High Insulin Concentrations Repress Insulin Receptor Gene Expression in Calf Hepatocytes Cultured *in Vitro*

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Key Words

Insulin • Insulin receptor • Gene expression • Calf hepatocytes • Dairy cows

Abstract

The major role of insulin and the insulin receptor (InsR) in the liver is to mediate glucose uptake into hepatocytes to synthesize glycogen and to maintain blood glucose homeostasis. In this study, we investigated the effects of high insulin concentrations on InsR gene expression in calf hepatocytes cultured *in vitro*. After the cells were cultured for 72 h, insulin was added to the culture solution at final concentrations of 0, 1, 10, 100 or 1000 nM. InsR mRNA expression was determined by semi-quantitative RT-PCR. The results showed that InsR mRNA expression in hepatocytes, adjusted for β -actin expression, decreased dose dependently with increasing insulin concentration. InsR mRNA expression was similar at 1 and 10 nM insulin, but was significantly lower than that in the control. InsR expression was similar at 100 and 1000 nM insulin, but was significantly lower than that in the control, 1 and 10 nM insulin groups. These data suggest that high concentrations of insulin significantly repress InsR mRNA expression in calf hepatocytes, and this inhibition occurs in a dose-

dependent manner. Further studies are needed to investigate the mechanisms underlying these effects of insulin.

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Introduction

The liver is the main gluconeogenic organ, and 90% of the glucose in ruminants is provided by hepatic gluconeogenesis. The main role of insulin in the liver, muscle and fat, by acting through the cell surface insulin receptor (InsR), is to stimulate glucose uptake into hepatocytes to promote glycogen synthesis and to maintain blood sugar homeostasis [1, 2]. The regulation of energy metabolism, including gluconeogenesis, fat mobilization and glycogen storage/breakdown in ruminants is mainly regulated by insulin and glucagons [3]. Accordingly, the regulation of the InsR has become a focus of ruminant feeding and energy metabolism research.

In dairy cows selected for high milk production, peripartum insulin resistance seems to play a pivotal role in the adaptation to postpartum negative energy balance (NEB) and in the pathogenesis of some NEB-related metabolic disorders and organ diseases [4], including excessive lipid accumulation in the liver and ketosis [5], abomasal displacement [6], and cystic ovarian disease [7].

A low dose of slow-release insulin (0.14 IU/kg body weight) could be considered for prophylactic use to prevent hepatic lipidosis and ketosis, but continued administration of higher concentrations of insulin was reported to decrease hepatic glyconeogenesis and glycogen output [8].

However, the effects of high concentrations of insulin on InsR expression in hepatocytes from cows remain unknown. Therefore, the aim of this study was to determine the effects of insulin on InsR mRNA expression in neonatal calf hepatocytes cultured *in vitro*.

Materials and Methods

Reagents and hepatocyte culture medium

HepatoZYME medium, RPMI-1640 medium, fetal bovine serum (FBS) and collagenase IV were purchased from Gibco. All other chemicals were purchased from Sigma Aldrich.

RPMI-1640 basic culture medium was prepared according to the manufacturer's instructions and 26 mM NaHCO₃, 10 mM HEPES and 20 mM NaCl were added and pH was adjusted to 7.2. Perfusate A comprised 140 mM NaCl, 6.7 mM KCl, 10 mM HEPES, 2.5 mM glucose, and 0.5 mM EDTA, with pH adjusted to 7.4. Perfusate B comprised 140 mM NaCl, 6.7 mM KCl, 30 mM HEPES, 2.5 mM glucose, and 5 mM CaCl₂, with pH adjusted to 7.4. Perfusate C comprised perfusate B plus 50 g/L collagenase IV. The adherent medium comprised RPMI-1640 basic medium supplemented with 10% newborn calf serum, 10⁻⁶ mol/L insulin, 10⁻⁶ mol/L dexamethasone, 10 µg/mL vitamin C, 100 U/mL benzylpenicillin and 100 µg/mL streptomycin.

Isolation and culture of hepatocytes

Hepatocytes were cultured by the collagenase perfusion method. Briefly, livers (caudate process) were obtained under sterile conditions from three Hostein newborn calves anesthetized with thiamylal sodium respectively, and were divided into three group according to calf. The liver was quickly transferred into a superclean bench and bloodstains on the surface were removed using perfusate A (37°C) to reveal blood vessel on the cross-section of the caudate process. A moderate-size blood vessel was cannulated and the liver was perfused with perfusion solution A (37°C) at a flow rate of 50 mL/min for 15 min. The liver was then perfused with solution B (37°C) at the same infusion rate for 3 min. When the liquid became clear, the liver was perfused with perfusate C (37°C) at 20 mL/min. Before the final perfusion, all large vessels were partially ligated to slow perfusate flow through the organ. After digestion for 15 min, the caudate process was transferred to a sterile flat plate.

Then, 50 mL of basic medium containing 0.2% BSA was added to the plate and the blood vessels, fat and connective tissue were removed. After tearing the liver amacula with tweezers, the remainder of the liver was cut into pieces and filtered sequentially through 100-mesh (150 µm), 200-mesh (75 µm) and 500-mesh (30 µm) cell sieves. The resulting hepatocyte sus-

pension was washed twice with basic medium (4°C) by centrifugation for 2 min at 50 xg, resuspended in adherent medium, and viability was determined by trypan blue staining and cell counting with a hemocytometer.

The hepatocyte cell density was adjusted 5×10⁵ cells/mL with adherent culture medium, and 4 mL of cells was inoculated onto a flat plate, and incubated at 37°C in a humidified atmosphere of 5% CO₂. After 4 h, the culture medium was replaced with growth medium containing 5% newborn calf serum, benzylpenicillin and streptomycin. The hepatoZYME-SFM was subsequently exchanged every 24 h.

Primer design and DNA amplification

Primer design and DNA amplification were performed as previously described [9, 10].

Insulin treatment and RNA extraction

After culture for 72 h, the hepatocytes were treated with insulin at final concentrations of 0, 1, 10, 100 or 1000 nM [11-13], with six replicates per group. After incubation for a further 12 h, total RNA was isolated using a Trizol RNA purification kit (Invitrogen) according to the manufacturer's instructions. The RNA extract was diluted 1:40 and analyzed at 260 and 280 nm by spectrophotometry. Only samples with an optical density ratio at 260/280 nm >1.9 were used in further analyses.

Complementary DNA synthesis and expression analysis by semi-quantitative RT-PCR

Complementary DNA synthesis and InsR mRNA semi-quantitative RT-PCR were performed as previously described [10].

Data analysis

Data are presented as means ± SEM. Statistical significance was determined by one-way analysis of variance followed by Duncan's least significant difference test. P < 0.05 was considered to be significant. All data analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA).

Results

Agarose gel electrophoresis of hepatocytes total RNA

As shown in Fig. 1, three bands of total RNA were extracted, corresponding to 28S, 18S and 5S. The RNA had not degraded, and met the requirements of reverse transcription. The A260/A280 values were > 1.9 and achieved the specified purity requirements.

As shown in Fig. 2 and Table 1, as the insulin concentration increased, so the InsR mRNA expression level in hepatocytes, adjusted for β-actin expression, decreased. Comparatively, the relative InsR mRNA expression in hepatocytes was similar at 1 and 10 nM insulin, but was significantly lower than that in the control group. Meanwhile, the relative InsR mRNA expression in hepatocytes

Insulin (nM)	0	1	10	100	1000
InsR mRNA abundance relative to β -actin (mean $\pm$ SD)	0.57 $\pm$ 0.13 ^A	0.40 $\pm$ 0.16 ^B	0.37 $\pm$ 0.10 ^{Bb}	0.26 $\pm$ 0.13 ^{Cb}	0.23 $\pm$ 0.12 ^{Ca}

Table 1. Relative abundance of InsR mRNA in primary hepatocytes treated with 0–1000 mM insulin (n=18). Different superscript letters indicate significant differences at $P < 0.05$.

was similar in the 100 and 1000 nM groups, but was significantly lower in these groups than in the control or 1 and 10 nM groups.

Discussion

Insulin, a principal regulator of glucose homeostasis, promotes glucose uptake in the liver, fat and muscle, and also plays an important role in the synthesis and storage of lipids and proteins [1]. Insulin exerts its effects in its target cells by binding to the InsR, which is expressed on the cell surface [14]. The InsR is a membrane-bound glycoprotein that has specific and high affinity binding to insulin [15], and plays a pivotal role in the metabolism of glucose and fat by initiating the intracellular insulin signaling pathways [2, 16, 17]. Upon binding to its receptor, insulin induces autophosphorylation of tyrosine residues within the B subunit of the receptor. This increases tyrosine kinase activity of the InsR and phosphorylates the cellular substrates [18, 19].

The relative abundance of InsR on a cell is regulated by a balance between synthesis and degradation, which can be influenced by various physiological and pathological factors [9, 20, 21]. Impaired InsR signaling will result in impaired insulin-stimulated glucose uptake and decreased hepatic glucose production [22].

Insulin resistance is a condition in which higher than normal insulin concentrations are needed to achieve normal metabolic responses [23]. One general effect of impaired insulin function in man is fatty infiltration in the liver, which may give rise to numerous pathological changes. Obesity and fatty liver are also common in dairy cows, and result in the fat cow syndrome [24, 25]. Insulin resistance is closely associated with impaired glucose homeostasis and is generally accompanied by a compensatory increase in insulin levels [26, 27].

The expression of InsR mRNA in the liver of ketotic dairy cows and cows with fatty liver was reported to be lower than that in the liver of healthy cows, and might be due to insulin resistance [9]. Insulin is an anabolic hormone that plays numerous roles in the metabolism of carbohydrates, lipids and proteins, acts to preserve nutrient availability, and is a potent regulator of feed intake regulator [28]. Insulin is administered to alleviate

Fig. 1. Agarose gel electrophoresis of hepatocyte total RNA. Effects of insulin on InsR mRNA expression in hepatocytes

Fig. 2. Effects of insulin on insulin receptor (InsR) mRNA expression in calf hepatocytes treated with 0–1000 mM insulin for 12 h. M: DL 2000 marker.

severe NEB, and its administration in combination with dextrose is an effective treatment for hepatic lipidosis and ketosis. However, data on use of insulin to prevent these lipid-related metabolic disorders are limited, warranting further studies in this field.

Impaired insulin regulation of energy metabolism is an etiologic factor underlying metabolic disturbances [3, 29]. In humans, obesity is associated with reduced insulin sensitivity concomitant with an increased incidence of metabolic diseases [30]. Cows overfed during dry periods have a high body condition score and showed reduced glucose disappearance during a glucose tolerance test, suggesting that a high body condition score is associated with insulin resistance as compared with thinner cows [29].

As shown in this study, adding insulin to the culture solution of calf hepatocytes cultured *in vitro*, the expression of InsR mRNA decreased with increasing insulin concentration. This indicates that the expression of InsR is downregulated by insulin itself, which may decrease insulin sensitivity in hepatocytes and may thus adversely affect blood glucose homeostasis.

In conclusion, high concentrations of insulin significantly reduced InsR mRNA expression in calf hepatocytes cultured *in vitro* in a dose-dependent manner. Further studies are needed to investigate the underlying mechanisms.

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