HYPOGLYCEMIA AND EMBRYONIC HEART DEVELOPMENT

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1. ABSTRACT

Abnormal embryonic development is a complication of the diabetic pregnancy, and heart defects are among the most common and detrimental congenital malformations of the diabetic embryopathy. Hypoglycemia is a common side effect of diabetes therapy and is a potential teratogen. An association between hypoglycemia and congenital defects has been difficult to demonstrate in humans, but in vivo and in vitro animal studies have illustrated the importance of glucose as a substrate for normal development. Hypoglycemia alters embryonic heart morphology, producing abnormal looping and chamber expansion, decreased myocardial thickness, disorganized layers, and decreased overall size. Hypoglycemia decreases embryonic heart rate and vascularity, and it alters embryonic heart metabolism by increasing glucose uptake and glycolysis. Hypoglycemia also affects protein expression in the embryonic heart, increasing the expression of glucose regulated proteins, hexokinase, and glucose transport protein. Thus, hypoglycemia interferes with normal cardiogenesis and alters morphology, function, metabolism, and expression of certain proteins in the developing heart. It is likely that these factors contribute to heart defects observed in the diabetic embryopathy, but the definitive link has yet to be made. Future studies are expected to further elucidate mechanisms mediating hypoglycemia-induced cardiac dysmorphogenesis.

2. INTRODUCTION

Hypoglycemia is only one of many candidate teratogenic factors potentially responsible for cardiac defects observed in the diabetic embryopathy. However, the importance of glucose as a metabolic substrate in the organogenesis-stage embryo and embryonic heart makes hypoglycemia a potent disrupter of normal development during this critical stage. Experimental evidence has now accumulated, mainly through in vitro animal studies, that hypoglycemia alters the morphology, function, and metabolism of the organogenesis-stage embryonic heart.

This review summarizes historical information regarding the diabetic embryopathy, pathogenesis of hypoglycemia in diabetes, and early animal studies pointing to the potential teratogenicity of hypoglycemia. It also reviews more recent information regarding morphologic,
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function, and metabolic alterations resulting from hypoglycemia, specifically in the embryonic heart. Despite the recent progress in this field, there is still much to understand about the potential role of hypoglycemia, as well as its underlying mechanisms, in cardiac dysmorphogenesis.

3. THE DIABETIC EMBRYOPATHY

Abnormal development of the human embryo and fetus has long been recognized as a complication of the diabetic pregnancy. Improved management of the pregnant insulin-dependent diabetic patient during the past several decades has reduced the incidence of spontaneous abortions and neonatal mortality (1,2), but the frequency of congenital anomalies in the infants of diabetics remains three to four times higher than in the general population (3-5). In fact, malformations have become the leading cause of perinatal deaths in the offspring of diabetic patients (6,7).

The diabetic embryopathy is characterized by a wide variety of congenital defects that affect many organ systems and may occur singly or in combination. Abnormalities described in the offspring of diabetic patients most commonly involve developmental defects of the cardiovascular system, central nervous system, as well as gastrointestinal and urogenital systems and generalized growth disturbances (8,9). Recent attempts to evaluate patterns of birth defects associated with the diabetic embryopathy have demonstrated that major cardiovascular system defects (10), and a combination of cardiovascular and vertebral malformations (11) are most predictive of the disease. The types of defects produced in the diabetic embryopathy are indicative of teratogenic insult during the period of organogenesis and before the seventh week of human gestation (12).

Cardiovascular defects described in the offspring of diabetic patients include transposition of the great vessels, ventricular and atrial septal defects, coarctation of the aorta, tetralogy of Fallot, double outlet right ventricle, and patent ductus arteriosus (3,5,13-16). The wide spectrum of defects, as well as the multiple organ systems affected, most likely represent the manifestation of multiple etiologic factors.

4. HYPOGLYCEMIA AND DIABETES

Hypoglycemia has long been recognized as a common side effect of exogenous insulin therapy in diabetics (17), but this condition has become more common because of recent clinical practices. For example, the current approach to the management of the pregnant insulin-dependent diabetic patient favors strict glycemic control instituted before conception and maintained throughout pregnancy in an attempt to reduce the incidence of malformations and perinatal mortality (18-21). Strict control is often attained with intensive insulin therapy, using multiple daily insulin injections or continuous subcutaneous insulin infusion (22). Such intensified treatment programs place the insulin-dependent diabetic patient at increased risk of severe and prolonged hypoglycemia (23-25). Constant insulin therapy maintains blood glucose at lower levels than conventional therapy, making the patient more susceptible to hypoglycemia from changes in eating or exercise schedules (26).

Strict glycemic control in diabetic patients has also been found to lower the threshold for glucose counter-regulatory responses, resulting in delayed and prolonged recovery from hypoglycemic episodes (27-30). Glucagon released from pancreatic alpha cells is typically diminished by an unknown mechanism early in the course of diabetes (27,31). Other counter-regulatory hormones, such as epinephrine, growth hormone, and cortisol, are thus activated to correct insulin-induced hypoglycemia (32-33). Of these, epinephrine plays a major role in insulin-dependent diabetic glucose counter-regulation (33-35) and the threshold for its release is lowered in the well-controlled diabetic (36). The delayed release of epinephrine is associated with a decrease in autonomic sensations, and thus a blunted awareness of hypoglycemia, especially in the patient under strict glycemic control (36,37).

In addition to its importance as a side effect of diabetes mellitus therapy, hypoglycemia is also a result of other disease processes. These include starvation, pancreatic tumors, and alcoholism, among others (38).

5. TERATOGENICITY OF HYPOGLYCEMIA

Many factors are likely involved in the diabetic embryopathy (39), but alterations in glucose levels represent an important and predictable metabolic derangement of this disease.

Compared to other metabolic alterations of diabetes, hypoglycemia has only recently received serious attention as a potential teratogenic factor. Early reports suggested an association between hypoglycemic coma induced by insulin shock therapy and fetal malformations and mortality (40-42), but the number of cases reported was small. In one study of nineteen pregnant women receiving insulin shock therapy, six cases of “fetal damage” resulted, including four deaths and two malformed infants. In five of these six, treatment was begun before ten weeks of gestation. No fetal damage resulted when treatment was started after thirteen weeks of gestation (42). Considering that insulin does not cross the human placenta (43), it is tempting to speculate that hypoglycemia played a significant role in these cases of embryopathy.

In general, an association between hypoglycemia and congenital malformations has been difficult to demonstrate in human studies. Glucose tolerance tests and estriol excretion measurements during pregnancy demonstrated a weak association between hypoglycemia and developmental anomalies, including growth retardation, perinatal deaths, and malformations (44). Two reports have suggested an association between maternal hypoglycemia and growth retardation, but hypoglycemia was diagnosed during the third trimester of pregnancy, after
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the period when malformations are typically induced (45,46).

Several investigators have disputed the teratogenic effect of diabetic hypoglycemia. In one study, only eight women reported first-trimester insulin reactions out of a total of fifty-five diabetic women with malformed infants (47). In another study, diabetic women who produced normal infants reported hypoglycemia four times more frequently than diabetic women with malformed infants (14). However, these arguments were based on retrospective data that relied on patients’ memory of hypoglycemic episodes occurring several months earlier. It is now known that episodes of hypoglycemia frequently occur nocturnally (25,48) and therefore often go unnoticed. Thus, an association between hypoglycemia and diabetes-induced embryopathy has been difficult to support or refute in human studies.

5.1. In Vivo Animal Studies

In vivo studies in laboratory animals have demonstrated a variety of malformations in the offspring of mothers made hypoglycemic by fasting or treatment with insulin or tolbutamide. Skeletal malformations and exencephaly were observed in offspring after maternal insulin injection in rats (49-55) during early gestation. However, maternal blood glucose levels were not measured in these early studies, and it can only be supposed that malformations resulted from insulin-induced maternal hypoglycemia. An argument for direct action of insulin on the embryo was proposed (56) before it was known that the placenta is impermeable to insulin during organogenesis (57). In a subsequent study, pregnant rats were fasted and concurrently injected with insulin on gestational days (gd) 7-11, and maternal glucose was monitored. Glucose levels were lower with this dual treatment than with either fasting or insulin alone, and these were correlated with increased defects, especially on gd 9 and 11 (58). Similarly, fasting of pregnant mice for 24-30 hours between gd 7 and 10 produced defects, with the highest incidence on gd 9 (59). A disadvantage of these in vivo studies is the inability to adequately quantify or control the duration and level of embryonic exposure to hypoglycemia. In addition, the in vivo approach does not allow the evaluation of individual factors for their role in the pathogenesis of malformations.

5.2. In vitro Animal Studies

The in vitro method of whole-embryo culture (60) has made it possible to determine the direct effect of hypoglycemia on embryos at specific stages of development. In an early study, gd 10 rat embryos undergoing organogenesis were cultured for 24 hours in extensively-dialyzed rat serum to assess their requirement for various nutrients (61). It was found that nutrient-free medium supported minimal growth and that addition of glucose alone to the medium, to achieve a level of 150 mg/dl glucose, restored growth and differentiation to control levels (61). Rat embryos cultured on gd 9.5 for 45 hours in hypoglycemic serum (78 mg/dl glucose) from fasted rats were growth retarded compared to embryos grown in the same medium to which glucose was added to achieve a level of 120 mg/dl glucose (62). Severity of defects was correlated with degree of hypoglycemia in fasted rat serum used to culture rat embryos for 48 hours in vitro (63). Serum from insulin-injected rats was used to produce hypoglycemic medium for the culture of neurulating mouse (64) and rat embryos (65). Defects were produced in mouse embryos exposed for 28 hours to severe hypoglycemia (40 or 60 mg/dl glucose) and were prevented by glucose supplementation to control levels (64). Rat embryos exposed to hypoglycemia in vitro for 48 hours at the head-fold stage were mainly affected during the first 24 hours in culture (65). Rat embryos exposed in vitro to as little as 1 hour of hypoglycemia on gd 10 demonstrated growth retardation and increased malformation rates compared to controls (66). Exposure of mouse embryos to 4 hours of hypoglycemia on gd 7.5 resulted in an increased rate of malformations and decreased overall embryonic growth (67). Embryos exposed on gd 8.5 demonstrated a dose- and time-dependent response to hypoglycemia when evaluated for malformation rate, protein content, and somite number (Figure 1) (67). Interestingly, concurrent hypothermia (32°C or 35°C) partially protected these embryos against the dysmorphic effects of short-term hypoglycemia. The impact of short-term hypoglycemia was more severe in embryos exposed on gd 7.5 compared to gd 8.5 (67). Therefore, even brief hypoglycemia appears to interfere with normal development if the exposure occurs during the critical period of organogenesis.

6. HYPOGLYCEMIA AND THE DEVELOPING HEART

6.1. Importance of Glucose to the Embryo and Embryonic Heart

Glucose is used rapidly by the embryo during the period of organogenesis (68), and the vast majority of this substrate is metabolized by glycolysis for the production of ATP (68-70). The embryonic heart demonstrates a similar dependence on glucose and glycolytic metabolism. Glucose uptake into isolated fetal rat hearts was highest at the earliest stage tested (gd 15) and declined with decreasing age to gd 22 (71). Glucose was necessary to maintain maximal heart rate under anaerobic conditions in gd 11 rat embryos, followed by a shift at gd 13 to include extra-glycolytic energy sources and aerobic metabolism (72). Similarly, inhibition of glycolysis by iodoacetate depressed heart rate in gd 11-12 rat embryos, whereas the Krebs cycle inhibitor, malonate, and the oxidative phosphorylation uncoupler, 2,4-dinitrophenol, selectively depressed contractile rate in older hearts (73). Glucose is also important in the embryonic heart for the synthesis of cell-surface glycoconjugates involved in developmental regulation of intercellular interactions (74). Thus, an adequate supply of glucose to the embryonic heart during organogenesis is critical to normal development of cardiac morphology, function, and metabolism.

6.2. Hypoglycemia alters Morphology of the Embryonic Heart

Several studies have examined the effects of hypoglycemia on embryonic development, and many of these reported cardiac malformations after hypoglycemic exposure in vivo (75) or in vitro (64-67), even after brief
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A recent study focused specifically on cardiac defects resulting from in vitro exposure to hypoglycemia in the embryonic mouse (76). Morphological abnormalities of the heart and pericardium occurred in a dose-dependent manner in mouse embryos exposed in vitro for 6 hours to 80 mg/dl glucose on gd 9.5 and 40 mg/dl glucose on gd 10.5 (Figure 2). These abnormalities included pericardial edema and incomplete looping and expansion of the chambers of the heart (Figure 3). Histologic evaluation demonstrated decreased thickness of the myocardium, disorganization of cell layers, and the presence of pyknotic nuclei in hypoglycemia-treated hearts (Figure 4). These results suggest that hypoglycemia induces cell death in the developing myocardium. Morphometric analysis demonstrated decreased luminal volume in hearts exposed to hypoglycemia for 6 hours on gd 9.5. Total protein content was decreased in gd 9.5 hearts exposed to severe (20 mg/dl glucose) hypoglycemia for 6 hours in vitro (Figure 5).

6.3. Hypoglycemia alters Function of the Embryonic Heart

In addition to morphological defects, alterations in contractile rate of the embryonic heart and vascular perfusion of the embryo and visceral yolk sac are produced by hypoglycemic exposure in vitro. Heart rate was decreased after as little as 2 hours of hypoglycemia in gd 9, 9.5, and 10.5 mouse embryos in vitro (76). Glucose levels of 40 mg/dl consistently reduced heart rate in gd 9 and 9.5 embryos exposed for 2 hours, whereas heart rate was decreased in gd 10.5 embryos exposed to 20 mg/dl glucose for 2 or 4 hours, or 40 mg/dl for 6 hours (Figure 6). Visible pooling of blood and changes in visceral yolk sac vascularity were observed in gd 9.5 embryos exposed to hypoglycemia for 6 hours in vitro (Figure 7). Alterations in vascular perfusion are likely secondary to hypoglycemia-mediated decrease in heart rate.

6.4. Hypoglycemia alters Metabolism of the Embryonic Heart

The embryonic heart has been examined for changes in glucose metabolism in response to hypoglycemic exposure (76). Glucose uptake, as measured using the glucose analog, $^3$H-2-deoxy-D-glucose, was increased in gd 9.5 hearts exposed to 40 mg/dl glucose, and in gd 10.5 hearts exposed to 80 mg/dl glucose, for 6 hours in vitro (Figure 8).

Glycolytic metabolism, as measured by the conversion of $^{14}$C-glucose to $^{14}$C-lactate, was increased in gd 9.5 and gd 10.5 hearts exposed for 6 hours to 40 mg/dl glucose in vitro (Figure 9) (76). A similar increase in cardiac glycolysis was seen in mouse hearts exposed to 60 mg/dl glucose for 4 hours in vitro on gd 9.5 (77). ATP level was increased only in hearts from gd 9.5 embryos exposed for 6 hours to 20 mg/dl glucose in vitro (76).

Figure 1. Response of gd 8.5 mouse embryos to 2, 4, 6, and 24 hours of hypoglycemia (20, 40, 60, 80 mg/dl glucose) in vitro. Control value in each graph is represented by horizontal line. * different from controls at $p<0.05$. a. Malformation rate, b. Embryonic protein content, c. Increased somite number
6.5. Hypoglycemia alters Protein Expression in the Embryonic Heart

6.5.1. Glucose regulated proteins

Glucose regulated proteins (GRPs) are members of the stress protein family and are induced in several cell types in response to hypoglycemic stress (78). GRPs are also constitutively expressed and function as molecular chaperones within the endoplasmic reticulum. The two most prevalent GRPs, GRP78 and GRP94, demonstrate strong expression in the embryonic heart during organogenesis (79,80).

GRP78 and GRP94 have also been evaluated in the embryonic heart after hypoglycemic exposure (80,81). GRP78 levels increased in hearts from gd 9.5 mouse embryos exposed in vitro to increasing durations (2, 6, and 24 hours) of 40 mg/dl glucose or 6 and 24 hours of 80 mg/dl glucose when examined after 24 hours (Figure 10) (81). Hearts evaluated for GRP78 immediately after 2, 6, or 12 hours of hypoglycemic exposure (40 mg/dl glucose) did not have elevated GRP78 levels (Figure 11), suggesting that a >12 hour lag period after hypoglycemic stress is required for GRP expression to be manifested in the embryonic heart (80).

GRP94 levels increased in the embryonic heart only after 24 hours of exposure to 40 mg/dl or 80 mg/dl glucose in vitro (Figure 12) (81).

6.5.2. Hexokinase

Hexokinase (HK) catalyzes the first step in glucose metabolism, in which glucose is converted to glucose-6-phosphate. Of the four HK isoforms, HK-I is ubiquitous in adult tissues and is the predominant isoform in embryonic and fetal tissues (82). Tissues dependent on glucose for energy typically demonstrate high HK-I activity, as seen in transformed cells that are glycolytically active (83).

Immunohistochemical analysis demonstrated HK-I protein expression in the normal gd 9.5 mouse embryo that was strongly localized to the myocardium of the developing heart (84). Immunoblots of heart homogenates demonstrated stronger HK-I protein expression in the embryonic heart on gd 13.5 than on gd 9.5, whereas HK-I mRNA expression was approximately threefold higher, and HK-I activity was approximately twofold higher, in gd 9.5 hearts than in gd 13.5 hearts (84). The mismatch in HK-I protein and mRNA expression between early and late organogenesis suggests a complex developmental regulation of HK-I expression and activity in the embryonic heart.

HK-I expression and activity were examined in embryonic mouse hearts following hypoglycemic exposure in vitro (85). HK-I protein levels in the embryonic heart were increased by hypoglycemia (40 mg/dl glucose), but only after a brief (2 hour) exposure; there was no difference in HK-I protein levels in hearts exposed to hypoglycemia and control medium for 6 hours (Figure 13). Similarly, HK-I enzymatic activity was increased after a 2
hour exposure to hypoglycemia in vitro, but there was no difference between treated and control hearts after 12 hours (Figure 14). These results suggest that hypoglycemia induces HK-I expression and activity in the embryonic heart as an acute compensatory response that does not persist beyond a few hours duration.

6.5.3. Glucose transport protein

The transport of glucose across the plasma membrane is rate-limiting for subsequent intracellular glucose utilization. Glucose is not freely permeable across the lipid bilayer but enters the cell by facilitated diffusion. This process is mediated by a family of transmembrane glucose transport (Glut) proteins, of which several isoforms have been identified (86). Glut-1 is the only isoform consistently identified within the embryonic heart during the early postimplantation period (87-89), and it presumably plays a critical role in delivering this important substrate to embryonic heart cells.

Glut-1 protein levels were compared by immunohistochemistry and immunoblot analysis in the embryonic heart throughout organogenesis (gd 9.5 to 13.5). Glut-1 protein levels in the embryonic heart were found to be highest on gd 9-10 and lowest on gd 13.5, and cardiac Glut-1 mRNA levels similarly declined between gd 9.5 and 13.5 (90). Thus, the stage of highest Glut-1 protein expression in the normal embryonic mouse heart corresponds to the period of greatest dependence on glucose as a metabolic substrate.

The Glut inhibitor, cytochalasin B, produced a dose-dependent decrease in glucose uptake in isolated hearts exposed to hypoglycemia for 30 minutes or 6 hours, implicating a role for Glut in cardiac glucose uptake. Levels of Glut-1 protein in the gd 9.5 heart were unchanged after 2 or 6 hours of hypoglycemia but increased after 12 and 24 hours of hypoglycemia (40 mg/dl glucose) in vitro (Figure 15) (90). Glut-1 mRNA expression in the embryonic heart was unchanged after exposure to 40 mg/dl glucose for 24 hours. These results suggest that post-transcriptional regulation may determine the level and activity of Glut-1 protein in the embryonic heart in response to hypoglycemia.

7. PERSPECTIVE

The metabolic complexity of diabetes mellitus makes it difficult to isolate factors responsible for undesirable sequelae of this disease, including the diabetic embryopathy. Serum glucose concentrations that are significantly outside of normal ranges are the most characteristic metabolic derangements of diabetes and likely contribute to congenital defects, which occur at increased rates in the offspring of diabetics. Heart defects comprise a large percentage of diabetes-induced congenital malformations and contribute significantly to perinatal mortality in these patients. The developing heart is dependent on glucose as a metabolic substrate during early organogenesis, and it is during this stage that hypoglycemia likely has its most significant effect on cardiogenesis.

This manuscript has reviewed historical information characterizing the diabetic embryopathy and the pathogenesis of hypoglycemia in diabetes. Early evidence for the teratogenicity of hypoglycemia in humans has been described, along with animal studies that more definitively support the role of hypoglycemia in abnormal development.

Consideration of the developing heart as a target of hypoglycemia is a relatively recent focus of research in this area. In vitro studies using the rodent embryo model have demonstrated that hypoglycemia alters heart morphogenesis, both at gross and histologic levels. In addition, heart rate and vascular perfusion of the embryo are decreased by hypoglycemia, probably due to insufficient energy for myocardial contractile function. Increased glucose uptake and glycolysis in the embryonic heart presumably represent compensatory
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Figure 6. Heart rate in gd 9, 9.5, and 10.5 mouse embryos exposed in vitro to hypoglycemia (20, 40, 80 mg/dl glucose) or control medium (150 mg/dl glucose). * different from controls at p<0.05. a. 2 hr, b. 4 hr, c. 6 hr

Figure 7. Mouse embryos exposed on gd 9.5 to control medium (right) or hypoglycemia (left) for 6 hours in vitro. Note indistinct yolk sac vessels (arrowhead) and pooling of blood within the embryo (arrow) after hypoglycemic exposure. EC = ectoplacental cone; vv = yolk sac vessels.

Figure 8. Uptake of 2-DG in gd 9.5 and gd 10.5 mouse embryos exposed in vitro to hypoglycemia (20, 40, 80 mg/dl glucose) or control medium (150 mg/dl glucose) * different from controls at p<0.05.

Figure 9. Glycolytic metabolism in gd 9.5 and gd 10.5 mouse embryos exposed in vitro to hypoglycemia (20, 40, 80 mg/dl glucose) or control medium (150 mg/dl glucose) * different from controls at p<0.05.
Figure 10. GRP78 protein levels in hearts of gd 9.5 mouse embryos exposed in vitro to hypoglycemia at 40 mg/dl glucose (left) or 80 mg/dl glucose (right) for 2, 6, or 24 hours compared to controls (C). All hearts were evaluated at 24 hours. * different letters are significantly different at p<0.05.

Figure 11. GRP78 protein levels in hearts of mouse embryos exposed in vitro to hypoglycemia (40 mg/dl glucose) or control medium (150 mg/dl glucose) for 2, 6, 12, or 24 hours. Hearts were evaluated immediately after hypoglycemic exposure. * different from controls at p<0.05.

Figure 12. GRP94 protein levels in hearts of gd 9.5 mouse embryos exposed in vitro to hypoglycemia at 40 mg/dl glucose (left) or 80 mg/dl glucose (right) for 2, 6, or 24 hours compared to controls (C). All hearts were evaluated at 24 hours. *different letters are significantly different at p<0.05.

Figure 13. HK-I protein levels in hearts of mouse embryos exposed in vitro to hypoglycemia (40 mg/dl glucose) or control medium (150 mg/dl glucose) for 2, 6, 12, or 14 hours.* different from controls at p<0.05.
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Figure 14. Hexokinase activity in hearts of mouse embryos exposed in vitro to hypoglycemia (40 mg/dl glucose) or control medium (150 mg/dl glucose) for 2 or 12 hours. * different from controls at p<0.05.

Figure 15. Glut-1 protein levels in hearts of mouse embryos exposed in vitro to hypoglycemia (40 mg/dl glucose) or control medium (150 mg/dl glucose) for 2, 6, 12, or 14 hours. * different from controls at p<0.05.

metabolic alterations in response to insufficient glucose substrate. Evaluations of altered expression patterns in the embryonic heart in response to hypoglycemia have only just begun. Results thus far suggest that proteins involved in response to hypoglycemic stress (the GRPs) and those involved in compensatory metabolic responses (HK-I, Glut-1) are increased by hypoglycemia. It is expected that future research will uncover a myriad of alterations in protein and gene expression that will help elucidate the mechanisms involved in hypoglycemia-mediated cardiac dysmorphogenesis.

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