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ORIGINAL ARTICLE

The glucose-induced synthesis of insulin in liver

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Abstract Pancreatic β cells, stimulated by glucose, are known to synthesize and secrete insulin. As liver diseases are reported to cause diabetes mellitus, studies were conducted to determine the possibility of glucose-induced insulin synthesis in the liver cells. The glucose-induced insulin synthesis was determined by in vitro translation of mRNA from the hepatocytes. The cDNA from mRNA was prepared and sequence analysis was performed. Incubation of hepatocytes from the liver of adult mice ($n = 10$) with glucose (0.02 M) resulted in the insulin synthesis [0.03 (mean) $\pm$ 0.006 (S.D.) μunits/mg/h] compared to the pancreatic β cells [0.04 $\pm$ 0.004 μunits/mg/h]. Immunohistochemical study also demonstrated the glucose-induced synthesis of insulin in liver cells. Incubation of the mice hepatocytes with glucose resulted in the synthesis of insulin mRNA. The purified mRNA which was used to prepare cDNA resulted in the formation of proinsulin I and proinsulin II genes corresponding to 182 and 188 base pairs, respectively. Sequence analysis of the cDNA indicated that proinsulin I as well as proinsulin II gene could be involved in the synthesis of insulin by hepatocytes. These results suggested that insulin synthesis in both hepatic and pancreatic cells could be involved in the control of diabetes mellitus.

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Introduction

Insulin, an essential hormone for carbohydrate metabolism is believed to be produced in the β cells in the islets of Langerhans in the pancreas in response to the increased glucose levels in the circulation [1]. It has been reported that glucose has a critically important role not only in the synthesis of insulin in the pancreatic β cells, but the sugar is also reported to release the preformed insulin from the granules in these cells [2]. It has also been reported that in the process of insulin synthesis in the mice pancreatic β cells, two non-allelic proinsulin I and proinsulin II genes are involved [3]. Subsequently, both proinsulin I and proinsulin II are converted to insulin by the removal of a peptide chain known as C-peptide, by the proteolytic cleavage of the precursors. Both insulin and C-peptide are kept in the secretory granules of the pancreatic β cells, and both are released in the circulation in equimolar quantities [4]. The glucose-dependent stimulation of insulin synthesis and release of the hormone in the pancreatic β cells are crucially important in the maintenance of the glucose homeostasis in the system.

It is generally accepted that no other cells possess the unique ability of the pancreatic β cells which can synthesize and release insulin when stimulated by glucose.

Although insulin is an essential hypoglycemic hormone in carbohydrate metabolism, the liver is known to play a critically important role in maintaining the systemic glucose homeostasis both for long and short periods of time [5]. Furthermore, diabetes mellitus is known to occur in

various liver diseases [6]. For example, chronic hepatitis has been reported to be a risk factor for diabetes mellitus [7]. The development of diabetes mellitus in these conditions could, however, be related to the impaired pancreatic insulin synthesis or to the development of insulin resistance as a consequence of the existing pathologic condition itself. Alternatively, it could also be hypothesized that the development of the systemic hyperglycemic state in these liver diseases is due to the impairment of insulin synthesis in the liver cells which under normal condition synthesize and release the hormone when stimulated by glucose.

In this context, it should be also mentioned here that although the autoimmune disease has been reported to be the major cause of Diabetes mellitus I, it is known that even in those Diabetes mellitus I patients, after the onset of the disease, no insulin or very low insulin is required, referred to as the "honeymoon" period, indicating the possible existence of extrapancreatic availability of insulin. The occurrence of proinsulin gene in the hepatic cells has been reported before [8]. It has also been hypothesized that rat hepatic oval stem cells are capable of producing insulin when cultured in high glucose environment [8, 9]. We report herein, the glucose-induced synthesis and secretion of insulin in adult mice hepatocytes similar to or even more than that in the case of pancreatic β cells on mg/mg basis.

Results

The staining of protein bands from different cell suspension incubated with glucose in the SDS-polyacrylamide gels after electrophoresis by Coomassie blue and by immunoblot technique using anti insulin antibody

Since hepatocytes comprise 70–80% of the parenchymal liver cells' mass [10], to determine whether liver is capable of synthesizing insulin when stimulated by glucose, the hepatocytes suspension from the liver of adult mice was prepared [11]. The hepatocyte suspension [11] was incubated with different amounts of glucose (0–0.11 M) at 37°C for different periods. After incubation, the production of insulin, if any, was determined by immunoblot technique using anti insulin antibody (Fig. 1) [12]. It was found that the treatment of either hepatocyte suspension (Fig. 1, panel E_1 and E_2) [11] or the islets of Langerhans [13] (Fig. 1, panel C_1 and C_2) with 0.2 M glucose (optimal concentration as determined in separate experiment), resulted in the appearance of insulin in the supernatant of the incubation mixtures. Treatment of muscle cell suspension [14], well known target cells for insulin effect [15], with glucose under identical conditions failed to show the presence of insulin in the supernatant (Fig. 1, panels F_1 and F_2).

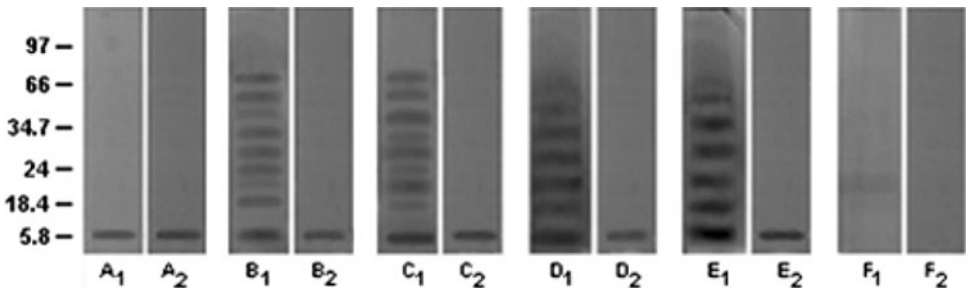

Fig. 1 The staining of protein bands from different cell suspension incubated with glucose in the SDS-polyacrylamide gels after electrophoresis by Coomassie blue and by immunoblot technique using anti insulin antibody. Hepatocytes, islets of Langerhans and epitroclearis muscle cells were prepared from adult mice [11, 13, 14]. The hepatocytes (25 mg/ml, suspended in HEPES) or islets of Langerhans (25 mg/ml, suspended in Krebs bicarbonate buffer) or epitroclearis muscle cells (30 mg/ml, suspended in Tyrod's buffer) were incubated with 0.02 M glucose for 30 min at 37°C. The supernatant was collected and subjected to SDS polyacrylamide gel electrophoresis. After the electrophoresis, protein bands were stained with Coomassie blue [33] and immunoblot technique [32] using anti insulin antibody. The figure shown is a typical representative of at least five different experiments using five different mice. A_1 Pure insulin stained by Coomassie blue. A_2 Insulin stained by immunoblot. B_1 Supernatant from the reaction mixture containing pancreatic islets stained by Coomassie blue. B_2 The same supernatant B_1 stained by immunoblot. C_1 Supernatant from the reaction mixture containing the pancreatic islets treated with glucose, stained with Coomassie blue. C_2 The supernatant C_1 stained by immunoblot. D_1 Supernatant from the reaction mixture containing hepatocytes stained by Coomassie blue. D_2 The same D_1 supernatant stained by immunoblot. E_1 The supernatant from the reaction mixture containing hepatocytes treated with glucose, stained by Coomassie blue. E_2 The same supernatant E_1 stained by immunoblot. F_1 Supernatant from the reaction mixture containing epitrochlearis muscle cells treated with glucose, stained with Coomassie blue. F_2 The same supernatant F_1 stained with immunoblot. Figure shown is a typical representation of five similar experiments using five different animals

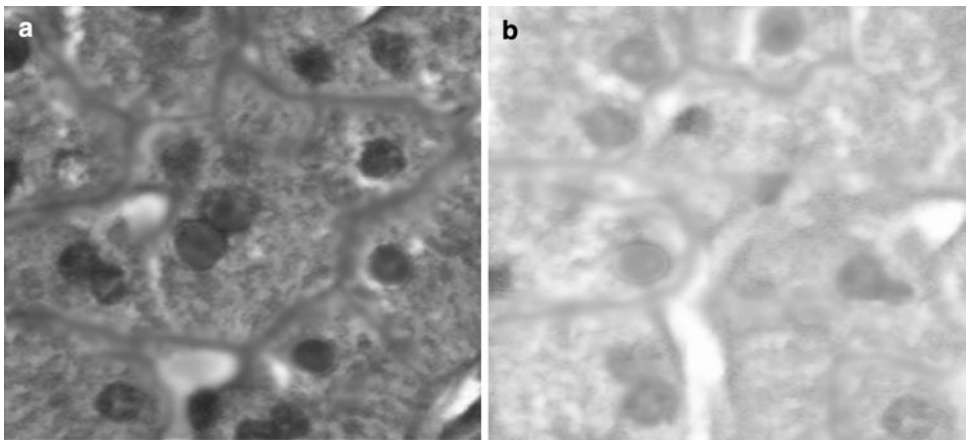

Fig. 2 Immunohistochemical study of insulin secretion in mice hepatocytes incubated with and without glucose. **a** represents insulin production in hepatocytes incubated with glucose, and **b** represents the same hepatocytes not treated with glucose. The same anti insulin antibody was used for the immunohistochemical studies in both cases. The non-weighted images were captured in a high resolution digital color camera and are typical representatives of six other similar experiments using six different mice

Immunohistochemical study of insulin secretion in mice hepatocytes incubated with and without glucose

In a parallel immunohistochemical study [16], using anti insulin antibody [12], it was found that the treatment of hepatocytes with glucose as described under Fig. 1 demonstrated the presence of insulin in hepatocytes (Fig. 2, Panel A) when compared to the appropriate control using the hepatocytes preparation not treated with glucose that showed little or no synthesis of insulin as demonstrated by the immunohistochemical study (Fig. 2, Panel B).

Kinetics of release of insulin from the pancreatic islets of Langerhans and from the hepatocytes of liver of adult mice

In parallel experiment, the rate of appearance (release) of insulin in the incubation mixture was determined by enzyme-linked immunosorbent assay (ELISA) as described [17] (Fig. 3). For comparison, in parallel experiment the release of insulin from the mice pancreatic islets of Langerhans [13] was also similarly determined (Fig. 3). It was found that the rate of insulin release in the reaction mixture in the case of hepatocytes was comparable to that in the case of islets of Langerhans. It was found that the rate of insulin release both in the case of islets and the hepatocytes was maximally achieved after 30 min of incubation with the sugar at 37°C. It was noted that the initial rate of release of insulin in the pancreatic islets was greater than that in the hepatocytes. However, it was also noted that the rate of ebbing of the hormone release in the case of hepatocytes was much slower than that in islets, and thereby hepatocytes provide a more sustainable release of insulin compared to that in the pancreatic islets at least in vitro.

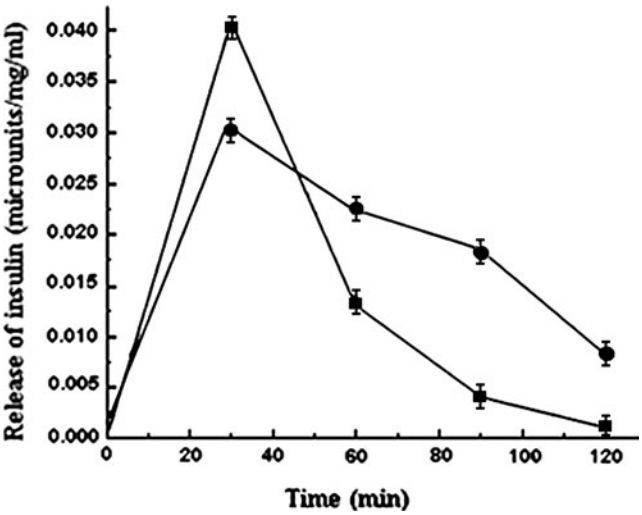

Fig. 3 Kinetics of release of insulin from the pancreatic islets of Langerhans and from the hepatocytes of liver of adult mice. The islets of Langerhans from the pancreas and the hepatocytes from the liver were prepared [11, 13] and suspended in the reaction mixture in the presence of 0.02 M glucose as described. After different periods of incubation as indicated, the supernatants were collected by centrifugation, and insulin was quantitated by ELISA. *The solid squares* represent the islets of Langerhans and *the solid circles* indicate hepatocytes. Each point represents mean ± SD (standard deviation) of at least five experiments using five different animals each in triplicate

Synthesis of insulin in the mice hepatocytes suspension and the quantitation of the hormone by ELISA and by bioassay

To determine whether the glucose-induced production of insulin in the reaction mixture was due to the release of the

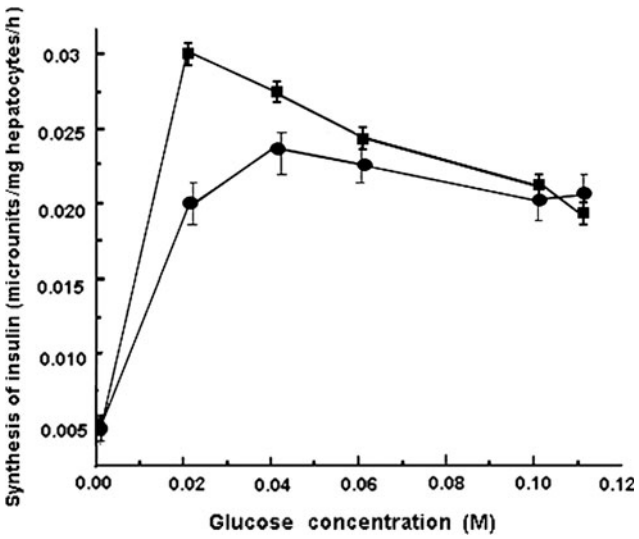

Fig. 4 Synthesis of insulin in the mice hepatocytes suspension and the quantitation of the hormone by ELISA and by bioassay. The hepatocytes suspension of the liver of adult mice was prepared and incubated with different amounts of glucose as indicated for 30 min at 37°C. The messenger RNA (mRNA) was isolated and translated in vitro for the synthesis of insulin. The synthesized hormone was quantitated by both ELISA [17] and by bioassay using diabetic mice [20]. While *the solid squares* represent ELISA for insulin, *the solid circles* indicate bioassay for insulin. Each point represents mean ± SD of five different experiments using five different mice each in triplicate

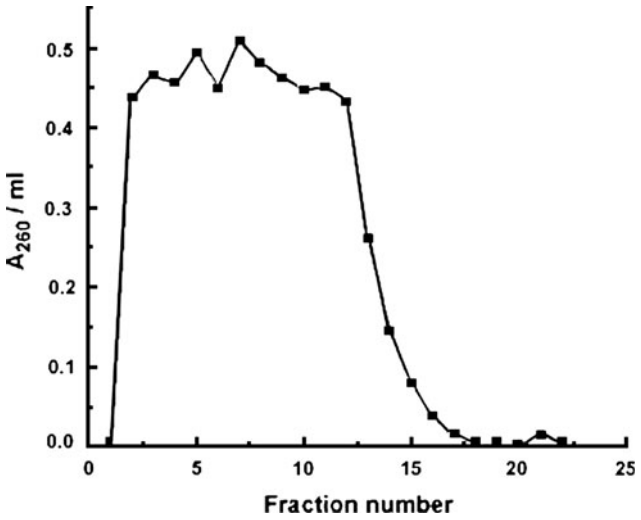

Fig. 5 Purification of insulin mRNA by oligo(dT)-cellulose from the total isolated mRNA from mice hepatocytes. Hepatoctyes were isolated from the adult mice as described [11]. Hepatocytes were suspended in buffer consisting of 10 mM HEPES solution (pH 7.4) and incubated with glucose for 30 min at 37°C. After incubation the mRNA was isolated by Trizol method. Total glucose treated mRNA was purified by oligo(dT)-cellulose. The fractions were eluted with eluting buffer containing 10 mM Tris (pH 7.5); 1 mM EDTA and 0.05% SDS. Each fraction was translated in vitro using plant ribosomes as described [19]. The fractions [5–10] containing the highest activity for the in vitro translation of insulin were pooled

preformed insulin into the medium from the hepatocytes or due to the glucose stimulated actual synthesis of the hormone in these cells, the hepatocytes suspension was treated with different amounts of glucose for 30 min at 37°C. The mRNA was subsequently isolated [18] from the hepatocytes and was translated in vitro for the synthesis of insulin as described [19]. The amount of insulin synthesized in the reaction mixture was determined both by ELISA as described above and by the bioassay of insulin [20] using diabetic mice (Fig. 4). To determine whether the insulin so detected in glucose treated hepatocytes is functionally active, bioassay was performed where diabetic mice was injected with the supernatant of the glucose treated hepatocytes supernatant. As shown in the Fig. 4, there was a marked lowering of the blood glucose level as confirmed by the determination of the blood glucose. It was found that the blood sugar level in the alloxan-treated diabetic mice that weighs 600 mg/ml before the injection of supernatant lowered to 350 mg/dl ($P < 0.0001$; $n = 5$) after the injection of 0.25 ml of the supernatant that was quantitatively equal to 0.022 ± 0.002 μunits of insulin (monocomponent human insulin of r-DNA origin).

Both assays demonstrated the glucose-stimulated insulin synthesis in hepatocytes as determined by in vitro translation of the mRNA. It was found that the maximal in vitro synthesis of the hormone in the isolated hepatocytes occurred in the presence 0.02 M glucose in the reaction mixture. Addition of higher amounts of glucose in the hepatocytes suspension, however, resulted in the decreased synthesis of insulin in these cells.

Purification of insulin mRNA by oligo(dT)-cellulose from the total isolated mRNA from mice hepatocytes

In order to purify the insulin mRNA from mice hepatocytes, the glucose-treated mRNA from hepatocytes was applied to an oligo(dT)-cellulose column and optical density measured at 260 nm [21]. The fractions were eluted with eluting buffer containing 10 mM Tris (pH 7.5); 1 mM EDTA and 0.05% SDS. The fractions were translated in vitro using plant ribosomes as described [19]. The fractions [5–10] showing the highest activity for the in vitro translation of insulin were pooled (Fig. 5).

Agarose gel electrophoresis of proinsulin I and proinsulin II gene (cDNAs) prepared from the mRNA of liver hepatocytes incubated with glucose

It has been reported before that in the synthesis of insulin in the pancreatic β cells of the mice, 2 proinsulin genes (viz. proinsulin I and proinsulin II) are involved [3]. To characterize these genes [22], the purified mRNA was used and

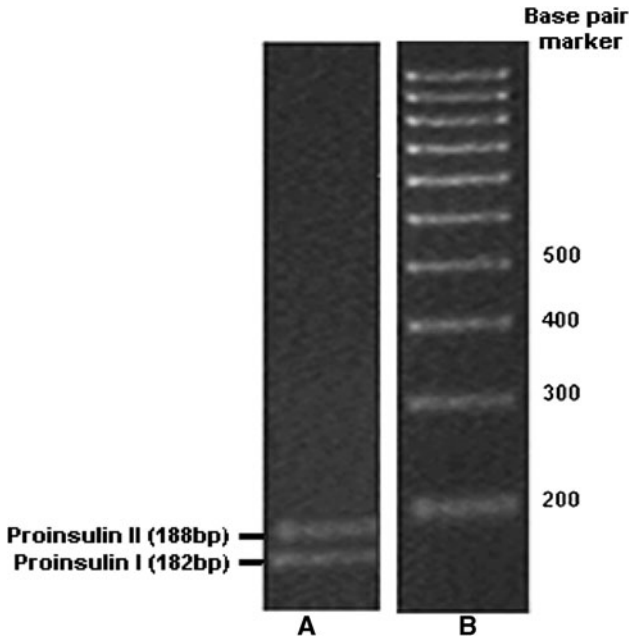

Fig. 6 Agarose gel electrophoresis of proinsulin I and proinsulin II gene (cDNAs) prepared from the mRNA of liver hepatocytes incubated with glucose. The fractions containing mRNA for the highest synthesis of insulin was pooled, concentrated and used for the synthesis of cDNAs by RT-PCR as described and the RT-PCR products were subjected to gel electrophoresis using 1% agarose gel. cDNAs were prepared from the purified mRNA. *Panel A* represents two bands, proinsulin I gene (182 bp) and proinsulin II gene (188 bp) [3]. *Panel B* represents the standard base pair marker. The bands were visualized under UV light as described [23]

a reverse transcriptase-polymerase chain reaction (RT-PCR) method which has been reported to allow identification and estimation of the amounts of mRNA transcribed from each of the proinsulin genes [3] was used. The RT-PCR product obtained from the insulin mRNA of the glucose-treated mice hepatocytes were subjected to agarose gel electrophoresis. This included the incorporation of ethidium bromide in the sample which allowed the clear visualization of the two proinsulin bands immediately after the gel was run upon illumination with UV light [23]. It was found that as in the case of mice pancreatic β cells, in the hepatic insulin synthesis, 2 different genes, presumably proinsulin gene I (182 bp) and proinsulin gene II (188 bp) were involved (Fig. 6).

The sequence of the cDNA from the liver of mice representing proinsulin II (A) and I gene (B)

The nucleotide sequence [22] of the presumed proinsulin I and proinsulin II genes were determined (Fig. 5, panel A) and alignment of the sequences were matched with that of the known DNA sequence of mice insulin genes (Pearson) [24], it was found that in the mice hepatocytes the proinsulin II gene alignment score was 80% compared to mice proinsulin I gene (Fig. 7, panel A), but the proinsulin I sequence of the hepatocytes scored 66% when compared to the mice pancreatic proinsulin I gene (Fig. 7, panel B). These results suggested that like the pancreatic β cells in the mice [3], the glucose-induced synthesis of insulin in hepatocytes was probably mediated both through the proinsulin I gene as well as proinsulin II gene.

Discussion

Although various cells have been reported to express proinsulin genes [8, 9, 25], it is currently believed that only the pancreatic β cells which when stimulated by glucose are able to synthesize and secrete insulin for the maintenance of systemic glucose homeostasis. In this context, it should be mentioned as reported above that after the initial onset of Diabetes mellitus I, there is the evidence of the existence of a "honeymoon" phase marked by a control of the hyperglycemia in the presence of very low doses of externally administered insulin or even no insulin at all [26]. However, we have found the plasma insulin level in Diabetes mellitus I was not that low but was 5–8 μunits/dl ($n = 29$) (unpublished) that could be due to the methodology used. It might be suggested that the euglycemia during the "honeymoon" phase was due to the possible existence of extrapancreatic insulin synthesis and secretion, and at least in this phase there might be the hepatic insulin synthesis. Our results as presented above strongly suggested that besides the pancreatic β cells, the adult hepatocytes from the liver were capable of synthesizing and secreting insulin when stimulated by physiological amounts of glucose usually present in the circulation.

That liver cells can make insulin when stimulated by glucose and in greater quantities than that of pancreas itself, demonstrate a hitherto unknown role of liver in the glucose-stimulated insulin production which is currently believed to be a unique property of pancreas. The glucose-induced synthesis of insulin in the hepatocytes has been demonstrated by four independent methods: (I) The production of insulin in liver cells could be shown by ELISA using anti insulin antibody which showed an analytical precision of the assay by more than 95% (Fig. 3). (II) The glucose-induced synthesis of insulin in the liver cells could be shown by the bioassay in diabetic mice (Fig. 4) indicating that the ELISA assay was not merely due to a cross reactivity of insulin antibody against unknown protein containing similar epitopes of insulin. (III) Immunohistological studies of the liver cells exposed to glucose showed the active synthesis of insulin in comparison to identical preparation of liver cells in the absence of sugar under similar experimental conditions (Fig. 2). Had the presence of insulin in the glucose-treated liver cells was due to the

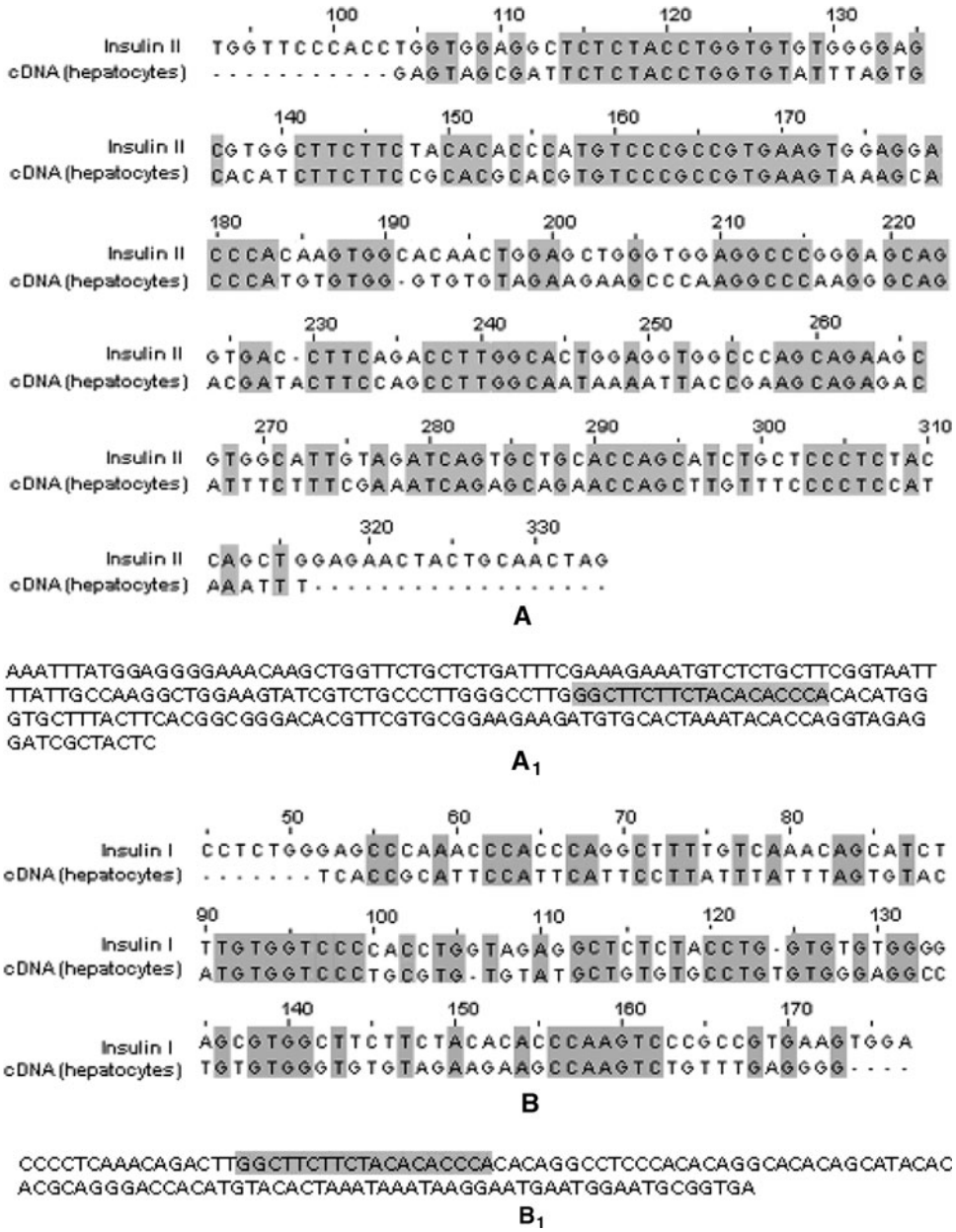

Fig. 7 The sequence of the cDNA from the liver of mice representing proinsulin II (**a**) and I gene (**b**). The aligned sequences of the cDNAs obtained from the liver represents proinsulin I and II gene of mice [24]. The alignment score was 80 in case of proinsulin II gene and 66 in case of proinsulin I gene. The sequences were matched with the known DNA sequence of mice insulin. The *shaded areas* represent the matched alignment sequence. Reverse Primer was used for sequencing and the region of primer have been illustrated in the amplified complementary strand of the cDNAs. The same experiment of sequencing was carried out using the forward primer also with similar results.
A Alignment of the sequence of the cDNA from the glucose treated hepatocytes matched with proinsulin II. A_1 Amplified complementary strand of the cDNA of proinsulin II. The *shaded region* represents the primer. *B* Alignment of the sequence of the cDNA from the glucose treated hepatocytes with proinsulin gene I. B_1 Amplified complementary strand of the cDNA of proinsulin I. *The shaded region* represents the primer

transport of the preformed hormone in the pancreatic cells to the liver was true, then the control liver cell preparation where only glucose was absent should have shown the active synthesis of insulin. (IV) Finally, not only glucose-induced synthesis of insulin mRNA could be used to synthesize insulin in in vitro translation, but the use of the insulin mRNA when cloned by using RT-PCR clearly demonstrated the presence of proinsulin I and II as in the case of pancreatic β cells which are involved in the synthesis of insulin in the pancreas (Fig. 7).

These results also explain at least, partly, how liver diseases can lead to the Type I diabetes mellitus in that damage of liver cells can cause diabetes mellitus due to impaired insulin synthesis. More interestingly, in contrast to the currently held belief that pancreas is the major source for systemic insulin production, our results demonstrated that liver was probably a more efficient organ for the systemic insulin synthesis in the presence of glucose than that by pancreas itself.

As discussed above, liver has an important role for glucose homeostasis both for long and short periods of time [5]. Our results demonstrated that glucose-induced insulin synthesis in the liver might itself be involved in the maintenance of glucose homeostasis, and not merely due to the supply of glucose in the circulation from the liver produced by the break down of glycogen.

As 50% of the insulin produced in the pancreas is known to be destroyed by the liver [27], it could be suggested that the

hormone produced in the hepatocytes might compensate for the hormone destroyed in the organ which was necessary for the systemic carbohydrate metabolism. It is well known that although hepatocytes possess insulin receptor on their surface [28], the uptake of glucose in these cells is known to be insulin independent process [29]. Since glucose was found to be capable of inducing synthesis and release of insulin from the hepatocytes as described above, it is possible that the insulin produced in the liver was acting like an autocrine or paracrine hormone or both.

Our results indicated that the amounts of insulin synthesized in the pancreatic islets (0.04 ± 0.004 μunits/mg/h) was roughly similar if not a little higher than that produced in the hepatocytes (0.03 ± 0.006 μunits/mg/h). Alignment of cDNAs with proinsulin I and II genes indicated that while the alignment of proinsulin II was 80%, the alignment score in case of proinsulin I was 60% (Fig. 7). Alignment score between 60 and 80% in DNA sequence is accepted to be highly significant in the 100% homology of the gene involved [30] for the presence of proinsulin I and proinsulin II genes in the hepatocytes. It must be noted that as demonstrated in Fig. 5, mRNAs produced by proinsulin I and II genes can be translated to insulin and both proinsulin I and II are capable of synthesizing insulin in the hepatocytes. It must, however, be mentioned that the mRNAs produced by these proinsulin genes as isolated by oligo-dt-cellulose chromatography could not separate the two different population of mRNAs. These mRNAs nevertheless were translated in vitro to produce insulin as determined by ELISA and by bioassay. As shown in the Fig. 6, two bands representing proinsulin I and proinsulin II genes of 182 and 188bps, respectively, are observed when the cDNAs from the mRNAs were subjected to agarose gel electrophoresis. Therefore, it is apparent that Oligo-dT cellulose was not capable of isolating the two different proinsulin mRNAs.

The liver, in mice as in the case of man, is much heavier (8gm) than the pancreas (0.7 g), and 70–80% of the liver mass is due to the hepatocytes, and, as such, the total amounts of insulin produced in the liver could be substantially higher than that synthesized by the pancreatic islets.

Studies by numerous investigators have implied that many liver diseases may cause diabetes mellitus [6, 7]. However, the mechanism for the development of insulin-dependent diabetes mellitus remains speculative [31]. Our results that hepatocytes when stimulated by glucose were capable of producing insulin, might offer an explanation for the development of diabetes mellitus due to the hepatic impairment of insulin synthesis and might suggest the development of insulin-dependent diabetes mellitus (Type I) that may not be related to the impaired insulin synthesis in pancreas only, but might also be due to the derangement of hepatic insulin synthesis as well.

Although in mg/mg basis, the amount of insulin produced in the hepatocytes (0.03 ± 0.006 μunits/mg/h) is lower than that in the pancreas (0.04 ± 0.004 μunits/mg/h), however, as the mass of liver in mice, as in the case of human, is much heavier than pancreas, the total amount of insulin synthesized by the liver is more than 8-fold greater than that synthesized by the pancreas. If this data could be extended to man, another mammalian group, it could be inferred that liver is the major source of insulin compared to pancreas. However, the correlation between the liver and pancreas to meet the demand of the system is not known. In this context, probably there must be the existence of some "cross talk" for the production of insulin between the two organs. Although the cause of Type II diabetes, the most common form of diabetes remains obscure, it perhaps could be hypothesized that the production of glucose-induced insulin synthesis in the liver has an important role in the development of diabetes mellitus II.

Materials and methods

Ethical clearance

The protocol was approved by the Internal Review Board, Sinha Institute of Medical Science and Technology, Calcutta. This study used normal white mice (*Mus musculus*) and adult New Zealand rabbit. Appropriate permission was also obtained from the IRB.

Chemicals

Goat anti-rabbit immunoglobulin G-alkaline phosphatase, HEPES(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, insulin specific primers, and Revert Aid M-MulV reverse transcriptase (MBI Fermentas) were obtained from Sigma Chemical Co. ELISA Maxisorb plates were from Nunc, Roskilde, Denmark. All other chemicals were of analytical grade.

Preparation of islets of Langerhans from the mice pancreas

The islets of Langerhans were prepared as described [13]. The islets were suspended in Kreb's bicarbonate buffer (pH 7.4) and used within 1 h of the preparation.

Preparation of hepatocytes from the mice pancreas

Typically, adult mice were killed by cervical dislocation and the liver was perfused by collagenase in a buffer consisting of 10 mM HEPES solution (pH 7.4) without glucose as described [11].

Immunoblot analysis of insulin in the supernatant of mice hepatocytes

The presence of insulin in the crude supernatant of hepatocytes treated with glucose and hepatocytes alone was identified by immunoblot technique [32]. The samples were subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue [33]. The transfer of the separated protein bands were next carried out electrophoretically to a nitrocellulose membrane. Insulin was identified by using anti insulin antibody as described elsewhere [12].

Immunohistochemical localization of insulin in mice hepatocytes

Mice liver was sliced into 6–8 mm sections in a cryostat in order to demonstrate the presence of insulin in the hepatocytes. These sections were incubated with anti-rabbit polyclonal antibody against insulin (1:100 dilutions) and identified by using anti-rabbit immunoglobulin G-alkaline phosphatase as described elsewhere [16]. Following immunohistochemistry, sections were imaged using an upright microscope attached to a high resolution digital color camera which was used to capture the non-weighted images to avoid biasedness.

Determination of the synthesis and release of insulin

The glucose-stimulated insulin synthesis was determined by incubating the islet preparation with different amounts of glucose in Tyrod's buffer (pH 7.4) for different time at 37°C. After incubation, the nucleic acid which contained insulin mRNA was isolated [18] and translated in vitro using plant ribosomal particles as described [19]. The synthesized insulin was determined by ELISA as described below. The release of insulin from the islets of Langerhans in the assay medium was determined by centrifuging the assay mixture at 10,000×g for 10 min at 0°C, and the clarified supernatant was used for the assay of insulin by ELISA.

ELISA for insulin

Polyclonal antibody against pure insulin was raised in adult New Zealand rabbit by intradermal injection of insulin emulsified with Freund's adjuvant as described [12]. The antibody titer was determined by Ouchterlony method [34].

The feasibility of the determination of insulin by ELISA was tested by immunoblot technique [32]. Typically, the upper and lower limits of insulin that could be determined by ELISA assay was carried out by constructing a standard curve using pure insulin. For insulin assay, only the linear portion of the standard curve was used. In the linear portion of the constructed curve, as little as 0.008 μunits/100 µl and as high as 2.44 μunits/100 µl of insulin could be determined with high reproducibility and confidence. However, in actual assay it was sometimes necessary to dilute the concentrated sample to fit into the linear portion of the curve. In the analytical recovery assay, a known quantity of pure insulin in the probable ranges was added, that could be present, in the assay mixture for the determination of insulin by the recovery experiments.

The analytical precision of the assay for insulin was determined by "recovery" experiments which indicated >95% of the added insulin to the sham reaction mixtures for the insulin synthesis that could be accounted for by the ELISA.

Bioassay of insulin using diabetic mice

Adult healthy mice as examined by a licensed veterinarian, irrespective of gender, were used for the study. A standard pellet diet and water were given ad libitum. Prior to the induction of diabetes, the mice were fasted for 18 h. Intraperitoneal injection of 150 mg/kg body weight of alloxan dissolved in normal sterile saline was administered to induce diabetes as described [20]. Confirmation of diabetes was made by the determination of blood glucose levels on the third day of administration of alloxan.

Ten mice used were divided into five equal groups:

1. Diabetic control group
2. Diabetic group injected with glucose treated hepatocytes supernatant.

The fasting blood glucose level in both groups of mice was measured by a glucose analyzer by collecting blood from the tip of the tail vein.

RNA isolation and cDNA preparation

The mRNA was purified from total mRNA isolated from the glucose-treated hepatocytes. The total mRNA was purified by oligo (dT)-cellulose chromatography as described [21]. Each fraction was separately translated to determine the mRNA synthesis of insulin. Two fractions showing the highest activity for the synthesis of insulin was pooled, concentrated and used for RT-PCR. RT-PCR was done using the primer 5′(5′-GGCTTCTTCTACACACC CA-3′) as forward and 3′(5′-CAGTAGTTCTCCAGCTG GTA-3′) as reverse was purchased. Each cycle for PCR consisted of 1 min at 95°C, 1.5 min at 55°C, and 1.5 min at 72°C, and 35 to 40 cycles were carried out. The cDNA was synthesized from the purified mRNA using Revert Aid M-MulV reverse transcriptase (MBI Fermentas) as instructed by the manufacturer.

Sequencing and analysis

Sequencing of the PCR products were performed by ABI prism automatic DNA sequencer (PerkinElmer). Sequence alignment and data analysis were done using ClustalW software [24].

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