Alteration of the Gene and Protein Levels of Insulin-Like Growth Factors in Streptozotocin-Induced Diabetic Male Rats

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ABSTRACT. Insulin-like growth factor (IGFs: IGF-I and IGF-II) systems have been reported to be associated with the onset of diabetic mellitus. Therefore, we investigated the effect of diabetes on regulation of the IGF system in the liver, kidneys and heart, which are important organs in the pathogenesis of diabetes. The experimental groups were subdivided into three groups: 1) controls, 2) streptozotocin (STZ)-induced untreated diabetic group, and 3) an insulin-treated group (plus diabetic rats). In the present study, starting on the second day after STZ treatment, the diabetic group exhibited hyperglycemia, polyuria, and polydipsia, which are characteristic of diabetes mellitus. Serum levels of IGF-I were decreased, but those of IGF-II were increased in the diabetic group compared with the controls. The expression levels of IGF-I and IGF-II protein in the livers of the diabetic group had a similar pattern to the serum. In addition, the expression levels of liver IGF-I mRNA and IGF-II mRNA were decreased in the diabetic groups. In the heart, IGF-I levels were decreased, but IGF-II levels were increased in the untreated diabetic groups, which was consistent with the expression levels of their mRNA. However, both the IGF-I and IGF-II levels in the kidneys were increased in the untreated diabetic groups, but the mRNA levels were decreased. Insulin treatment ameliorated the changes of IGF system in the serum, liver, kidneys, and heart. In conclusion, diabetes induced alteration of the IGF system tissue-specifically, and this was blocked by insulin treatment.

KEY WORDS: diabetes mellitus, heart, insulin like growth factor, kidney, liver.

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Diabetes mellitus is associated with growth retardation and abnormality in a wide range of diverse organs [17]. The primary factor in regard to etiologic effect is known to be hyperglycemia [36]. Diabetic nephropathy is a serious complication that is characterized by renal hypertrophy and hyperfiltration [3]. In our previous reports [29, 31] and other report [24], dysfunction of the proximal tubule cells and glomeruli was implicated in the development of diabetic nephropathy. These reports suggest that kidneys are important in diabetic nephropathy. Hyperglycemia in diabetes results from a combination of increased hepatic glucose production and decreased metabolic clearance of glucose [9]. Holstein *et al.* [14] reported the clinical implications of hepatogenous diabetes in liver cirrhosis, suggesting an important role for the liver in diabetes. Diabetes mellitus is frequently associated with dysfunction of the heart [11]. A recent report demonstrated that serum biological markers are useful tools for assessing diabetic complications [18].

Insulin-like growth factors (IGFs) are major endocrine and paracrine regulators of tissue growth and metabolism. IGFs have been implicated as potentially important regulatory factors in insulin-dependent diabetes mellitus (IDDM) [2]. IGFs are mostly produced by the liver, and are also synthesized in many other tissues such as the kidneys and heart [5, 34]. Insulin-like growth factors (IGFs) mediate the early renal changes in diabetes mellitus, i.e. hypertrophy and hyperfiltration [6]. These findings suggest that serum, liver, heart, and kidneys are very important sites in the regulation

of the IGF system under diabetic conditions. Until now, a lot of evidences have been produced from studying the effect of diabetes on IGFs in the serum, liver, and kidneys [13, 26, 39]. However, the regulation of IGF-II secretion in diabetes has not been fully elucidated. Furthermore, the involvement of the heart in diabetes-induced alteration of the IGF system has not been examined. Therefore, we investigated the effect of diabetes on IGF-I and IGF-II gene and protein expression in the serum, liver, kidneys, and heart.

MATERIALS AND METHODS

Experimental animals: Eighteen male Sprague-Dawley rats (190–225 g, initial body weight) were purchased from Dae Han Experimental Animal Co, Ltd. (Chungju, Korea). The rats were housed at 24°C in a 12-hr day/12-hr light cycle and supplied with a standard pellet diet and tap water *ad libitum*. The rats were divided into following three groups of 6 animals each: Group 1: non-diabetic; Group 2: untreated streptozotocin-diabetic; and Group 3: insulin-treated streptozotocin-diabetic rats (treated with insulin for 21 days). The Principles of Laboratory Animal Care (NIH publication no. 85–23, revised 1985) were followed throughout the duration of the experiment and instructions given by our institutional ethics committee were followed regarding injections and other treatments of the experiment. Diabetes was induced by intravenous administration of streptozotocin (STZ) (65 mg/kg body weight; Sigma, ST. Louis, Mo., U.S.A.), dissolved in sodium citrate buffer (0.1 mol/l, pH 4.5) at a concentration of 20 mg/ml immediately before use. The control rats, which were matched for age

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and weight at time of STZ administration, received an equal volume of the vehicle. Rats were considered diabetic if their blood glucose levels were greater than 6.7 mmol/l 24 hr after injection. Human recombinant insulin (Humulin N, Eli Lilly, Indianapolis, Ind., U.S.A.; 10 IU/day) was administered subcutaneously once daily (at 10:00 hr). Insulin treatment began 1 day after STZ injection. In all groups, blood glucose levels were measured at the indicated times via tail vein sampling. The animals were individually housed in metabolic cages, and urine samples were collected at the indicated times. The rats were anaesthetized using sodium pentobarbital (35 mg/kg) and sacrificed by decapitation after cervical dislocation 21 days after administration of STZ. The liver and kidneys were removed quickly, rinsed in phosphate buffered saline, and frozen in liquid nitrogen for subsequent RNA isolation. The separated tissues were stored at -70°C until assayed. Serum was taken by centrifugation $1600 \times g$ for 30 min at 4°C and stored at -70°C until used for the experiment. In the serum glucose analysis experiment, a commercially available enzyme kit (Wako, Osaka, Japan) was used that made use of the O-toluidine method.

IGF-I/II radioimmuno assay (RIA): Rat IGF-I/II (GroPep Ltd.) were iodinated to a specific radioactivity of 150–300 $\mu\text{Ci/g}$ [^{125}I] (Amersham, Birmingham, England) using a modified chloramin-T (Kodak, U.S.A.) method, as described in our previous report [30]. The specific activity of the iodinated IGFs was approximately 60–110 $\mu\text{Ci}/\mu\text{g}$ protein. The iodination mixture was purified on a Sephadex G-50 column (1×50 cm) and pre-equilibrated with phosphate buffered saline (0.1 mol/L; pH 7.4). Serum and tissue IGF binding proteins (IGFBPs) were separated by the method of Lee and Henricks [22]. The IGF-I results were expressed in terms of nanograms of pure human IGF-I per ml assuming equal cross-reactivity of rat and human IGF-I in the RIA. Fifty microliters of rat polyclonal IGF-I/II antibodies (Santa Cruz, U.S.A.) diluted to 1:250 or 1,000 were added to 100 μl of each sample/standard, which was then incubated for 1 hr at room temperature. Subsequently 20,000 cpm of [^{125}I]-IGFs was added to the sample/standard, and then it was incubated for an additional 18 hr at 4°C . Fifty microliters of horse serum (Sigma, U.S.A.) was then added to the incubated sample, which was then centrifuged at $3,000 \times g$ for 30 min. The supernatant was discarded, and the radioactivity of the precipitate containing bound [^{125}I]-IGF was counted in a gamma scintillation counter (Wallac, Finland). All assays were performed in duplicate. Intra-assay and extra assay coefficients of variation for IGFs were 8% and 10% respectively.

IGF-I/II mRNA expression assay by quantitative competitive reverse transcription and polymerase chain reaction (QC RT-PCR): Total RNA was extracted from homogenised tissues using TRI reagent (Molecular Research Center, Cincinnati, OH, U.S.A.) according to the manufacturer's instructions. Reverse transcription (RT) was then performed in a final volume of 20 μl with 4 μg RNA and 200 U murine leukemia virus transcriptase (GIBCO BRL Life

Technologies, Gaithersburg, MD, U.S.A.) at 42°C for 60 min. PCR amplification was performed with 5 μl of RT product, 10 pmol of each primer, 1.25 U Taq polymerase (Promega, Madison, WI, U.S.A.), and 1 mM dNTP. After an initial incubation at 95°C for 3 min, 30 amplification cycles consisting of 95°C (1 min), 55°C (1 min), and 72°C (1 min) were performed, followed by 10 min of final extension at 72°C . The products were electrophoresed in $1 \times$ TBE buffer on 2% agarose gel containing ethidium bromide. Specific primer sets were designed from the following published cDNA sequences: IGF-I sense, 5'-CAC AGG GTA TGG CTC-3'; IGF-I antisense, 5'-CTT CTG GGT CTT GGG-3'; IGF-II sense, 5'-CGA TGC TGG TGC TTC TCA-3'; and IGF-II antisense, 5'-GGG GTC TTG GGT GGG TAG-3'. To quantify the expression of IGF-I and IGF-II gene copies, we established a quantitative competitive PCR assay using a deleted internal standard. The internal standard of IGF-I, a 56 bp-deleted from a 175 bp IGF-I PCR product, was made by digestion with Alu I and ligation in T-easy PCR cloning vector (Promega, Madison, WI, U.S.A.). The internal standard of IGF-II was made by partial digestion with Alu I from human IGF-II cDNA which deleted 113 bp, base pair numbers 108 to 222 in the translation site, of a 476 bp IGF-II PCR product, and ligation in T-easy PCR cloning vector (Promega, Madison, WI, U.S.A.). These internal standards were amplified with the same primers, and the copy numbers were determined by comparison of the band expressions and internal standards.

Statistical analysis: All values are expressed as group means $\pm$ SEM. Statistical significance of differences between groups was determined by Student's *t*-test and analysis of variance using SAS version 6 (SAS Institute, Cary, NC, U.S.A.). When the F test indicated differences between groups, the differences were separated using Duncan's multiple range test.

RESULTS

Basic physiologic parameters of diabetic animals: The level of blood glucose in the normal rats was about 5 mmol during the experiment periods. However, all animals given STZ (65 mg/kg, ip) demonstrated blood glucose concentrations above 20 mmol after 1 day (Fig. 1A). The changes in body weight are shown in Fig. 1B. We also examined the fractional urine glucose levels. As shown in Fig. 1C, there was a remarkable increase of urine glucose in the diabetic rats. These results suggest that the diabetic animals exhibited polyuria, proteinuria, and glucosuria. Insulin treatment ameliorated diabetes-induced dysfunction of physiologic parameters. Indeed, STZ-induced diabetic rats exhibited a decrease of plasma insulin concentration, which was blocked by insulin treatment (controls: 2.97 ± 0.40 ; STZ-induced untreated diabetic rats*: 0.50 ± 0.20 ; insulin-treated STZ-induced diabetic rats **: 2.33 ± 0.41 ng/ml; * $p < 0.05$ vs. control, ** $p < 0.05$ vs. STZ-induced diabetic rats).

The expression of IGF-I gene and protein in the serum, liver, kidneys and heart of diabetic rats: To investigate the

Fig. 1. The effect of insulin deficiency on blood glucose (A), body weight (B), and fractional urinary glucose (C). Rats were treated with streptozotocin, and insulin was administered to diabetic rats after 24 hr. Values are means $\pm$ SEM. * $P < 0.05$ vs. controls, ** $P < 0.05$ vs. diabetic rats.

effect of diabetes on IGF-I, IGF-I was measured in the serum, liver, heart, and kidneys of control and STZ-treated rats. As shown in Fig. 2, there was a significant decrease in the total IGF-I serum level in the STZ-induced diabetic rats compared with control rats. The IGF-I level in the liver was also decreased in STZ-induced diabetic rats. In addition, the IGF-I level in the heart was decreased in STZ-induced diabetic rats. In contrast, the IGF-I level in the kidneys was

increased in STZ-induced diabetic rats. Insulin treatment ameliorates diabetes-induced alteration of IGF-I levels. In the experiment to examine IGF-I mRNA expression in these tissues using QC-RT-PCR, there was an ethidium bromide staining pattern in the amplified RT-PCR product, demonstrating that the IGF-I mRNA corresponded to the length expected from the primer design (Fig. 3). Figure 3A shows that STZ-induced diabetic rats exhibited a decrease in liver IGF-I mRNA expression. IGF-I mRNA expressions in the heart and kidney were also decreased in the diabetic rats (Fig. 3B, 3C).

The expression of IGF-II gene and protein in the serum, liver, heart, and kidneys of diabetic rats: Next, we investigated the effect of diabetes on IGF-II gene and protein levels. Unlike IGF-I, the IGF-II level in the serum was increased in the STZ-treated diabetic rats (Fig. 4A). In addition, the IGF-II level in the liver, heart, and kidneys were also increased in the STZ-treated diabetic rats (Fig. 4B, 4C, 4D). The expression of IGF-II mRNA was also examined in the liver, heart, and kidneys. As shown in Fig. 5A and 5B, an inhibitory effect was shown for liver and kidney IGF-II mRNA expression in the STZ-treated rats. However, heart IGF-II mRNA expression was increased in the diabetic rats (Fig. 5C). Insulin treatment ameliorated diabetes-induced mRNA and protein expression of IGF-II in the liver, heart, and kidneys.

DISCUSSION

To investigate the effect of diabetes mellitus (DM) on the IGF system, we used accepted DM model rats injected with STZ. In accordance with the expected changes observed in rats with DM, the diabetic rats had higher plasma glucose levels, and urinary volumes, but lower total body weights compared to the controls. The decrease in body weight of diabetic rats is due to excessive breakdown of tissue proteins [1]. In the present study, the diabetic groups exhibited a loss of body weight and increase of blood glucose that was completely ameliorated by insulin treatment. This result is not in accordance with the reports of other investigators who demonstrated that insulin treatment slightly or negligibly ameliorates diabetes-induced weight loss [23, 36]. However, several studies have reported similar results, with insulin completely blocking diabetes-induced loss of body weight and increasing blood glucose [33, 36]. Although the reasons for this discrepancy are not clear, it may be due to different dosages of insulin and insulin treatment time after the onset of diabetes. Recently, Tang *et al.* [38] reported that a high dosage insulin therapy (6–10 U/kg), the concentration in this study, but not a low dosage insulin (2 U/kg), effectively reversed the loss of body weight.

A number of growth factors are involved in the development of diabetes mellitus. Among them, the IGF system may be associated with the onset of early diabetes mellitus [34]. In the present study, there was a decrease of IGF-I concentration in the serum of the diabetic rats. This result mimics the condition of patients with type I DM [37], who

Fig. 2. Effect of insulin deficiency on insulin-like growth factor (IGF)-I peptide content in the serum (A), liver (B), heart (C), and kidneys (D) of rats. Rats were treated with streptozotocin, and insulin was administered daily to diabetic rats after 24 hr. Values are means $\pm$ SEM. * $P < 0.05$ vs. controls, ** $P < 0.05$ vs. diabetic rats.

Fig. 3. Effect of insulin deficiency on insulin-like growth factor (IGF)-I mRNA by quantitative competitive reverse transcription polymerase chain reaction (QC-RT-PCR) in the liver (A), heart (B), and kidneys (C) of rats. Rats were treated with streptozotocin, and insulin was administered daily to diabetic rats after 24 hr. 1 denotes control, 2 diabetic rats, and 3 insulin-treated diabetic rats. The example shown is representative of four independent experiments.

exhibit reduced circulating level of IGF-I. The liver is a major site of IGF-I production [32]. In accordance with our hypothesis, the level of liver IGF-I was decreased in the STZ-induced diabetic rats. In the present study, the IGF-I level of the heart was decreased in the diabetic groups. This result suggests that the heart is the one of the candidates for reducing plasma IGF-I, although the liver is the main source. This result is not consistent with the report of Leaman *et al.* [21], who demonstrated that heart IGF-I protein was not altered in diabetes-induced pigs. However, several other investigators support our results. Norby *et al.* [27] reported that IGF-I attenuates diabetes-induced cardiac contractile dysfunction in ventricular myocytes. Kajstura *et al.* [16] reported that IGF-1 overexpression inhibited development of diabetic cardiomyopathy. The kidneys also are source of significant synthesis of IGF-I, some of which is released into the circulatory system. In the present study, the kidney IGF-I level in the diabetic rats was increased, unlike the serum IGF-I levels. Phillip *et al.* [32] suggested that transient accumulation of IGF-I in the kidneys of STZ-diabetic rats may not be due to an increase in local synthesis of IGF-I, but rather may be due to an increase in IGF-I uptake from the circulatory system. In the present study, the IGF-I mRNA content was significantly decreased in the liver of the diabetic animals. This result is supported by evidence that the decrease in IGF-I mRNA is accompanied by a direct reduction in the rate of IGF-I gene transcription in diabetes [28]. Recently, Yu *et al.* [40] also reported similar result that liver-specific IGF-I gene deficient mice exhibit accelerated diabetes in response to streptozotocin. In addition, IGF-I mRNA in the heart and kidneys was decreased in the diabetic group, which is in line with the report of Born-

Fig. 4. Effect of insulin deficiency on insulin-like growth factor (IGF)-II peptide content in the serum (A), liver (B), heart (C), and kidneys (D) of rats. Rats were treated with streptozotocin, and insulin was administered daily to diabetic rats after 24 hr. Values are means $\pm$ SEM. * $P < 0.05$ vs. controls, ** $P < 0.05$ vs. diabetic rats.

Fig. 5. Effect of insulin deficiency on insulin-like growth factor (IGF)-II mRNA by quantitative competitive reverse transcription polymerase chain reaction (QC-RT-PCR) in the liver (A), heart (B), and kidneys (C) of rats. Rats were treated with streptozotocin, and insulin was administered daily to diabetic rats after 24 hr. 1 denotes control, 2 diabetic rats, and 3 insulin-treated diabetic rats. The example shown is representative of four independent experiments.

feldt [4]. However, there are reports showing no change [10] or a STZ-dependent increase in kidney IGF-I mRNA levels [20]. It is likely that these inconsistencies reflect variations in the duration and severity of the diabetes, the sex and age of the studied animals, and the dose of STZ used to induce diabetes.

Up to now, IGF-II regulation of diabetic adult rats was not fully understood. Diabetes-induced changes in IGF-II have been reported in the serum of adult diabetic rats [2] and in the serum, liver, and kidneys of neonatal diabetic rats [7, 12, 33]. To our knowledge, no data has been reported concerning adult liver, kidney, and heart IGF-II during diabetes. In the present study, IGF-II serum level was increased. In addition, liver, kidney, and heart IGF-II level was increased in the diabetic rats. It is interesting to note the liver and kidney IGF-II mRNA level was decreased, but the heart IGF-II mRNA level was increased in the diabetic rats. These findings suggest that accumulation of IGF-II in the serum during diabetes results from increasing synthesis of IGF-II gene in tissues, such as the heart and other tissues, but not in the liver and kidneys. This obscure phenomenon may be due to differences in IGF-II regulation (local or endocrine). Indeed, diabetic neonatal rats also showed a similar result, exhibiting an increase in IGF-II renal concentration and a decrease in IGF-II mRNA [7]. Devedjian *et al.* [8] reported that transgenic mice overexpressing insulin-like growth factor-II in beta cells develop type 2 diabetes, suggesting a role for IGF-II. Our present results revealed the possibility that IGF-II is also involved in the development of diabetes mellitus. The major finding of the present study was that the IGF-II content in the heart was increased in the diabetic group. The involvement of IGF-II in diabetic cardiomyopa-

thy also has not been elucidated. However, a recent report revealed that IGF-II induces hypertrophy of adult cardiomyocytes, which is the characteristic of diabetic cardiomyopathy [15]. Therefore, in this study, we demonstrated evidence that IGF-II in the adult rat heart may be involved in development of diabetes mellitus. We also showed evidence that the increase of IGF-II in the adult kidneys might be a factor that contributes to development of diabetic nephropathy.

In the present study, insulin treatment ameliorated IGF-I and IGF-II protein and gene alteration in the serum, liver, kidneys, and heart, suggesting that these changes are dependent on insulin. The hypothesis that insulin plays a major role in IGF-I mRNA regulation is supported by evidence that an increase in the physiological concentration of insulin triggers the interaction of insulin-responsive nuclear binding protein with the IGF-I region V sequence, which is critical for transcriptional regulation based upon diabetes status [19, 28]. It has also been reported that IGF-I genomic templates containing region V demonstrate increased transcriptional activity using nuclear extracts from normal compared with diabetic animals [28]. Since, the level of insulin in the present study was decreased in the STZ-induced diabetic rats, the IGF-I region V sequence may have been downregulated. The levels of gene and protein expression between IGFs were mismatched in the liver and kidneys of the diabetic rats. Although several studies have reported mismatches between IGF protein and gene expression [7, 9, 34], the reasons for them have not been clarified. Thus, more precise time courses of the changes in the proteins and mRNAs needs to be obtained through further study. In summary, the results of the present study demonstrate that diabetic rats exhibited a decrease of IGF-I in the blood, and this may be contributed by the decrease of liver and heart IGF-I synthesis and secretion. Furthermore, the increase of serum IGF-II levels in diabetic rats is, in part, due to regulation of the heart and other tissues.

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