Enhanced proteolytic activity directed against the N-terminal of IGF-I in diabetic rats

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Abstract

We have recently identified in serum an acid protease which is capable of generating des(1–3)IGF-I from intact IGF-I. Here we have utilized a synthetic substrate with the sequence, biotin-G-P-E-T-L-C-BSA which contains the N-terminal sequence of IGF-I, to investigate the levels of this protease activity in streptozotocin-diabetic rats. Protease activity, quantified in terms of the amount of the biotin label lost, was determined in serum and hepatic extracts from normal control rats, diabetic rats and insulin-treated diabetic rats. Both the serum protease activity and protease activity in hepatic extracts were significantly increased in diabetic rats compared with control rats ($P<0.02$ and $P<0.005$). Following acute administration of insulin, a rapid and marked reduction in serum protease activity was observed; with an $\sim 50\%$ reduction apparent at 30 min ($P<0.001$). Chronic insulin treatment of dia-

betic rats also significantly reduced the serum and hepatic protease activity to the levels seen in control rats. A positive correlation between protease activity and serum glucose level was observed ($r=0.58$, $P<0.005$). The abundance of Spi 2.1 mRNA, a serine protease inhibitor, capable of inhibiting the IGF-I protease activity *in vitro*, was significantly decreased in the liver of diabetic rats and insulin treatment of diabetic rats did not normalize Spi 2.1 mRNA levels.

These data suggest that the conversion of IGF-I to the more active des(1–3)IGF-I variant may be enhanced in diabetic animals. Since serum IGF-I levels are reduced in diabetic rats, increased des(1–3)IGF-I-generating protease activity would enhance the functional activity of the circulating IGF-I.

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Introduction

The majority of insulin-like growth factor (IGF)-I and -II present in the circulation is bound to high affinity proteins, the IGF-binding proteins (IGFBPs). These binding proteins modulate the egress of the IGFs out of the vascular space and may serve to limit the hypoglycemic effects of the IGFs (Meuli *et al.* 1978, Bar *et al.* 1990, Clemmons 1990). We have previously identified an acid protease activity present in rat serum and tissue extracts which is capable of catalyzing the conversion of IGF-I to the des(1–3) variant (Yamamoto & Murphy 1994). This naturally occurring IGF-I variant is more potent than IGF-I in a variety of *in vitro* and *in vivo* assays, most likely because of its reduced affinity for IGFBPs (Ballard *et al.* 1987, Bagley *et al.* 1989, Gillespie *et al.* 1990, Tomas *et al.* 1991). Des(1–3)IGF-I has a modestly reduced binding affinity for IGFBP-3, the major binding protein in serum, but a markedly reduced affinity for IGFBP-1 (Clemmons *et al.* 1990). Although the concentration of the latter is approximately tenfold less than IGFBP-3, IGFBP-1 may

have a more important role in modulating free IGF-I levels and, in particular, the effects of IGF-I on glucose homeostasis. Unlike IGFBP-3, where serum concentrations are relatively stable, the levels of IGFBP-1 show marked fluctuations in relationship to food intake (Cotterill *et al.* 1988, Suikkari *et al.* 1988). Expression of IGFBP-1 is inversely regulated by insulin (Suikkari *et al.* 1988, Suwanickul *et al.* 1993). This effect is mediated via the protein kinase B/Akt pathway and a negative insulin response element located ~ 140 bp 5' of the RNA cap site (Unterman *et al.* 1992, Suwanickul *et al.* 1993, Robertson *et al.* 1994, Cichy *et al.* 1998). Serum IGFBP-1 concentrations are increased in diabetic rats (Unterman *et al.* 1989). Consistent with this reduced affinity of des(1–3)IGF-I for the binding proteins, the plasma clearance rate of des(1–3)IGF-I is approximately fivefold greater than IGF-I (Ballard *et al.* 1991).

Previous studies with protease inhibitors indicate that the protease which converts IGF-I to des(1–3)IGF-I is trypsin-like with an acidic pH optimum (Yamamoto & Murphy 1994). In addition, this protease activity is

inhibited by the growth hormone-dependent, α 1-antitrypsin-like protease inhibitor, serine protease inhibitor 2:1 (Spi 2:1) (Maake *et al.* 1997). The latter may account for the elevated levels of protease activity present in hypophysectomized rats (Yamamoto & Murphy 1995).

Uncontrolled diabetes mellitus is characterized by poor growth and development in both humans and rodents. A number of perturbations in the growth hormone/IGF/IGFBP system occurs in diabetic animals with the end result that both circulating IGF-I levels and somatomedin activity, as measured in cartilage bioassays, are low (Phillips & Young 1976, Phillips & Orawski 1977). The low levels of circulating IGF-I are likely to be a major cause of growth retardation, since administration of IGF-I can restore growth in diabetic rats without normalizing blood glucose (Scheiwiller *et al.* 1986, Tomas *et al.* 1993). Since proteolytic conversion of IGF-I to des(1-3)IGF-I represents a potential site at which the biological activity of IGF-I could be modulated, we utilized a protease assay based upon an immobilized biotinylated peptide to measure the protease activity directed against the N-terminal of IGF-I. We have previously shown that this assay measures the protease activity which is capable of generating des(1-3)IGF-I (Yamamoto & Murphy 1994). In addition, hepatic Spi 2:1 mRNA expression was measured in diabetic rats.

Materials and Methods

Animals and experimental design

Adult male Sprague-Dawley rats of 250–300 g body weight were obtained from the University of Manitoba breeding facility. They were housed under controlled environmental conditions (22 °C; lights on between 0700 and 1900 h). Diabetes was induced by a single i.p. injection of streptozotocin (STZ; 75 mg/kg body weight) dissolved in citrate buffer (pH 4.5). Control rats received an equivalent volume of 0.9% (w/v) NaCl. Saline-treated control rats and STZ-treated rats were killed by decapitation 6 days after treatment and trunk blood was collected. Insulin pellets (~2 U/day; Linplant; Linshin Canada, Inc., Scarborough, Ontario, Canada) were implanted in the dorsal subcutaneous tissue 5 days after STZ administration in another group of diabetic rats. These rats were killed 3 days after implantation of the insulin pellets.

To investigate the acute effect of insulin treatment on IGF-I protease activity, diabetic rats were anesthetized with a mixture of 20 mg ketamine hydrochloride and 3 mg xylazine (MTC Pharmaceuticals, Cambridge, Ontario, Canada) administered by an i.p. injection. Blood (100 μ l) was sampled via a heparinized femoral artery catheter and 5 U regular insulin (Novo Laboratories Ltd, Willowdale, Ontario, Canada) was administered i.p. at time zero. Blood samples were subsequently withdrawn after 15, 30, 60, 120 and 180 min.

In a separate experiment, saline-treated control rats and STZ-treated rats were killed by decapitation 6 days after treatment. A group of five STZ-treated rats was killed 2 h after receiving a single i.p. injection of regular insulin (Humulin R; Eli Lilly Canada, Ontario, Canada). Serum and liver tissue were collected from these rats and processed as described below.

All the blood samples were immediately put on ice, allowed to clot, and serum was separated by centrifugation. Serum glucose level was measured within 2 h of blood collection using a YSI 2300 glucose analyzer (Yellow Springs Instrument Co., Yellow Springs, OH, USA) and the residual serum was stored at –20 °C for up to 3 days prior to assay of protease activity. Liver tissue was rapidly frozen and stored at –70 °C until processed for protease extraction or mRNA preparation. All animal experimentation was performed in accordance with protocols approved by the Animal Care Committee of the Faculty of Medicine, University of Manitoba.

Assay of protease activity

Serum IGF-I protease activity was measured using a synthetic substrate with the sequence, biotin-G-P-E-T-L-C coupled to BSA (Biochem Immunosystems Inc., Montreal, Canada) as described previously (Yamamoto & Murphy 1994). The sequence of this peptide corresponds to the N-terminal end of IGF-I and includes the site of cleavage of intact IGF-I which results in the generation of des(1-3)IGF-I. We have previously demonstrated that the enzyme activity partially purified from rat serum using this assay system is able to convert IGF-I to des(1-3)IGF-I (Yamamoto & Murphy 1994).

The peptide-BSA conjugate (the equivalent to 50 ng peptide) was coated onto 96-well microtiter plates (Nunc, Kamstrup, Denmark) in 200 μ l 0.1 M carbonate buffer (pH 9.6). The plates were subsequently blocked with 0.5% BSA in the same buffer and washed twice with Tris-buffered saline (TBS; 0.5 M, pH 7.5 containing 0.05% Tween 20). Serum samples diluted 1/4 in TBS containing 0.1% BSA were added in a total volume of 200 μ l to each well and the microtiter plate was incubated at 37 °C for 16 h. Enzymatic cleavage of the synthetic peptide resulted in loss of biotin label. Residual biotin label was identified using a streptavidin alkaline phosphatase conjugate (Gibco BRL, Burlington, Ontario, Canada) and p-nitrophenyl phosphate as a substrate. Appropriate controls, e.g. buffer blanks and heat-inactivated serum, were included to account for the small amount of leaching of the biotinylated peptide-BSA conjugate from the plates. The assay was performed in the presence of BSA at a final concentration of 0.1% to exclude proteolytic activity directed against the BSA moiety. The addition of biotin had no effect on the assay. Protease activity was calculated from the absorbance, measured at 405 nm, using the formula: activity = $100 - 100 \times (\text{OD of tested sample} / \text{OD of control})$.

Figure 1 Serum IGF-I protease activity is enhanced in diabetic rats. Protease activity was measured in serum from normal control rats, diabetic rats and insulin-treated diabetic rats as described in Materials and Methods. Insulin pellets which released ~ 2 U/day were implanted in the dorsal subcutaneous tissue in the insulin-treated diabetic rats group. The number of animals per group and the serum glucose levels (mean $\pm$ S.E.M.) are shown below the histograms.

of buffered control) where OD is optical density. In this assay a value of 100 would indicate complete loss of the biotinylated label from the peptide.

A portion of the liver from each animal was homogenized in 4 volumes of ice-cold 0.05 M TBS with 0.1% BSA (pH 7.5). The homogenate was clarified by centrifugation at 10 000 *g* for 10 min at 4 °C. The supernatant was added to the protease assay directly without dilution.

RNA extraction and dot-blot analysis of Spi 2.1 mRNA

Total RNA was extracted from individual rat liver using the acid guanidinium thiocyanate–phenol–chloroform (Chomczynski & Sacchi 1987). A rat Spi 2.1 cDNA obtained from Dr LeCam, Montpellier, France (Paquereau *et al.* 1992) was radiolabeled by nick-translation using 32 P- α dCTP (New England Nuclear, Lachire, Quebec, Canada). For quantitation of Spi 2.1 mRNA, serial dilutions of RNA (5–20 μ g) from individual rat liver samples were immobilized on nitrocellulose paper using a filtration vacuum manifold (Schleicher and Schuell, Keene, NH,

Figure 2 IGF-I protease activity correlates with serum glucose levels. The relationship between IGF-I protease activity and glucose level in serum from the three groups of rats is shown. The control rats are shown as circles, the triangles represent untreated STZ-injected rats and the squares represent insulin-treated STZ-injected rats. The positive correlation between serum IGF-I protease activities and glucose levels was determined by least squares linear regression analysis. The line of best fit is shown; $y = 0.47x + 32.7$; $r = 0.58$, $P < 0.005$, $n = 39$.

USA) and hybridized under stringent conditions. Filters were also hybridized with a 28S ribosomal probe (Gonzalez *et al.* 1985) as a control for gel loading. Autoradiography was performed using Kodak X-Omat AR film and the results were quantified by densitometry. Appropriate exposures were chosen to yield a linear densitometric signal with increasing amounts of RNA and the slope of the line of best fit was compared with a standard RNA pooled from the liver of ten normal rats and arbitrarily attributed a value of 100%.

Statistical analysis

Statistical differences between groups were determined by analysis of variance followed by Dunnett's *t*-test. A value of $P < 0.05$ was considered significant.

Results

The level of protease activity, as determined by the amount of biotin label lost from the immobilized peptide–BSA conjugate, was increased approximately 20% in serum from untreated diabetic rats compared with normal control rats. Serum glucose levels were also significantly elevated in diabetic rats. The results of the first experiment are shown in Fig. 1. This difference in protease activity between diabetic and control animals was statistically

Figure 3 Acute effect of insulin treatment on IGF-I protease activity in diabetic rats. Blood was withdrawn via a femoral artery catheter from diabetic rats ($n=5$) at various time-points after an i.p. injection of insulin. (A) Individual data for each rat; (B) means $\pm$ S.E.M. * $P<0.05$ and ** $P<0.01$ for the difference in protease activity from the basal, time-zero sample.

significant. Chronic insulin treatment of diabetic rats, administered by subcutaneous implants for 3 days, significantly reduced the levels of protease activity in the serum. There was no statistical difference between the level of protease activity in serum from insulin-treated diabetic rats and that seen in serum from normal rats. The serum glucose in these insulin-treated diabetic rats was significantly reduced compared with the normal rats.

A positive correlation ($r=0.58$, $P<0.005$) was found between protease activity and serum glucose when all three groups of rats were considered (Fig. 2). Normal control rats and untreated diabetic rats tended to form discrete populations; however, the positive correlation was apparent when only the insulin-treated diabetic rats were considered.

The acute effect of insulin on serum protease activity in five individual diabetic rats is shown in Fig. 3A. In each case there was a decline in protease activity within the first 30 min after insulin administration. The magnitude of the decrease in protease activity following insulin was highly variable and ranged from a 22 to 83% decrease from basal values. In three of the five rats a biphasic response was apparent with a rise and further fall after the initial decline

in protease activity. When considered as a group the decline in protease activity had a $t_{1/2}$ of ~ 45 min and a nadir of $\sim 50\%$ of basal levels was reached after 120 min (Fig. 3B). The level of protease activity subsequently rose back towards normal levels. Even after 3 h, protease activity remained significantly less than basal values. Blood glucose declined from a baseline level of 30.1 ± 1.0 mmol/l to 9.7 ± 3.1 mmol/l to a nadir at 120 min. However, no significant correlation between blood glucose and enzyme activity was apparent when all data points were considered in these acute insulin studies.

Since the IGF-I protease was abundant in the liver tissue we examined the effects of diabetes on hepatic protease levels. Protease activity was significantly increased in hepatic extracts from diabetic rats whereas protease activity in hepatic extracts from diabetic rats which received a single injection of insulin 2 h prior to death was not significantly different from that seen in hepatic extracts from control rats (Fig. 4). The relationship between hepatic protease activity and serum glucose was also examined. A significant positive correlation ($r=0.68$, $P<0.001$) was apparent when all rats were considered, although each of the groups formed distinct populations.

Figure 4 Hepatic IGF-I protease activity is enhanced in diabetic rats. Protease activity was measured in hepatic extracts from normal control rats, diabetic rats and insulin-treated diabetic rats as described in Materials and Methods. Insulin-treated diabetic rats received a single i.p. injection of regular insulin 2 h prior to death. The number of animals per group is shown below the histograms.

However, a significant correlation was also observed between serum glucose and hepatic protease activity within the largest group, the untreated diabetic rats (Fig. 5). Because of the small number of rats in the control and insulin-treated group this relationship was not observed when each of these groups was considered separately.

Hepatic Spi 2.1 mRNA abundance was markedly reduced in diabetic rats compared with normal rats (Fig. 6). In diabetic rats a single injection of insulin 2 h prior to death had no significant effect on hepatic Spi 2.1 mRNA levels.

Discussion

A number of important changes in the IGF/IGFBP system occur in diabetic rodents which may impact on the functional activity of IGF-I. First, there is attenuation of growth hormone secretion (Tannenbaum 1981) with the consequent reduction of the acid-labile subunit and IGF-I expression and serum concentrations of these proteins (Tamborlane *et al.* 1981, Salamon *et al.* 1989, Dai & Baxter 1994). This results in the reduced concentration of IGF-I present in the serum and a reduction in the relative amounts of IGF-I present as a ternary complex. IGFBP-3 expression appears to be IGF-I dependent in the rat and

Figure 5 Hepatic IGF-I protease activity in diabetic rats correlates with serum glucose levels. The relationship between hepatic IGF-I protease activity and glucose level is shown. The positive correlation between hepatic IGF-I protease activities and glucose levels was determined by least squares linear regression analysis. The line of best fit is shown; $y = 0.78x + 48.4$; $r = 0.56$, $P < 0.01$, $n = 20$.

thus the levels of IGFBP-3 expression are also reduced in diabetic rats (Zapf *et al.* 1989). Furthermore, both receptor and post-receptor defects in growth hormone action have been reported in diabetic animals (Baxter *et al.* 1980, Maes *et al.* 1986).

A role for insulin in the growth hormone signal transduction mechanism has been proposed by a number of investigators (Daughaday *et al.* 1976, Scott & Baxter 1986, Griffen *et al.* 1987, Salamon *et al.* 1989) and the lack of response to growth hormone by diabetic animals may be an indirect consequence of insulin deficiency.

IGFBP-1 expression is enhanced in diabetic animals (Unterman *et al.* 1989, Luo & Murphy 1991). Enhanced expression is thought to be due to insulin deficiency since insulin has a suppressive effect on IGFBP-1 transcription in the liver (Suwanickul *et al.* 1993, Unterman *et al.* 1992, Robertson *et al.* 1994). An effect which is mediated via the protein kinase B/Akt pathway and one or more insulin response elements (Cichy *et al.* 1998). This binding protein has been shown to raise blood glucose levels when administered acutely to normal rats (Lewitt *et al.* 1991) and transgenic mice which overexpress IGFBP-1 have fasting hyperglycemia and glucose intolerance (Rajkumar *et al.* 1995). This response is presumed to result from attenuation of the hypoglycemic effect of free IGF-I. These data suggest that the elevated IGFBP-1 level in diabetic animals could itself have a hyperglycemic effect and thus could perpetuate the hyperglycemia in diabetic animals.

Figure 6 Hepatic Spi 2.1 mRNA expression is reduced in diabetic rats. Spi 2.1 mRNA abundance was quantified by dot-blot hybridization and has been expressed as a percentage of that observed in an RNA pool from normal rats. The data represent means $\pm$ S.E.M. The number of animals per group are shown below the histograms.

Circulating inhibitors of IGF-I action in somatomedin bioassays and inhibitor(s) of serum-induced mitogenesis in cell culture assays have been reported (Phillips & Orawski 1977, Phillips *et al.* 1979, Murphy & Lazarus 1983, Taylor *et al.* 1987); however, the relationship between these inhibitor(s) and IGFBP-1 or other IGFBPs have not been delineated.

Here we report that the protease activity capable of generating des(1–3)IGF-I from intact IGF-I is enhanced in serum from diabetic rats. Des(1–3)IGF-I is more potent in stimulating growth than intact IGF-I when administered to rodents (Gillespie *et al.* 1990, Tomas *et al.* 1991). This enhanced potency is thought to be due to the reduced binding affinity of the IGFBPs, particularly IGFBP-1, for des(1–3)IGF-I (Bagley *et al.* 1989, Clemmons *et al.* 1990). The increased protease activity in diabetic serum would serve to enhance the functional activity of the low levels of circulating IGF-I, particularly in the presence of increased concentrations of IGFBP-1 (Unterman *et al.* 1989, Luo & Murphy 1991). This added complexity to the regulation of IGF-I bioactivity may allow for a much finer control of the biological action of IGF-I.

A weak but significant positive correlation was found between serum glucose and protease activity in the chronic study. Since the liver appears to be the most abundant source of this protease activity we also examined protease activity in hepatic extracts (Yamamoto &

Murphy 1995). Hepatic protease activity was increased in diabetic rats and was also correlated with serum glucose. Approximately one-third of the variance in hepatic protease activity could be attributed directly or indirectly to changes in blood glucose concentrations. Since no correlation was apparent between serum protease activity and glucose in the acute insulin study, it is unlikely that the protease activity is directly regulated by either insulin or glucose. The positive correlation found between protease activity and blood glucose most likely results from indirect effects of insulin on other metabolic perturbations which are known to occur in the diabetic state.

Since, as yet, the enzyme responsible for the des(1–3)IGF-I-generating protease activity has not been completely characterized, it is not clear whether the increased protease activity measured in serum from diabetic rats is due to increased enzyme concentrations, increased enzyme activity or reduced inhibitory activity. Of interest in this regard is the observation that Spi 2.1 inhibits this protease activity (Maake *et al.* 1997). This serine protease inhibitor is expressed predominantly in the liver and is growth hormone dependent (Paquereau *et al.* 1992). As discussed above, reduced growth hormone levels are observed in diabetic rats (Tannenbaum 1981). Although decreased hepatic Spi 2.1 mRNA was observed in diabetic rats acute insulin treatment had little effect on this parameter whereas a normalization of both serum and hepatic protease activity was observed. Thus, the increased protease activity in diabetic rat serum reported here is unlikely to result solely from diminished inhibitory activity. Furthermore, it is far from clear whether Spi 2.1 is the major inhibitor of the protease in serum or indeed physiologically important in regulating the proteolytic activity that generates des(1–3)IGF-I. The available polyvalent antisera against Spi 2.1 recognizes multiple proteins in serum which presumably represent other members of the serpin family (Paquereau *et al.* 1992). Thus, it is possible that changes in other inhibitors rather than changes in the enzyme concentration may account for the increased protease activity observed in diabetic rats. Further studies in this regard will have to await the development of specific molecular and immunological tools for measuring the protease.

Irrespective of the mechanism whereby the protease activity is enhanced in diabetic rats, the increased activity would favor formation of the more potent des(1–3)IGF-I. This may partially compensate for the reduced levels of IGF-I.

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