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Hepatic ALT isoenzymes are elevated in gluconeogenic conditions including diabetes and suppressed by insulin at the protein level

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Abstract

Alanine transaminase (ALT) plays an important role in gluconeogenesis by converting alanine into pyruvate for glucose production. Early studies have shown that ALT activities are upregulated in gluconeogenic conditions and may be implicated in the development of diabetes. Since ALT consists of two isoforms, ALT1 and ALT2, with distinctive subcellular and tissue distributions, whether and how they are regulated are largely unknown. In this study, we found that both ALT isoforms in the liver were increased in diabetic GK rats and during fasting. However in *ob/ob* mice, only ALT2, but not ALT1, protein levels were elevated and the increase of ALT2 is correlated with that of ALT activity. We further demonstrated that, *in vitro*, both ALT1 and ALT2 were induced by glucocorticoid dexamethasone but suppressed by insulin in Fao hepatoma cells. Finally, we showed that the over-expression of ALT1 and ALT2 in Fao cells directly increased glucose output. Correctively, we have revealed the similarity and difference in the regulation of ALT isoforms in gluconeogenic conditions at the protein level, supporting that ALT isoenzymes play an important role in glucose metabolism and may be implicated the development of insulin resistance and diabetes.

Introduction

Gluconeogenesis (GNG) is a metabolic pathway important for glucose homeostasis in physiological conditions and vital for animal survival by generating glucose from non-carbohydrate carbon substrates during fasting and starvation. However, unsuppressed gluconeogenesis is a major contributing factor to insulin resistance and type 2 diabetes (T2D) which is characterized by the inability of insulin to adequately suppress hepatic gluconeogenesis, leading to hyperglycemia, hyperinsulinemia and eventually to T2D [1-4].

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Alanine transaminase (ALT) is an intermediary enzyme that reversibly catalyzes transamination between alanine and α-ketoglutarate to form pyruvate and glutamate, through which it plays a pivotal role in glucose and protein metabolism. Physiologically, ALT is particularly important for energy homeostasis during fasting and prolonged exercise when muscle protein must first be broken down into its constituent amino acids. Blood draining from muscle is especially enriched with alanine and glutamine, which can each constitute up to 30% of the total amino acids released from muscle. Alanine released from muscle is subsequently taken up by the liver where it is converted to pyruvate by ALT for glucose production through gluconeogenesis [5]. Alanine is a main gluconeogenic precursor, in addition to lactate, glutamine, and glycerol in humans, together accounting for >90% of overall gluconeogenesis [6]. While there is still uncertainty about the actual contribution of ALT to hepatic glucose output in insulin resistance, many regulation and association studies indicate the enzyme may contribute to insulin resistance. For example, increased ALT activity is observed in rat liver after the administration of glucocorticoid hormone and in diabetic animals [7-9]. Higher ALT activity is often associated with insulin resistance as seen in obesity, diabetes and metabolic syndrome [10-13] Moreover, higher serum ALT activity is predictive of the development of insulin resistance and type 2 diabetes [10, 14, 15] in populations without apparent liver damage, suggesting that increased ALT activity may participate in the pathogenesis of insulin resistance. However, few studies have been conducted about the regulation of ALT at the transcription and protein levels in the liver.

We and others have cloned two ALT isoforms, ALT1 and ALT2 in mammalians and fish [16-19]. The two isoforms are encoded by separate genes, with distinctive cellular and tissue distribution in that ALT1 is a cytoplasmic protein and mainly expressed in intestine, liver, skeletal muscle and kidney, whereas ALT2 appears largely present in mitochondria and is mainly expressed in liver, muscle, brain, and white adipose tissue[16-18]. These dissimilarities suggest a difference in biological function as well as regulation of these two isozymes. Indeed, we reported that hepatic ALT2 mRNA is induced in *ob/ob* mice and muscle ALT2 is strongly induced by glucocorticoid in animals [18].

Since the two ALT isoforms catalyze the same biochemical reaction, the results of early ALT activity studies [7, 20-22], although informative, do answer the question of whether or how ALT isoforms are regulated under physiological and pathological conditions. The discovery of ALT isoforms has provided an opportunity to address this question. In view of the significance of ALT in gluconeogenesis and in the pathogenesis of diabetes in this study we used ALT isoform-specific antibodies to determine ALT1 and ALT2 regulation at the protein level in selective gluconeogenic conditions including diabetes.

Experiment procedures

Animal studies

Animal studies were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. Goto-Kakizaki (GK) breeding pairs were a gift from Dr R. V. Farese at the University of South Florida and expanded at the University of Maryland [23]. GK and Wistar male rats (Charles River Laboratories, Wilmington, MA.), lean C57BL/6J mice and *ob/ob* male mice (Jackson Laboratories, Bar Harbor, ME), Sprague

Dawley (SD) rats (Charles River Laboratories, Wilmington, MA.) were maintained in a temperature and light controlled environment and had free access to water and standard rodent chow. Experiments were carried out on animals at 8 weeks old. For an intraperitoneal glucose tolerance test (IPGTT), male rats were intraperitoneally injected with glucose (2 g/kg) after an overnight fast. Glucose levels were measured from venous tail blood using a glucometer (Accu-Check, Roche, Indianapolis, IN). For fasting experiments, SD rats were fasted for 36 hr with free access to water. To collect tissues, animals were euthanized with carbon dioxide and livers were excised for homogenization or snap-frozen in liquid nitrogen. Tissues were collected in fed state in all groups except the fasting one.

Cell culture and treatment

The rat hepatoma line of Fao cells (Sigma, Louis, MO) were cultured in a growth medium of DMEM (Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS, Invitrogen), 2 mM glutamine and penicillin and streptomycin antibiotics (Cellgro , Manassas, VA). For hormone treatment, the cell culture medium was changed to 1% fetal bovine serum in DMEM, and treated with insulin (Anventis Pharmaceuticals Inc, Bridgewater, NJ) and dexamethasone (Sigma) as indicated in the figures.

ALT activity assay

ALT activity was determined by using the alanine aminotransferase kit (Calchem, Huntington, NY) according to the manufacturer's instruction. Briefly, 10 μl of tissue or cell lysate was incubated with a 200 μl substrate mixture of reagent A and B containing L-alanine, NADH, LDH and 2-oxoglutarate at 25° C. Absorbance at 340 nm was recorded for 5 min at 30 second intervals after the addition of substrate mixture. The slope of absorbance decrease was proportional to ALT activity. Final ALT activities were corrected by protein concentration of cell lysates. One unit of ALT activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol/L of NAD per minute under conditions of the assay at 25° C.

Western analysis

Liver tissue or Fao cells were homogenized and lysed in lysis buffer containing 50 mM HEPES, 1 mM EDTA,150 mM NaCl, 1% Triton X 100 and a cocktail of proteinase inhibitors (Sigma). Lysates containing 20 μg protein were separated in 7.5% SDS-PAGE gel, and transfer to polyvinylidene difluoride membranes (Millipore corporation, Billerica, MA). The membranes were then incubated with affinity-purified, biotin labeled rabbit anti-ALT1 or rabbit anti- ALT2 polyclonal antibody [16] in TTBS buffer with 5% milk at 1:10,000 dilution at 4°C overnight. Streptavidin -conjugated alkaline phosphatase (Thermo Scientific, Rockford, IL) at 1:5000 dilution was used for detection. The blots were visualized by BCIP/NBT Western blot kit (Sigma) and quantified by measuring optical density values with ImageJ software.

Recombinant Adenovirus generation and infection

Rat ALT1 (rALT1), rALT2 or GFP cDNA was sub-cloned into pAdTrack-CMV [24]. The resulting adenoviral vector was digested with PacI and was transfected in HEK293A cells by

LipoD 293 (Signal Gene, Rockville, MD). Adenovirus-containing supernatant was collected 10 days after transfection to infect fresh HEK293 cells for virus amplification. After 3-4 rounds of amplification, the adenoviruses were produced in large-scale and purified by CsCl gradient ultracentrifugation [24].

Glucose output assay

To investigate the effect of Dex and insulin on glucose output [25], Fao cells were cultured in the growth medium, and then treated with Dex and/or insulin for 24 hr in DMEM/1%. The culture medium was replaced with glucose-free DMEM medium (Cat#11966, Invitrogen) with alanine (13.9 mg/L), but without pyruvate and lactate and further incubated for 4 hours. Glucose released into the medium was measured with Amplex® Red Glucose/ Glucose Oxidase Assay Kit (Invitrogen) and normalized with protein concentration. To determine the effect of ALT on glucose production, FBS Fao hepatoma cells were seeded on 12-well plates at ~90% confluency, infected with GFP, rALT1 or rALT2 recombinant adenoviruses with 10 ng/ml DEAE-dextron (SigmaA) at ~5 MOI for 12 hours, and then changed to the growth medium. The infection rate was approximately 90% 48 hours after infection by visualizing co-expressed GFP. The cells were further incubated for 4 hours for glucose measurement.

Statistics

Results are expressed as mean ± SE. Anova was employed to examine multigroup differences between means and Student *t*-test was used to examine the two group differences (GraphPad Software, San Diego, CA). Differences were considered statistically significant at $P < 0.05$.

Results

ALT isoenzymes are elevated in diabetic GK rats

The Goto-Kakizaki (GK) rat is a non-obese Wistar substrain which spontaneously develops T2D early in life [26]. We examined the total hepatic ALT activities and ALT expression in diabetic GK rats and wild-type controls. As anticipated, at the age of 8 weeks, the GK rats developed diabetes with fasting glucose levels of 136.3 ± 2.7 mg/dl and two-hour glucose levels of 333.0 ± 62.1 mg/dl in a glucose tolerance test, significantly higher than the wild-type ($p < 0.01$ for both, Fig 1A). We next measured ALT enzymatic activity in the liver lysate and found the total ALT activity was elevated by about 38.5% higher in GK rats compared to the wild type ($P < 0.01$, Fig 1B). The liver lysates were further analyzed for ALT1 and ALT2 protein content by immunoblotting. Compared with the control rats, hepatic ALT1 and ALT2 were higher by 72.3% ($P < 0.01$) and 58.0% ($P < 0.01$), respectively (Fig 1C, 1D). To evaluate which ALT isoform protein level is associated with the enzymatic activities, we conducted correlation analysis and found that the protein levels of both ALT1 and ALT2 were correlative with ALT activity (ALT1: $r^2 = 0.77$, $P < 0.01$; ALT2: $r^2 = 0.46$, $P < 0.05$) (Fig 1E). This experiment shows that both hepatic ALT1 and ALT2 protein expressions were increased in diabetic rats and were correlated with total ALT activities.

ALT isoenzymes are increased in obese and fasting states

Gluconeogenesis is enhanced in obesity as well as in a fasting state when insulin action is blunted. Thus, we examined ALT1 and ALT2 expression in these two conditions. In *ob/ob* mice, hepatic ALT activities were 34.1% higher than in lean mice (Fig. 2A). Protein analysis showed that ALT2 proteins were increased by 54.6 % whereas ALT1 remained unchanged (Fig. 2B, 2C). Moreover, the expression levels of ALT2, but not ALT1, were correlated with ALT activities (ALT2 $r^2 = 0.57$, $P < 0.01$; ALT1 $r^2 = 0.02$, $P = 0.64$) (Fig.2D). In fasting rats, hepatic ALT activity was increased by 31.6% at 36 hr ($P < 0.01$) (Fig. 3A) compared to fed rats. Likewise, ALT1 expression was increased by 68.6 % ($P < 0.01$) and ALT2 expression increased by 54.6 % ($P < 0.01$) (Fig 3B).

ALT isoforms are induced by glucocorticoid, but suppressed by insulin *in vitro*

Glucocorticoid and insulin are the major hormones modulating gluconeogenesis in an opposing manner in animals [16, 27], but few studies have been conducted to determine whether they regulate ALT directly or not. We thus set out to examine the possible effect of these hormones on ALT in Fao rat hepatoma cells. The cells were treated with dexamethasone (Dex), a glucocorticoid, for 24, 48 and 72 hr at the concentrations of 0.1, and 1.0 μM, and then assayed for ALT activities and protein levels. As shown in Fig 4A, ALT activities were induced by Dex in a dose-dependent manner in 24 hr, but were not further increased during the three days of treatment. At the protein level, ALT1 was not induced in 24 hr of treatment, but was increased in 48 and 72 hr at 0.1 μM Dex (4B, 4C). On the other hand, ALT2 was induced in 24 hr and remained higher up to 48 and 72 hr (4B, 4D).

We next examined the effect of insulin at 0.1 μM and 1.0 μM on ALT isoforms treated with or without Dex in Fao cells. As shown in Fig. 5A, significant decreases of 30.0% and 39.2% in ALT activities were observed by insulin for 24 hr in cells untreated or treated with Dex at 1 μM, respectively. The suppressive effect persisted for the 48 hr period of treatment. Remarkably, the suppression patterns by insulin were similar for both ALT1 and ALT2 (Fig 5B and 5C). Functionally, Dex increased glucose production, but the increase was blocked by insulin treatment (Fig 5D). Intriguingly, insulin alone did not suppress the *in vitro* glucose production despite the fact that it suppressed ALT protein expression (Fig 5). This experiment shows that insulin directly suppressed ALT1 and ALT2 protein levels and suppressed Dex-induced glucose production in hepatoma cells.

ALT directly contributes to glucose output in Fao cells

To investigate whether ALT isoforms directly contribute to glucose production, we over-expressed rat ALT1, ALT2 or control GFP by infecting Fao cells with respective recombinant adenoviruses. The expression of ALT1 and ALT2 proteins were confirmed by Western blot (Fig 6A). The infected cells were then incubated in glucose-free medium for 4 hours and the amount of glucose released into the medium was determined. As shown in Fig 6B, glucose concentrations were 49.5% and 42.4% higher in cells overexpressing ALT1 and ALT2, respectively, compared with GFP-expressing cells. No significant difference was noted in glucose production between ALT1 and ALT2 ($p = 0.55$). This work demonstrates that both ALT isoforms could directly promote glucose production.

Discussion

In this study, we investigated ALT isoform regulation in diabetic and other gluconeogenic conditions at the protein level. ALT is a well-studied enzyme because of its pivotal role in mediating carbohydrate and amino acid metabolism and its diagnostic value as a liver function marker. The presence of two ALT isoenzymes has raised a question of their similarity and difference in function and regulation. This study aimed to address the question of how they are regulated at the protein level.

We first investigated hepatic ALT isoenzyme expressions in diabetic GK rats, and found that both ALT1 and ALT2 proteins were significantly increased although ALT1 appeared elevated more than ALT2. The increase of both proteins was correlated with hepatic ALT activities, suggesting that both of them likely contribute to the total ALT activity increase. Similarly, in fasting rats, both isoforms were induced as well. Obesity is also a gluconeogenic condition where insulin action is blunted, and the hepatic glucose output is increased despite that insulin level is actually increased. We then determined ALT isoform levels in *ob/ob* mice, a genetically obese animal model, and found that hepatic ALT2, but not ALT1,was significantly increased, which is consistent with our previous observation of ALT2-specific induction at the mRNA level in the animal model [18]. Collectively, these studies indicate that both hepatic ALT1 and ALT2 are increased in gluconeogenic conditions such as diabetes and fasting. However, in ob/ob mice, only hepatic ALT2 is elevated, suggesting that this isoform may be more implicated in the obesity-related pathogenesis, e.g., fatty liver in obesity.

Glucocorticoid (GC) and insulin are two major hormones regulating hepatic gluconeogenesis in an opposite manner with the former inducing and the latter suppressing gluconeogenesis. GC excess is a prominent cause of insulin resistance. Twenty to 50% of patients with GC excess, resulting in Cushing's syndrome, develop diabetes and about 70% of the patients develops insulin resistance [28, 29]. GC induces hepatic production through both direct stimulation of gluconeogenesis and indirect inhibition of insulin action [30]. Animal studies have shown that GC can significantly induce ALT activity and proteins [31] but studies on the ALT regulation by in mammalian cells are scarce [8]. To further determine whether these actions are direct or not, we used Fao hepatoma cells to investigate the effect of Dex and insulin on ALT expression and activity. As a result, Dex treatment significantly induced ALT activity in the period of 24-72 hr examined. Interestingly, ALT1 was not induced within the first 24 hr at the lower concentration of 0.1 uM Dex but both the isoforms were elevated thereafter. Thus, ALT2 appeared a fast and sensitive responder to Dex and contributed to the initial phase of ALT activity increase, whereas both isoenzymes contribute to the late phase of ALT activity increase. Insulin is a potent suppressor of gene expression of gluconeogenic enzymes [27]. However, studies on the possible regulation of ALT by insulin are scarce. We showed that insulin decreased ALT activity and both isoenzymes at the protein level in the cells treated with or without Dex, which establishes that ALT1 and ALT2 are a target of insulin suppression. Intriguingly in hepatoma cells, despite that insulin suppressed ALT protein expression, insulin alone did not suppress glucose production, but could mitigate the Dex-induced increase of glucose production, suggesting that basal glucose production is not linearly dependent on ALT protein levels and

that other gluconeogenic substrates aside from alanine contribute the production. Nevertheless, our study shows that the insulin suppression mechanism is mediated in part by alleviating Dex's induction on ALT, which may be significant in vivo where glucocorticoids are consistently present. It is worth noting that although we show the suppressive effect of insulin on ALT protein expression, a possible direct effect of insulin on ALT activity through post-transcriptional modification via insulin substrates and PI3K/AKT pathway cannot be ruled out.

Although ALT has been considered a gluconeogenic enzyme, direct evidence is lacking for actual contribution of ALT to glucose production. We showed that cells overexpressing ALT1 and ALT2 produced more than 40% of glucose than the control cells and, therefore, directly confirmed their gluconeogenic function. Nevertheless, the degree of glucose increase is relatively small, compared to the large extent of 3-4 fold increase in ALT protein expression. One possible reason is that alanine as a gluconeogenic substrate might become limiting in the *in vitro* system, and another explanation is that gluconeogenesis is a complex, and ALT is just one of the many rate-limiting enzymes. Nevertheless, this study provides a piece of direct evidence that over-activity of ALT can be a mechanism for hyperglycemia in insulin resistance and diabetes. Indeed, elevated serum ALT activity levels are frequently observed in human subjects with insulin resistance but without apparent liver injury. Significantly, elevated ALT activity is predictive for insulin resistance and type 2 diabetes [10, 14, 15, 32-34]. Taken together, these studies support that increased ALT activity can be a contributing factor for insulin resistance and diabetes, and ALT may be an interventional target for these disease conditions. Future *in vivo* studies of modulating hepatic ALT levels by over-expressing or knocking-down the respective isoforms will help to address the presumption.

In summary, we showed in this study that ALT isoforms were increased in diabetes and other gluconeogenic conditions and suppressed by insulin at the protein level. Notably, these two enzymes appear to be regulated in a similar manner in response to GC and insulin treatment. Differentiated regulation is observed in *ob/ob* mice where hepatic ALT2, but not ALT1, is elevated. We have also demonstrated that both ALT isoforms contribute to glucose production and that ALT isoforms are elevated in diabetes and other gluconeogenic conditions at the protein level, supporting that ALT isoenzymes may play an important role in glucose metabolism and can be implicated the development of insulin resistance and diabetes.

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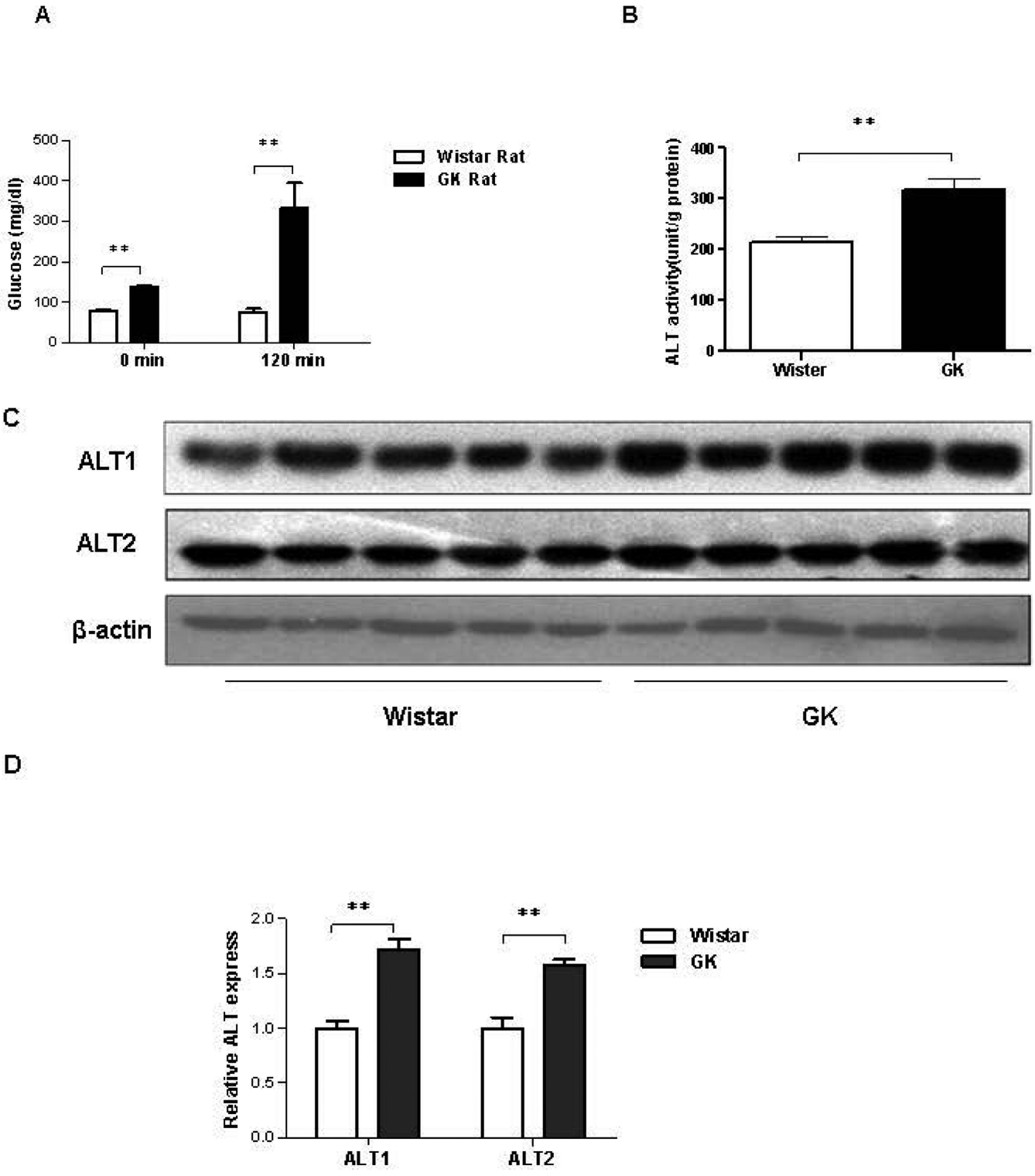


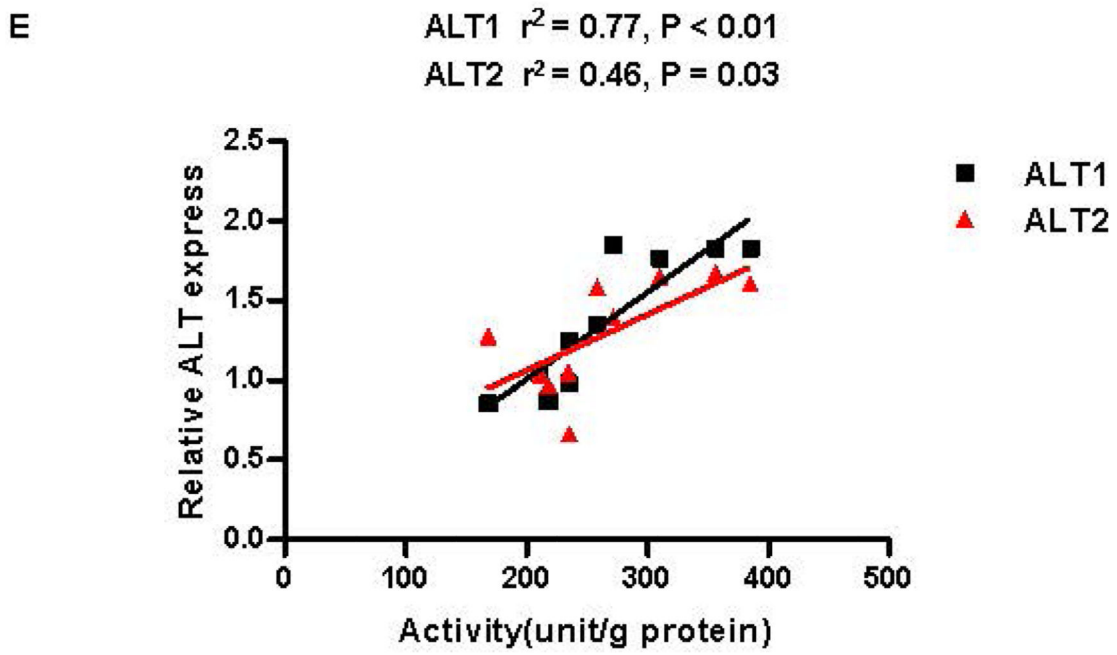
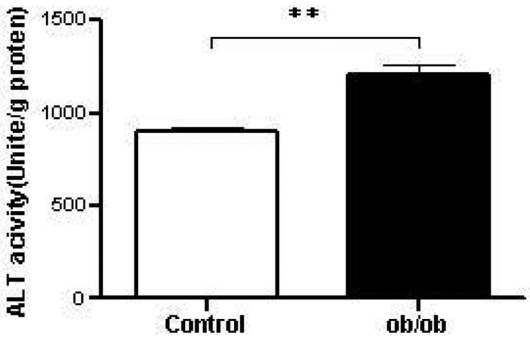


Figure 1.

Hepatic ALT activity and ALT isoenzyme protein expression in diabetic Goto Kakizaki (GK) and control Wistar rats. A. Blood glucose levels were measured before (0 min) and 120 min after intraperitoneal injection of glucose (2 g glucose/kg body weight) in eight-weeks old rats. B. Hepatic ALT activities in GK and control rats. C. Protein expression levels of ALT isoforms were analyzed by Western blotting using ALT1- and ALT2-specific antibodies. D. Quantification of ALT isoform protein expression level, adjusted to β-actin and relative to the hepatic ALT protein expression of Wistar rats. C. Correlation between ALT isoezyme protein levels and ALT activities. Data are expressed as mean ± S.E. (n = 5). **: $p < 0.01$; * $p < 0.05$

A



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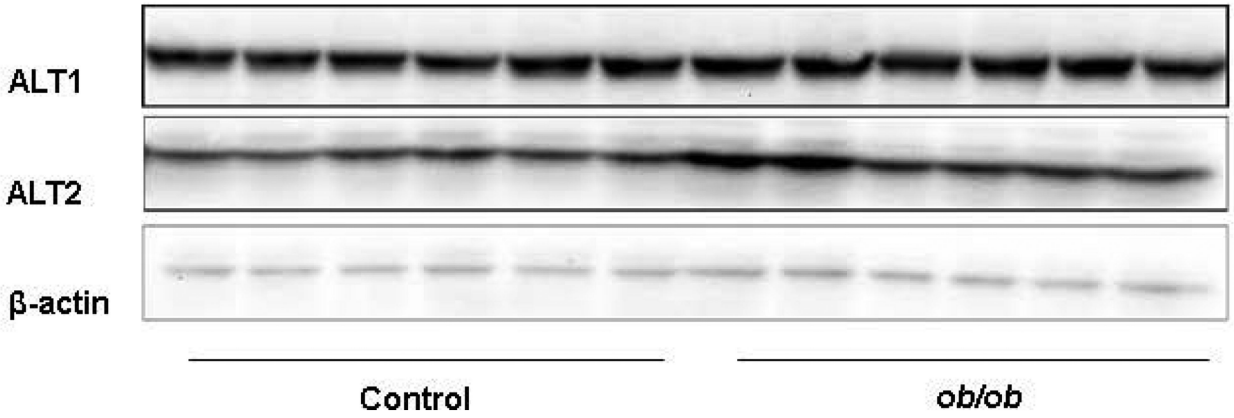


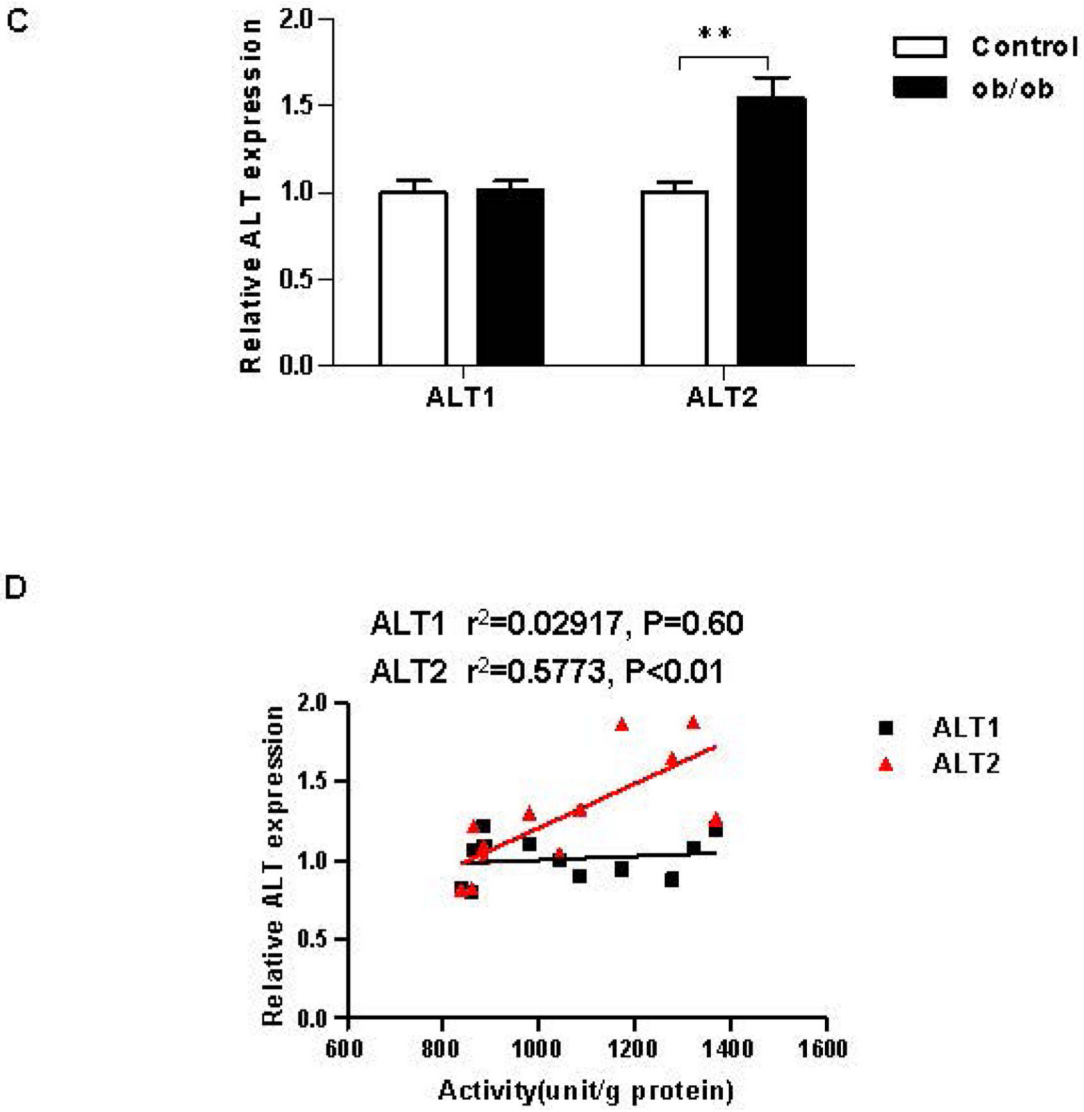


Figure 2.

Hepatic ALT activity and ALT isoenzyme expression in *ob/ob* mice. Liver lysates of ob/ob mice and lean controls were assayed for ALT activity (A) and for ALT1 and ALT2 protein expression by Western blotting (B). Relative protein expression levels were quantified by adjustment to β-actin and relative to the expression level of lean mice (C) and correlated with total ALT activities (D). Data are expressed as mean ± S.E. (n = 6). **: $p < 0.01$.

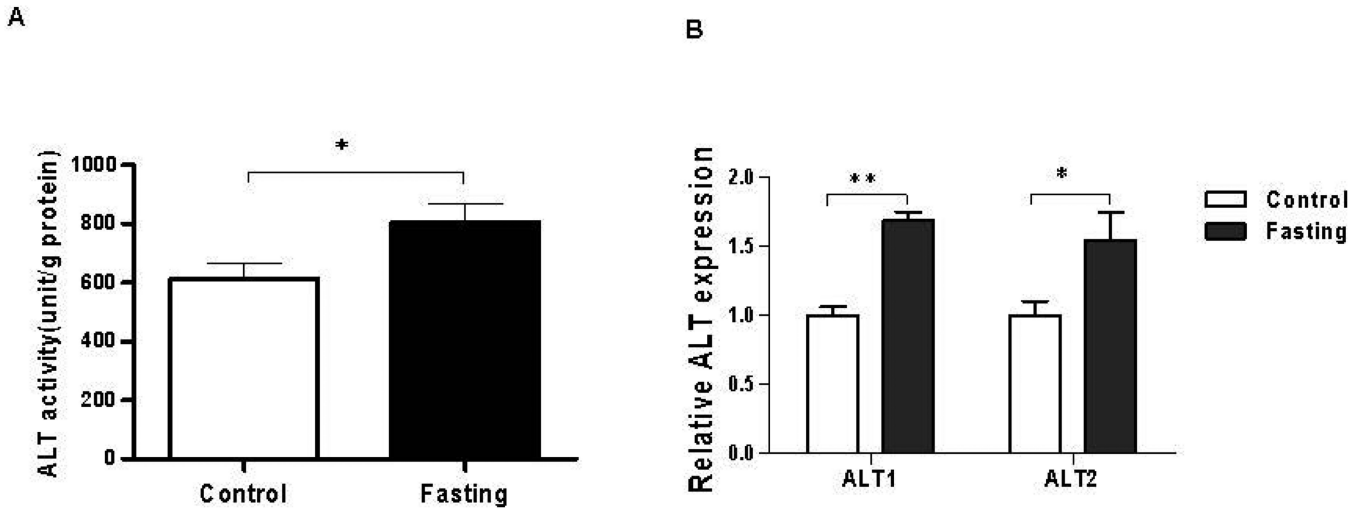
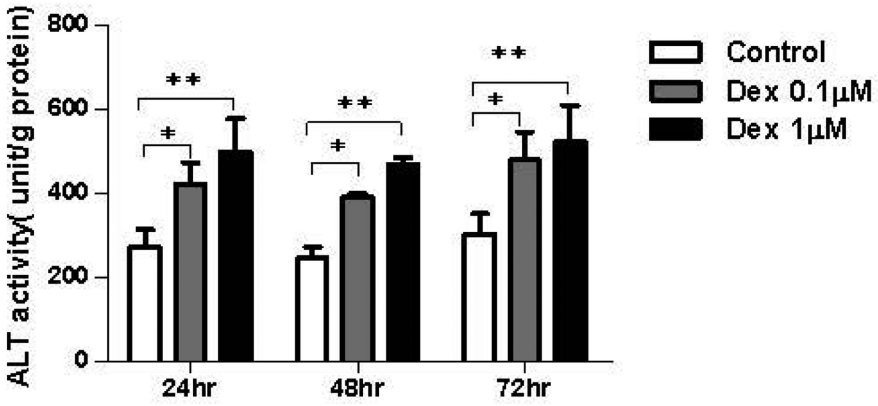


Figure 3.

Increase of hepatic ALT1 and ALT2 proteins in fasting rats. Liver lysates of rats fasted for 36 hr or fed *ad lib* were assayed for ALT activity (A) and for ALT1 and ALT2 protein expression by Western blotting. Relative protein expression levels were quantified by adjustment to β-actin and relative to the expression level of the non-fasting rats (B). Data is expressed as mean + S.E. (n = 5). **: $p < 0.01$; * $p < 0.05$

A



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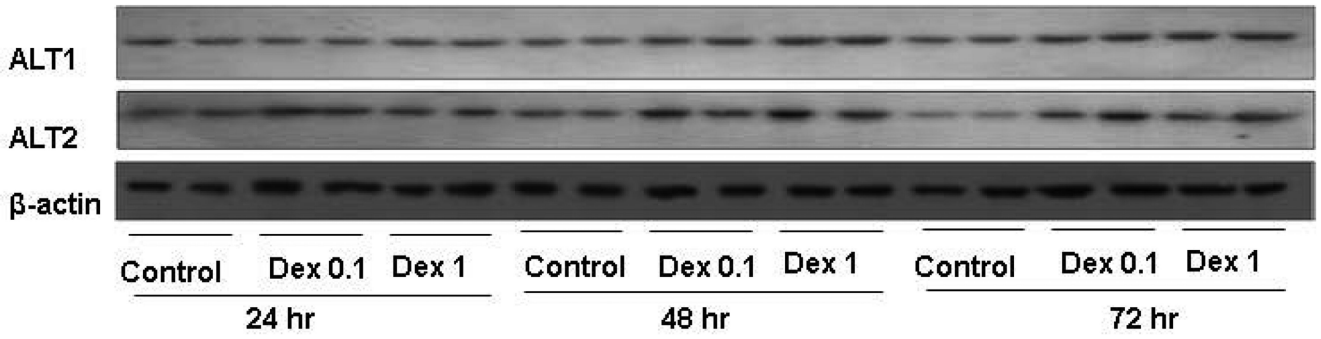


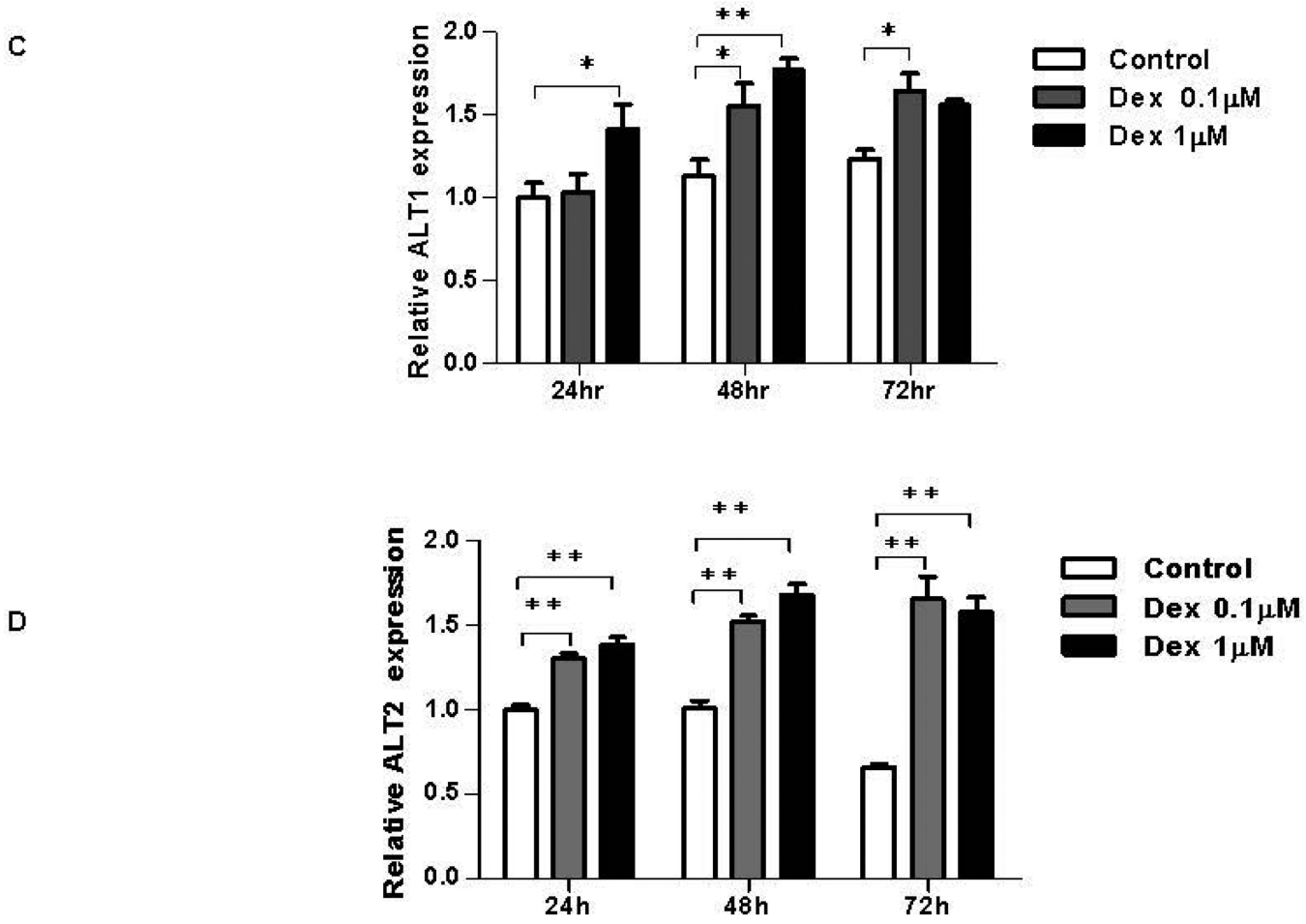
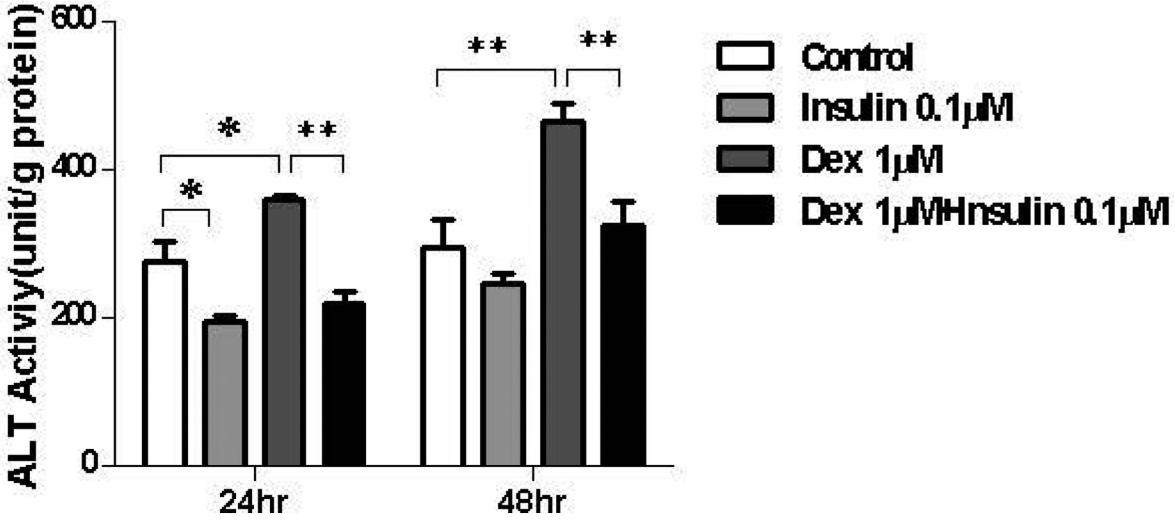


Figure 4.

Regulation of ALT1 and ALT2 by dexamethasone (Dex) in Fao hepatoma cells. Fao cells were treated with vehicle or dexamethasone at 0.1 and 1.0 μM for 24, 48 and 72 hr and assayed for ALT activities (A). Relative ALT1 and ALT2 protein expression was quantified by Western blotting (B) after being adjusted to β-actin and relative to the expression level of untreated control Fao cells (C) at 24 hr of the treatment. Data is expressed as mean + S.E. (n = 6). **: $p < 0.01$; * $p < 0.05$.

A



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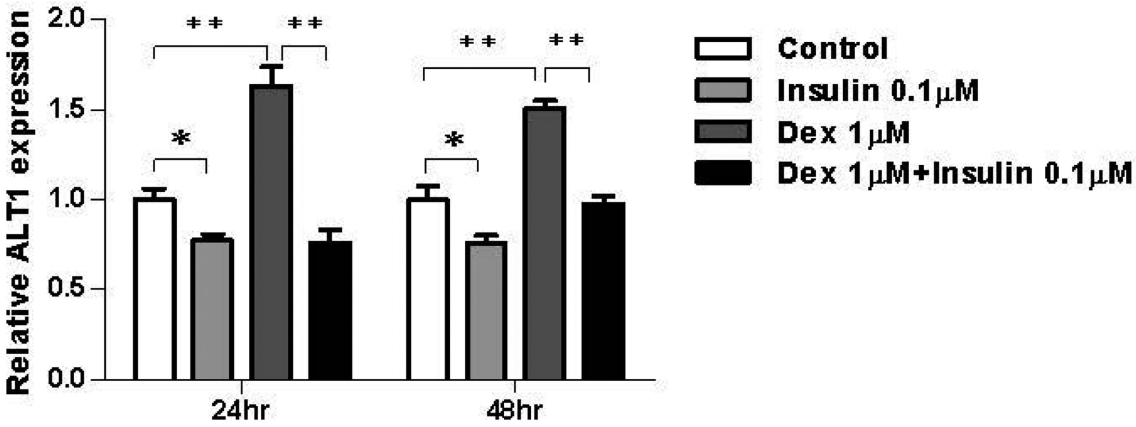
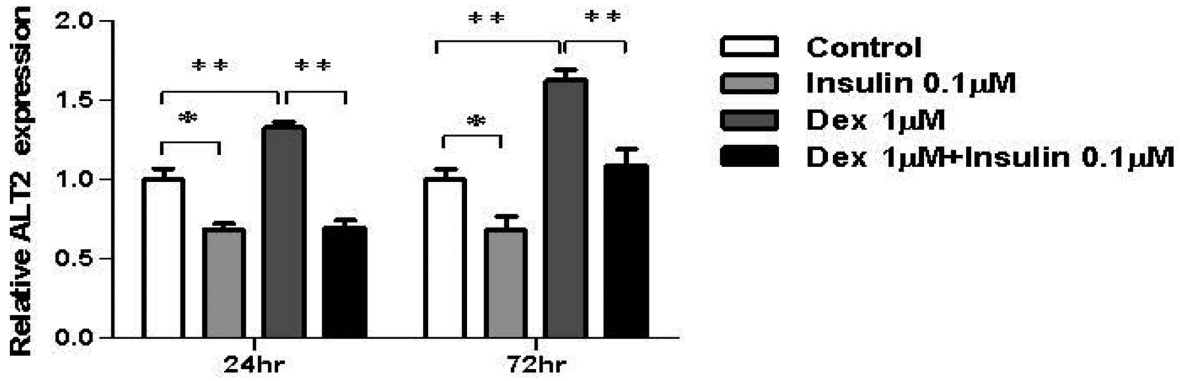


C



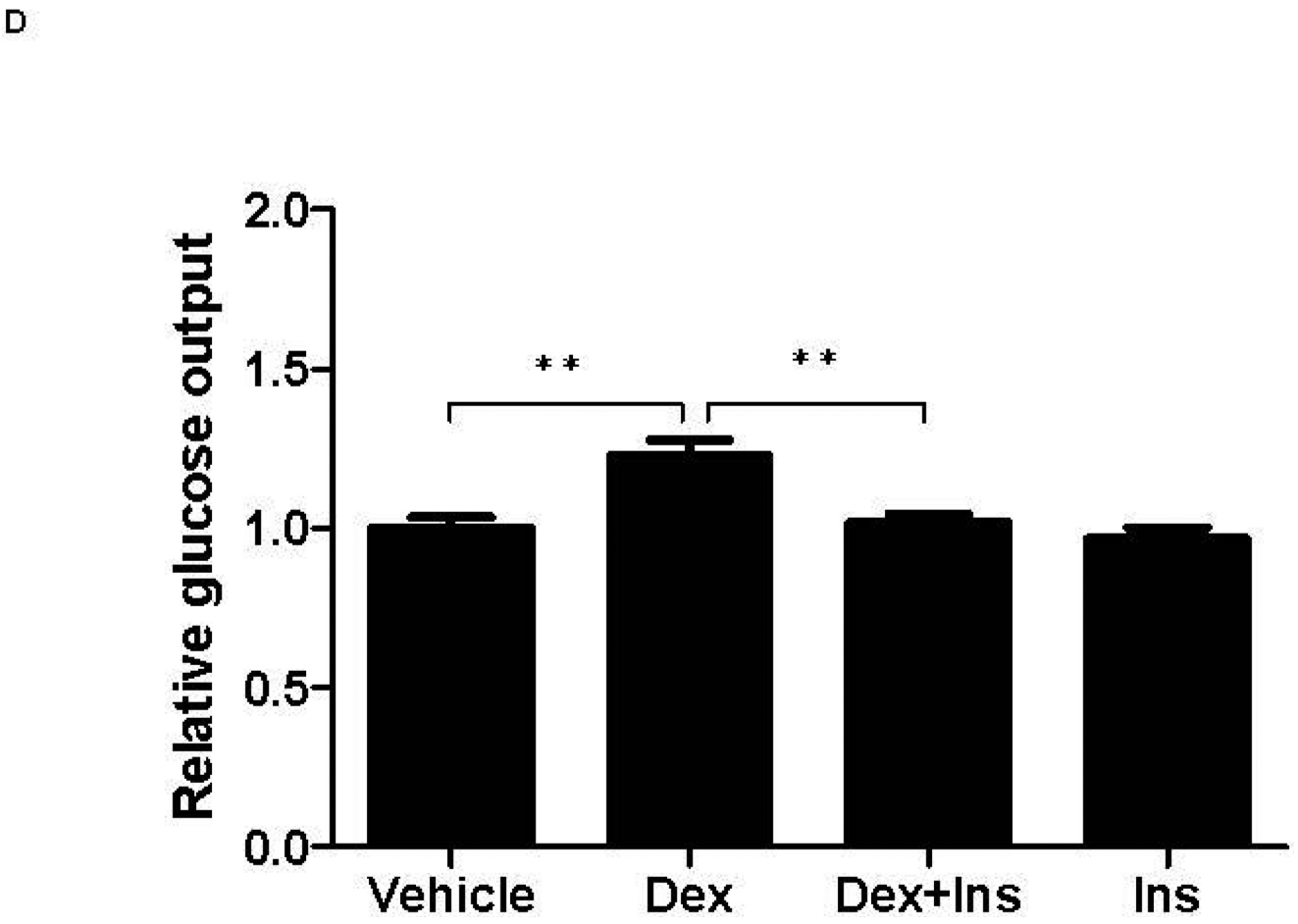


Figure 5.

Suppression of ALT and glucose production by insulin in Fao hepatoma cells. Fao cells were treated with insulin (0.1 μM) alone or insulin with Dex 1.0 μM for 24 and 48 hr and assayed for ALT activities (A) and for relative ALT1 and ALT2 protein expression (B, C) after adjustment to β -actin. The protein level is relative to that of untreated control Fao cells for the respective ALT isoform. D. Suppression of Dex-mediated glucose production by insulin. Glucose levels were measured in a medium of Fao cells which had cultured in glucose-free medium for 4 hr after pretreatment with Dex (1 μM) and/or insulin (0.1 μM) for 24 hours. Data were expressed as mean ± S.E. (n = 6). **: $p < 0.01$; * $p < 0.05$.

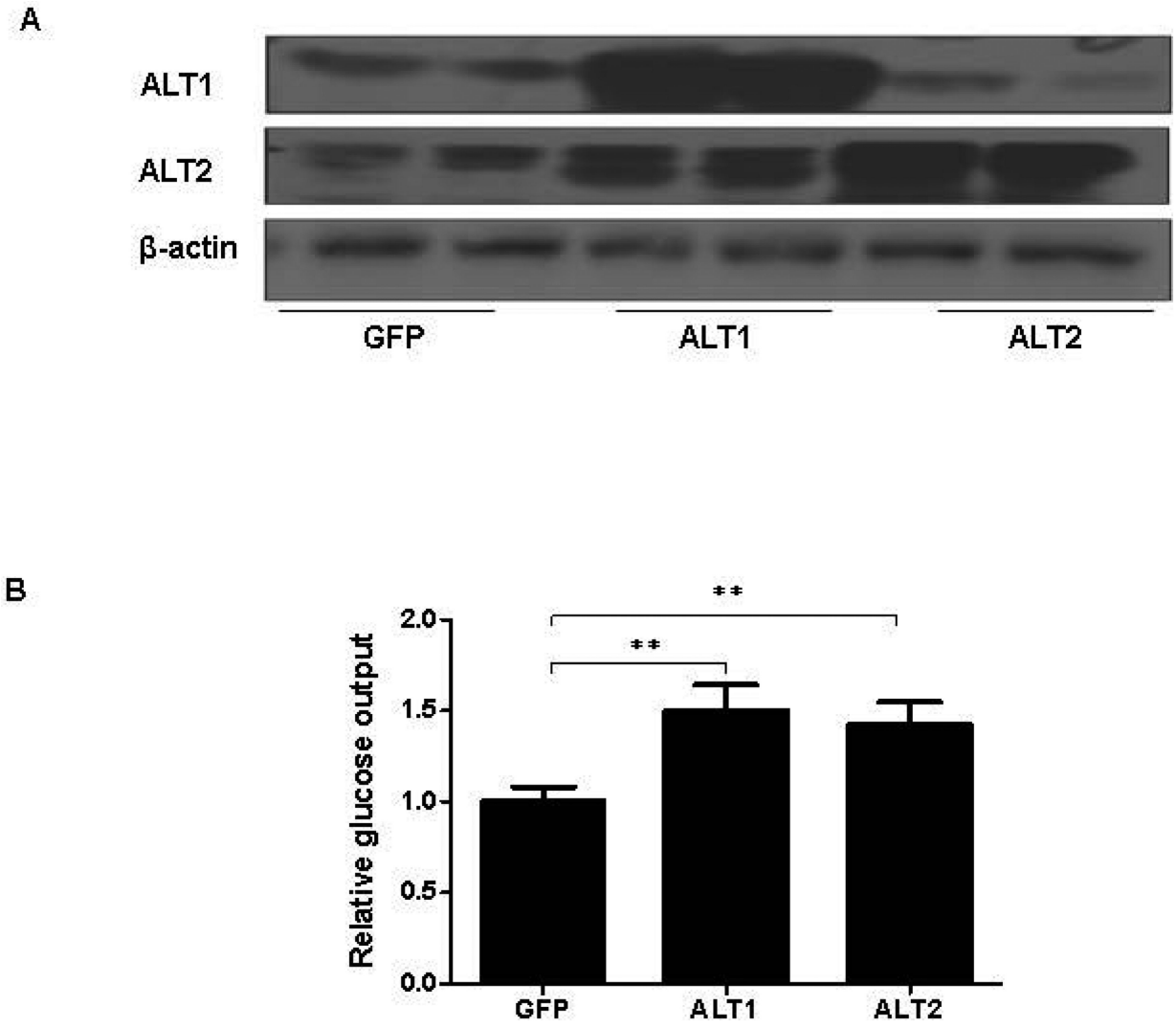


Figure 6.

Direct contribution of ALT1 and ALT2 to glucose production in Fao cells. Fao cells were infected with recombinant adenoviruses over-expressing ALT1, ALT2 or control GFP. Twenty-four hours post-infection, the regular glucose-containing culture medium was replaced with glucose-free medium and the cells were cultured for 4 hours. The amount of glucose in the culture medium was then measured, adjusted to the protein amount, and expressed relative to the GFP control. Data is expressed as mean ± S.E. (n = 6). **: $p < 0.01$; * $p < 0.05$.GFP-AD, ALT1-AD and ALT2-AD: cells infected with GFP, ALT1 and ALT2 adenoviruses (AD), respectively.