized in the gill of two lamprey species [35]. The focus of the present study is to examine the effects of two different control temperatures (9 and 13°C) on the ability to induce the HSR in liver, kidney, and intestine tissues of larval *P. marinus*, and to see whether the HSR is regulated in these tissues at post-embryonic intervals of the life cycle.

2. Materials and methods

2.1. Animals

Ammocoetes (larvae) of various pre-metamorphic stages [lengths < 120 mm (88–119 mm); weights < 3.0 g (1.02–2.50 g)] of landlocked *Petromyzon marinus* were collected from Fish Creek, NY from September 8–10, 1993 and from Putnam Creek, NY on June 3, 1994 using an electrofishing apparatus. Immediately pre-metamorphic larvae [lengths > 130 mm (130–162 mm); weights > 3.5 g (3.71–6.51 g)] [16,41] were obtained from the Putnam Creek collection, while metamorphosing and immediately post-metamorphic lampreys were obtained from the sample of ammocoetes collected from Fish Creek, NY which metamorphosed that summer. The metamorphosing lampreys used in this study were determined to be at stage 5 of metamorphosis [39].

2.2. Control temperatures

All lampreys were maintained in tanks supplied with continuously flowing, dechlorinated, aerated tap water at control temperatures resembling the ambient water temperatures at the time the animals were collected: 9°C for ammocoetes from Fish Creek and immediately post-metamorphic animals; and 13°C for immediately pre-metamorphic larvae and metamorphosing lampreys. Ammocoetes from Putnam Creek were acclimated to 13°C water for at least 16 weeks prior to

experimentation. Tanks containing ammocoetes or immediately pre-metamorphic larvae were provided with a 10 cm-deep substrate of river sand, and were fed a solution of baker's yeast once a week. Immediately post-metamorphic and metamorphosing animals were not fed because metamorphosis is a non-trophic interval, and immediately post-metamorphic lampreys do not initiate feeding this soon after metamorphosis.

2.3. Determination of temperature tolerance

To determine the maximum temperature that the animals could tolerate, one or two lampreys were placed in water elevated to the lethal temperature previously determined for ammocoetes (31.4°C) [32]. Animals were inspected for particular characteristics after 1 h at the elevated temperature: evidence of respiratory activity (branchial pumping), physical activity (swimming), and the ability to recover within 2 h after return to the control temperature. The exposure temperature was considered to be too high if any of these characteristics were not observed. All lampreys tolerated a maximum temperature of 29°C.

2.4. Heat shock protocol

The heat shock protocol was carried out as previously described [35], with the following modifications. Groups of ammocoetes maintained at 9°C were heat shocked for 1 h at 14, 19, 24, or 29°C, while those acclimated to 13°C were heat shocked for 1 h at 17, 21, 25, or 29°C. Due to limitations in the number of immediately pre-metamorphic larvae, metamorphosing lampreys, and immediately post-metamorphic lampreys, animals of these groups were exposed to only one or two elevated temperatures: groups of immediately pre-metamorphic larvae and metamorphosing animals were heat shocked at 21 or 29°C, and immediately post-metamorphic lampreys were exposed to a temperature of 29°C. The selection of 21°C as a heat shock temperature was based on fact that the incidence of metamorphosis is higher in immediately pre-metamorphic larvae maintained at 21°C as opposed to those maintained at 13°C [41]. Control animals were maintained at the respective pretreatment temperatures (9 or 13°C), and this experiment was repeated at least once for each interval of the life cycle.

All lampreys (experimentals and controls) were anaesthetized in 0.05% tricaine methanesulfonate (MS-222, Syndel) immediately after heat shock, and their lengths and weights were measured. Animals were injected intraperitoneally with [³⁵S]methionine (DuPont, NEN) diluted in 0.6% saline to a concentration of 25 μ Ci g⁻¹ fresh weight (1 Ci = 37 GBq), and returned to an aquarium maintained at the appropriate control temperature (9 or 13°C) for a 2 h recovery period. All

lampreys were then re-anaesthetized, killed by decapitation, and various tissue samples were removed, weighed, frozen on dry ice, and stored at -70°C . The kidneys of metamorphosing and immediately post-metamorphic animals were separated into an anterior portion containing the regressing larval opisthonephric kidney and a posterior portion containing the developing adult opisthonephros [38].

2.5. Gel electrophoresis

Frozen liver, kidney, or intestine tissues collected from groups of lampreys following each temperature trial were prepared for electrophoresis in the same manner as previously described [35]. Samples containing equivalent amounts of radioactivity (80 000 cpm for ammocoetes, immediately pre-metamorphic larvae, and metamorphosing animals, and 20 000 cpm for immediately post-metamorphic animals) were loaded onto SDS-polyacrylamide gels (10%) and electrophoresed [22]. The amount of radioactivity loaded onto the gels varied between life cycle intervals (since some animals incorporated the $[^{35}\text{S}]$ methionine better than others), but not between lanes on a particular gel. Molecular weight standards (Sigma, St. Louis, MO) were also loaded. Gels were impregnated with EN³HANCE (DuPont, NEN) according to the manufacturer's instructions, dried, and fluorographed at -70°C with X-ray film (X-OMAT AR, Kodak). Fluorography times were 4 days for gels loaded with 80 000 cpm lane⁻¹, and 16 days for gels loaded with 20 000 cpm lane⁻¹. This procedure was subsequently repeated using the same samples.

3. Results

3.1. Ammocoetes

To determine whether an increase in acclimation temperature had an effect on the induction of the lamprey HSR, the minimum temperature required for an induction of HSP synthesis (i.e. the threshold induction temperature) was estimated by exposing groups of ammocoetes acclimated to two different control temperatures (9 and 13°C) to a series of temperature elevations. The effects of heat shock were examined in liver, kidney and intestine tissues to ascertain whether lampreys respond to heat shock in a tissue-specific manner. The patterns of $[^{35}\text{S}]$ methionine incorporation in vivo by groups of heat shocked *P. marinus* ammocoetes revealed the enhanced synthesis of two HSPs in liver (Fig. 1), kidney (Fig. 2) and intestine (Fig. 3) in response to increases in water temperature. The molecular weights of these proteins were ≈ 90 and 70 kDa as determined by comparison to molecular weight stan-

Fig. 1. HSP induction profile in liver of *P. marinus* ammocoetes maintained at two different control temperatures following injection with $[^{35}\text{S}]$ methionine. (A) Four ammocoetes were heat shocked at the temperatures indicated above each lane. Control animals (C) were maintained at 9°C , the ambient water temperature at the time of year that samples were taken. Molecular mass markers (kDa) are noted to the left of the fluorogram. The bands corresponding to HSP70 and HSP90 are indicated. (B) Same as A except the animals were acclimated to a control temperature of 13°C . In Fig. 1A, a marked induction of the HSR was detected at 29°C . In Fig. 1B, the synthesis of HSPs was detected at 25 and 29°C in ammocoetes acclimated to 13°C . Note that the HSP70 and HSP90 bands in Fig. 1B appear as part of a protein doublet. In this case, the darker, upper bands are the HSPs, and the faint lower bands indicate a protein whose synthesis is not enhanced after heat shock.

dards, and were classified as members of the HSP90 and HSP70 families, respectively.

The synthesis of HSP70 and HSP90 was greatly enhanced in the liver (Fig. 1A), kidney (Fig. 2A) and intestine (Fig. 3A) of ammocoetes that had been maintained at a control temperature of 9°C following a heat

Fig. 2. HSP induction profile in kidney of *P. marinus* ammocoetes maintained at two different control temperatures following injection with $[^{35}\text{S}]$ methionine. (A) Four ammocoetes were heat shocked at the temperatures indicated above each lane. Control animals (C) were maintained at 9°C , the ambient water temperature at the time of year that samples were taken. Molecular mass markers (kDa) are noted to the left of the fluorogram. The bands corresponding to HSP70 and HSP90 are indicated. (B) Same as A except animals were acclimated to a control temperature of 13°C . In Fig. 2A, a slight enhancement of HSP synthesis was detected at 24°C in ammocoetes maintained at 9°C , and a marked induction of the HSR was detected at 29°C . In Fig. 2B, the synthesis of HSPs was detected at 25 and 29°C in ammocoetes acclimated to 13°C . Note that the HSP70 and HSP90 bands in Fig. 2B appear as part of a protein doublet. In this case, the darker, upper bands are the HSPs, and the faint lower bands indicate a protein whose synthesis is not enhanced after heat shock.

Fig. 3. HSP induction profile in intestine of *P. marinus* ammocoetes maintained at two different control temperatures following injection with [35 S]methionine. (A) Four ammocoetes were heat shocked at the temperatures indicated above each lane. Control animals (C) were maintained at 9°C, the ambient water temperature at the time of year that the samples were taken. Molecular mass markers (kDa) are noted to the left of the fluorogram. The bands corresponding to HSP70 and HSP90 are indicated. (B) Same as A except animals were acclimated to a control temperature of 13°C. In Fig. 3A, a slight enhancement of HSP synthesis was detected in the 24°C lane in ammocoetes maintained at 9°C, and a marked induction of the HSR was detected at 29°C. In Fig. 3B, the synthesis of HSPs was not detected at 25°C in ammocoetes acclimated to 13°C. The HSP70 band is darker than the HSP90 band in the 29°C lanes of both fluorograms.

shock to 29°C, although a slight increase in HSP synthesis was observed in kidney (Fig. 2A) and intestine (Fig. 3A) when water temperatures were elevated to 24°C. A detectable enhancement of HSP70 and HSP90 synthesis was observed in the liver (Fig. 1B) and kidney (Fig. 2B), but not the intestine (Fig. 3B) of ammocoetes acclimated to a control temperature of 13°C after a heat shock to 25°C, but a substantial increase in HSP synthesis was apparent in all tissues when the water temperature was increased to 29°C. The HSP70 and HSP90 bands on the liver (Fig. 1B) and kidney (Fig. 2B) fluorograms appear to be part of a doublet. In these figures the darker, upper bands are the HSPs, and the faint lower bands indicate a protein whose synthesis is not enhanced after heat shock. In the 29°C lanes of the intestine (Fig. 3), the band corresponding to HSP70 was considerably darker than the HSP90 band, suggesting that HSP70 synthesis may be more strongly induced in this tissue relative to HSP90 synthesis in response to heat shock.

3.1.1. Pre-metamorphic, metamorphosing, and post-metamorphic life cycle intervals

The responses of *P. marinus* liver (Fig. 4), kidney (Fig. 5) and intestine (Fig. 6) to increasing water temperatures were examined at various post-embryonic intervals of the life cycle (Fig. 4A, Fig. 5A, Fig. 6A = immediately pre-metamorphic larvae; Fig. 4B, Fig. 5B and C, Fig. 6B = metamorphosing lampreys; Fig. 4C, Fig. 5D and E = immediately post-metamorphic lampreys). Threshold induction temperatures could not be estimated due to the limited number of animals, and the intestine of immediately post-metamorphic lampreys was not analyzed due to the poor incorporation of [35 S]methionine.

An enhanced synthesis of HSP70 and HSP90 was observed following a heat shock to 29°C at all intervals of the lamprey life cycle and in all tissues (Figs. 4–6). In the intestine of immediately pre-metamorphic larvae (Fig. 6A) the synthesis of HSP70 was enhanced to a greater degree than that of HSP90, but differences in the enhancement of HSP70 and HSP90 synthesis were not observed in the intestine of metamorphosing lampreys (Fig. 6B). Also, anterior and posterior regions of the kidney did not differ in their responses to heat shock (Fig. 5B–E).

4. Discussion

The present study showed that a strong induction of HSP70 and HSP90 synthesis occurred in liver, kidney, and intestine tissues following a 16–20°C increase in temperature in ammocoetes of *Petromyzon marinus* maintained at a control temperature of 9°C. Further, some HSP synthesis was detected in kidney and intestine after the temperature was elevated to 24°C, suggesting that the HSR may have been induced by an increase in water temperature of 11–15°C. The liver data are consistent with the increase of 16–20°C required to induce the HSR in the gill of brook lamprey (*Lampetra appendix*) maintained at 9°C [35]. The results of these two studies demonstrate that lampreys require some of the largest temperature increases for induction of the HSR as compared to other aquatic poikilotherms ([18], see [35] for discussion).

The liver of *P. marinus* ammocoetes appeared to respond to an increase in the original control temperature (9–13°C) by decreasing its threshold induction

temperature. An induction of HSP synthesis was clearly apparent in the liver (Fig. 1A) when ammocoetes maintained at a control temperature of 9°C were exposed to a water temperature of 29°C. However, when the response of the liver (Fig. 1B) of ammocoetes acclimated to a control temperature of 13°C was examined, a heat shock to 25°C resulted in an obvious induction of HSP synthesis that was not observed at 21°C. By contrast, past studies on the Medaka (*Oryzias latipes*) [28] and goby fishes (*Gillichthys mirabilis* and *G. seta*) [4] reported that increases in acclimation or acclimatization temperatures resulted in increased threshold induction temperatures. However, an *in vitro* study using channel catfish (*Ictalurus punctatus*) hepatocytes demonstrated that the HSR is induced at the same temperature regardless of the original acclimation temperature [19]. Thus, the decrease in threshold induction temperature from 29 to 25°C that was demonstrated by the liver of *P. marinus* ammocoetes following an increase in the original control temperature from 9 to 13°C, respectively, would be a feature of the HSR which is unique to lampreys. However, additional experiments are required which would employ a greater number of heat shock temperatures and would assess HSP levels quantitatively.

The liver and kidney of *P. marinus* ammocoetes acclimated to a control temperature of 13°C appeared to be more sensitive to heat treatment than other tissues, such as the intestine and gill [35]. Induction of the HSR was detected in liver and kidney when ammocoetes were heat shocked to 25°C, but the gill [35] and intestine required an increase in temperature to 29°C to elicit HSP synthesis. A study conducted on four species of teleost fishes that were acclimatized to 10°C reported that threshold induction temperatures varied little between tissues of a particular species [5], but tissue-specificity for this parameter was reported for the fathead

minnow (*Pimephales promelas*) [6] and the killifish (*Fundulus heteroclitus*) [20].

The location of a particular tissue within the lamprey may have an effect on its HSR, for this has been an explanation for the different spectra of HSP70s synthesized in response to heat shock in gill and liver of *F. heteroclitus* [20]; the same may apply for threshold induction temperatures. Gills are directly exposed to the water and have a large surface area for gas exchange, and the ammocoete intestine is one of the first sites for exposure and accumulation of contaminants ingested during feeding due to its absorptive function [2,37]. The phenomenon known as acquired or induced thermotolerance [30] may account for the observed differences in threshold induction temperatures between liver and kidney versus intestine and gill [35] of *P. marinus* ammocoetes maintained at 13°C. That is, because of their constant exposure to contaminants and temperature fluctuations, cells of the intestine and gill increase their resistance to the effects of a subsequent extreme stress by increasing the expression of constitutive HSPs.

Induction of the HSR occurs in most organisms within a temperature range characteristic of their natural environments [24,33]. In cases where the threshold induction temperature lies outside of the range of temperatures commonly experienced in an organism's habitat, the HSR acts as an emergency response and is mandatory to ensure survival at these elevated temperatures [34]. The lamprey HSR was induced at water temperatures (25–29°C) not normally experienced by these animals in their natural environment [27]. It appears that during periods characterized by cooler water temperatures (i.e. the 9°C control animals), *P. marinus* ammocoetes are able to cope with naturally occurring increases in water temperature without mounting a HSR. Since this response was also observed in the gill

Fig. 4. HSP induction profile in liver of *P. marinus* at different life cycle intervals following injection with [35 S]methionine. (A) Two immediately pre-metamorphic larvae (PRM) were heat shocked at the temperatures indicated above each lane. Control animals (C) were maintained at 13°C, the ambient water temperature at the time of year that samples were taken. Molecular mass markers (kDa) are noted to the left of the fluorogram. The bands corresponding to HSP70 and HSP90 are indicated. (B) Same as A except data are for metamorphosing lampreys (META). (C) Same as A except data are for immediately post-metamorphic lampreys (PTM), and control animals were maintained at 9°C, which was the ambient water temperature during this life cycle interval. The enhanced synthesis of HSP70 and HSP90 was observed at 29°C for all intervals of the life cycle.

Fig. 5. HSP induction profile in kidney of *P. marinus* at different life cycle intervals following injection with [35 S]methionine. (A) Two immediately pre-metamorphic larvae (PRM) were heat shocked at the temperatures indicated above each lane. Control animals (C) were maintained at 13°C, the ambient temperature at the time of year that samples were taken. Molecular mass markers (kDa) are noted to the left of the fluorogram. The bands corresponding to HSP70 and HSP90 are indicated. (B) Same as A except data are for the anterior (regressing) portion of the kidney from metamorphosing lampreys (META-ANT). (C) Same as A except data are for the posterior (developing) portion of the kidney from metamorphosing lampreys (META-POST). (D) Same as B except data are for immediately post-metamorphic lampreys (PTM-ANT), and control animals were maintained at 9°C, the ambient water temperature during this life cycle interval. (E) Same as D except data are for the posterior (developing) portion of the kidney from immediately post-metamorphic lampreys (PTM-POST). The induction of HSP70 and HSP90 was observed in all 29°C lanes, and anterior (B and D) and posterior (C and E) sections of the kidney did not respond differently to heat treatment.

of another lamprey species, *L. appendix* [35], the HSR does not seem to be involved in the adaptation of ammocoetes to changes in water temperature until temperatures are elevated to such a degree that their survival is threatened.

The liver, kidney and intestine of lampreys responded to heat shock by eliciting the synthesis of only HSP70 and HSP90. This response is in contrast to the results of studies on other aquatic poikilotherms. For example, the in vivo heat shock of fathead minnows (*P. promelas*) resulted in the synthesis of six different HSPs in gill, four in striated muscle, and three in brain [6], while five different HSPs were synthesized by catfish (*I. punctatus*) hepatocytes [19]. Given that other fish synthesize a variety of HSPs in response to heat shock, it is possible that the bands corresponding to HSP70 and HSP90 on the lamprey fluorograms are composed of two or more proteins of different molecular weights that cannot be resolved using one dimensional electrophoresis.

Developmental regulation of the HSR during embryogenesis has been well documented in a number of organisms [12]; however, there are few studies that have dealt with the topic of HSR regulation during metamorphosis. Heat shock can induce metamorphosis in

the tunicate *Ciona intestinalis* [21], and HSP27 may be involved in liver development during metamorphosis of the bullfrog (*Rana catesbeiana*) [13]. The enhanced synthesis of HSP70 and HSP90 was observed at all intervals of the lamprey life cycle following heat shock, but no change in the types of HSPs synthesized was detected. However, the small HSPs (i.e. HSP27s) are commonly subject to developmental regulation [10,11], and the expression of these HSPs should be examined throughout the lamprey life cycle.

The synthesis of HSP70 appeared to exceed the synthesis of HSP90 in the intestine of ammocoetes and immediately pre-metamorphic larvae, but not in metamorphosing animals. This result suggests that HSP70 may be more important than HSP90 for repairing heat-induced damage to the intestine during the ammocoete and immediately pre-metamorphic intervals of the life cycle. That these differences in HSP70 and HSP90 synthesis patterns are not observed in metamorphosing lampreys may be related to the additional stress of tissue rearrangements and remodelling that occur during this interval of the life cycle [40]. Differences in the strength of HSP induction have also been observed in carp (*Cyprinus carpio*), where the strength of induction of HSP70 exceeded that of HSP60 [25]. However, dif-

Fig. 6. HSP induction profile in intestine of *P. marinus* at different life cycle intervals following injection with [35 S]methionine. (A) Two immediately pre-metamorphic larvae (PRM) were heat shocked at the temperatures indicated above each lane. Control animals (C) were maintained at 13°C, the ambient temperature at the time of year that samples were taken. Molecular mass markers (kDa) are noted to the left of the fluorogram. The bands corresponding to HSP70 and HSP90 are indicated. (B) Same as A except data are for metamorphosing lampreys (META). The intestine of immediately post-metamorphic lampreys was not analyzed due to the poor incorporation of [35 S]methionine by intestine at this interval of the life cycle. The HSP70 band was darker than the HSP90 band in the 29°C lane of immediately pre-metamorphic larvae (A), but not in metamorphosing lampreys (B).

ferences in the magnitude or strength of HSP induction as detected by radiolabelling do not necessarily result in differences in HSP protein levels [20,35].

In summary, the results of the present study indicate that the threshold induction temperature of the liver of *P. marinus* ammocoetes may be sensitive to differences in the original acclimation (control) water temperature. A decreased threshold induction temperature (from 29 to 25°C) in the liver of *P. marinus* ammocoetes in response to an increased control temperature (from 9 to 13°C) would be a feature of the HSR that is unique to lampreys among aquatic poikilotherms; however, further research is needed to confirm this suggestion. Lampreys responded to heat shock in a tissue-specific manner, but the HSR was not developmentally regulated at post-embryonic intervals of the life cycle. Finally, the synthesis of HSP70 exceeded that of HSP90 in the intestine of ammocoetes and immediately pre-metamorphic larvae, suggesting that HSP70 may be more important in dealing with heat-induced damage in this tissue at these intervals of the life cycle.

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