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Research Article

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Increased Secretory Demand Rather than a Defect in the Proinsulin Conversion Mechanism Causes Hyperproinsulinemia in a Glucose-Infusion Rat Model of Non–Insulin-dependent Diabetes Mellitus

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Abstract

Hyperproinsulinemia in non–insulin-dependent diabetes mellitus (NIDDM) is due to an increased release of proinsulin from pancreatic β cells. This could reside in increased secretory demand placed on the β cell by hyperglycemia or in the proinsulin conversion mechanism. In this study, biosynthesis of the proinsulin conversion enzymes (PC2, PC3, and carboxypeptidase-H [CP-H]) and proinsulin, were examined in islets isolated from 48-h infused rats with 50% (wt/vol) glucose (hyperglycemic, hyperinsulinemic, and increased pancreatic proinsulin to insulin ratio), 20% (wt/vol) glucose (normoglycemic but hyperinsulinemic), and 0.45% (wt/vol) saline (controls). A decrease in the islet content of PC2, PC3, and CP-H from hyperglycemic rats was observed. This reduction did not correlate with any deficiency in mRNA levels or biosynthesis of PC2, PC3, CP-H, or proinsulin. Furthermore, proinsulin conversion rate was comparable in islets from hyperglycemic and control rats. However, in islets from hyperglycemic rats an abnormal increased proportion of proinsulin was secreted, that was accompanied by an augmented release of PC2, PC3 and CP-H. Stimulation of the β cell's secretory pathway by hyperglycemia, resulted in proinsulin being prematurely secreted from islets before its conversion could be completed. Thus, hyperproinsulinemia induced by chronic hyperglycemia likely results from increased β cell secretory demand, rather than a defect in the proinsulin processing enzymes per se. (*J. Clin. Invest.* 1995. 95:1032–1039.) Key words: PC2 • PC3 • carboxypeptidase-H • proinsulin • pancreatic islets • diabetes

Introduction

Insulin is initially synthesized in the pancreatic β cell as a larger precursor molecule proinsulin which then undergoes limited proteolysis in the secretory granule to insulin and C-peptide (1–3). The proteolytic process involves endoproteolytic cleavages at Arg^{31}, Arg^{32}, and Lys^{64}, Arg^{65} by two distinct Ca^{2+}-dependent endopeptidases, named PC2 and PC3 (3–6). The newly exposed COOH-terminal basic amino acids are subsequently removed by the exopeptidase carboxypeptidase-H (CP-H)[1] (7). PC2 and PC3 (also known as PC1) (8) are members of the novel family of proprotein convertases (PCs) related to bacterial subtilisin and the yeast processing endopeptidase, Kex-2 (9, 10). PC3 is specific for the Arg^{31}, Arg^{32} site (11–14), and PC2 has a strong preference for the Lys^{64}, Arg^{65} site (11, 15). The bulk of the evidence for human proinsulin processing supports a sequential mechanism whereby PC3 cleaves intact proinsulin to *des* 31, 32 proinsulin followed by the PC2 cleavage (3). As such, we have proposed that PC3 is the controlling endopeptidase for human proinsulin processing (3).

Proinsulin conversion to insulin is normally very efficient, with > 90% of the secreted insulin-like molecules being native insulin and C-peptide (16, 17). However, in persons with non–insulin-dependent diabetes mellitus (NIDDM) the levels of proinsulin (and the conversion intermediates) relative to insulin in the blood are raised (for reviews see references 18–21). The metabolic clearance rate of proinsulin is unchanged in NIDDM (19, 22), making the cause a relative hypersecretion of the proinsulin-like peptides which agrees with our finding of higher levels of proinsulin relative to insulin in pancreas extracts from two hyperglycemic rat models (23, 24). In animal models an increased pancreatic proinsulin to insulin ratio tended to correlate with hyperglycemia, since it was found where plasma insulin levels were normal (23). Hypersecretion of incompletely processed forms of insulin worsens the insulin deficiency in NIDDM, since proinsulin and the conversion intermediates are biologically poorly active compared to insulin (22, 25). Two hypotheses to account for the hypersecretion of proinsulin have been suggested (3, 19). First, a heightened demand on insulin secretion from the hyperglycemia depletes the β cell of mature insulin granules, resulting in an enriched population of less mature (proinsulin-rich) granules for secretion. Alternatively, a defect in the enzymatic proinsulin processing mechanism has been proposed.

The present study investigated these two hypotheses using a rat model (48-h glucose infusion) (26) which is known to have a raised proportion of proinsulin relative to insulin in their β cells (24). The gene expression, biosynthesis, processing, and secretion of proinsulin, PC2, PC3, and CP-H were measured in pancreatic islets isolated from three groups of rats: (*a*) rats infused with 50% (wt/vol) glucose that are hyperglycemic and are known to have a raised pancreatic ratio of proinsulin to

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1. *Abbreviations used in this paper:* CP-H, carboxypeptidase-H; NIDDM, non–insulin-dependent diabetes mellitus; PC, proprotein convertase.

insulin (24, 26, 27); (*b*) rats infused with 20% (wt/vol) glucose that develop compensatory hyperinsulinemia while maintaining a normal basal level of blood glucose and a normal pancreatic proinsulin to insulin ratio (26, 27); 3) control rats infused with 0.45% saline (wt/vol).

Methods

Materials. L-[^{35}S]methionine (1.2 mCi/mmol) and α-[^{32}P]dCTP (3,000 Ci/mmol) were from DuPont-New England Nuclear (Cambridge, MA). Restriction endonucleases were from Stratagene (La Jolla, CA) or NE BioLabs (Cambridge, MA). All other analytical-grade biochemicals were from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Springfield, NJ).

Glucose-infused rat model. 200-g male Sprague-Dawley rats were infused for 48 h with either 0.45% NaCl, 20% glucose, or 50% glucose at a flow rate of 2 ml/h, as previously described (24, 26, 27). Blood samples obtained at the end of the infusion by tail snipping, were assessed for plasma glucose levels using a Beckman glucose analyzer II (Beckman Instruments, San Ramon, CA).

Islet isolation. Pancreatic islets were isolated from glucose/saline-infused rats by pancreas digestion with collagenase and islet isolation by Histopaque density gradient centrifugation, as previously described (28).

Pulse-chase [^{35}S]methionine-radiolabeling experiments. Batches of 100 islets were incubated for 40 min at 37°C under O_2/CO_2 (95/5%) in 1.5-ml microcentrifuge tubes in 0.1 ml of modified Krebs-Ringer bicarbonate buffer (115 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$), 20 mM Hepes, 0.1% bovine serum albumin, pH 7.4, containing varying glucose concentrations, as indicated for each experiment. Islets were recovered by centrifugation (800 *g* for 15 s), and pulse-radiolabeled at 37°C in 0.1 ml of the fresh incubation buffer containing 0.25 mCi of [^{35}S]methionine. After 20–30 min pulse medium was removed by centrifugation, islets washed with 1 ml of incubation buffer containing 2 mM L-methionine and chased for varying times in 0.1 ml of 2 mM L-methionine-containing buffer, at the glucose concentration indicated for each experiment. The medium was removed by centrifugation (800 *g* for 15 s) and the islets were resuspended in 250 μl of lysis buffer (50 mM Hepes, pH 8.0, 1% Triton X-100, 10 μM phenylmethylsulfonyl fluoride, 10 μM *trans*-epoxysuccinyl-L-leucyl-amido-(4-guanidino)butane, 10 μM pepstatin A, 10 μM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 0.1 mM leupeptin, 0.1% NaN_3) and sonicated (25 watts for 20 s). Islet lysates were centrifuged for 2 min at 10,000 *g* and supernatants collected. The presence of [^{35}S]methionine-radiolabeled proteins in islet lysates and incubation media was analyzed by trichloroacetic acid (TCA) precipitation in a 5-μl aliquot, as previously described (29).

Proinsulin, PC2, PC3, and CP-H immunoprecipitation and analysis. Islet lysates and incubation media were incubated for 2 h at room temperature with 25 μl of Cowan's strain *Staphyloccocus aureus* cells (Sigma Chemical Co.). The supernatant obtained after centrifugation (10,000 *g* for 2 min) was subjected to serial immunoprecipitation for PC2, PC3, proinsulin and CP-H. PC2 and PC3 were specifically immunoprecipitated as previously described (29). (Pro)insulin was immunoprecipitated, as described (29), using 10 μl of bovine insulin antiserum (Sigma Chemical Co.) for islet lysates, and 2 μl for the incubation media. CP-H was specifically immunoprecipitated with 4 μl of CP-H antisera (30) (generous gift of Dr. L. Fricker, New York). Immunoprecipitated proteins were analyzed by polyacrylamide gel electrophoresis and fluorography, as previously described (29). Fluorographs were subjected to densitometric scanner analysis (Computing Densitometer; Molecular Dynamics, Sunnyvale, CA).

Immunoblot analysis. Batches of 500 islets from glucose/saline-infused rats were resuspended in 20 μl of Laemmli sample buffer (31), 1 μl of 1 M DTT added, then heated at 96°C for 5 min and centrifuged (10,000 *g*, 2 min). Aliquots (5 μl) of supernatant were subjected to SDS-PAGE (29). Separated proteins were semi-dry blot transferred from the gel onto a nitrocellulose membrane and then subjected to immunoblot analysis for PC2, PC3, and CP-H, as described (29).

Northern blot analysis. Total RNA was extracted from glucose/saline-infused rat isolated islets using RNAzol™ (Biotec Laboratories, Inc., Houston, TX). Extracted total islet RNA (5 μg) were fractionated by 1% agarose/formaldehyde gel electrophoresis. RNA was capillary transferred onto a Biotrans™ nylon membrane (ICN, Irvine, CA) for 24 h and fixed to the membrane by long wave UV irradiation. The blot was prehybridized for 3 h at 42°C in 50 mM PIPES, 100 mM NaCl, 50 mM sodium phosphate buffer (pH 7.0), containing 1 mM EDTA, 5% SDS and 60 μg/ml denature salmon sperm DNA. Hybridization was carried out for 24–48 h at 42°C in fresh medium containing [^{32}P]-CTP-radiolabeled probes for PC2, PC3, CP-H, or insulin. The membrane was washed with 2X SSC, 0.1% SDS once at room temperature for 15 min and three times at 65°C for 10 min, and autoradiographed. cDNA probes were random primer radiolabeled, according to Prime-It™ II Stratagene protocol. The PC2 cDNA (obtained from Dr. Al Rehemtulla, The Genetics Institute, Cambridge, MA), comprising residues 137–2337, was obtained by Hind III digestion of PC2 cDNA clone (5), and PC3 cDNA (also from Dr. Al Rehemtulla), comprising residues 190–2701, was from PstI digested PC3 cDNA clone (6). The CP-H probe was obtained by EcoRI digestion of CP-H cDNA clone (32) (a gift from Dr. Lloyd Fricker, Albert Einstein College of Medicine, Bronx, NY). Proinsulin probe (424 base pair length) was obtained by HindIII and EcoRI digestion of rat insulin-II cDNA clone (33) (from Dr. Lydia Villa-Komaroff, Children's Hospital, Boston, MA). Digested cDNA was purified in 1% agarose gel, followed by Glassmilk® (Geneclean II®; BIO 101 Inc., La Jolla, CA) precipitation of the DNA from the appropriate agarose band.

Results

General characteristics of glucose-infused rats. The rats infused with 50% glucose were markedly hyperglycemic at the end of the infusion (16.4±1.6 [n = 8] mM glucose verses 7.6±0.2 [n = 8] mM glucose in saline infused controls) ($P < 0.002$ Student's *t* test), as opposed to the 20% glucose group in which the basal plasma glucose level was equal to the saline controls (8.1±0.2 [n = 8] mM; not significant verses saline-infused controls). Certain characteristics of this glucose-infusion rat model have been previously reported (24, 26, 27), that are germane to this study. In the 50% glucose-infused rats there is marked hyperinsulinemia, a reduced pancreatic insulin content to 30% of normal, and a raised proinsulin to insulin ratio in pancreatic extracts. In the 20% glucose-infused rats there is less severe hyperinsulinemia, a normal pancreatic insulin content, and a normal pancreatic proinsulin to insulin ratio when compared with saline-infused controls. Thus, comparison of the 20 and 50% glucose-infused rat groups allowed us to investigate effects on (pro)insulin synthesis, processing, and secretion under two quite different circumstances: (*a*) a severe model where hyperinsulinemia cannot compensate for overt chronic hyperglycemia (50% glucose-infused rat); and (*b*) a milder model where a heightened insulin secretory rate maintains normoglycemia (20% glucose-infused rat).

Islet content of proinsulin, PC2, PC3, and CP-H. The islet content of PC2, PC3, and CP-H by immunoblotting was markedly lower in the 50% glucose-infused rats compared to the saline-infused rats (Fig. 1). In contrast, the levels of these proteins were not different in the 20% glucose-infused islets and the control (saline-infused) islets. This observation generally parallels that previously observed for insulin content in the same glucose infused model (26, 27). In regard to the 50% glucose islets, the reduction in PC3 exceeded that of PC2 or CP-H. This

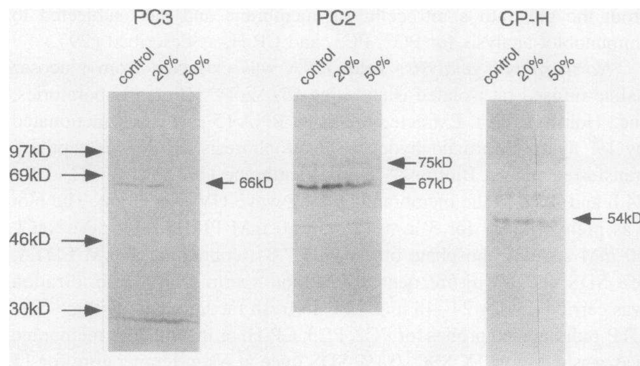

Figure 1. Immunoblot analysis of the proinsulin-processing enzymes. Isolated islets from rats infused for 48 h with 0.45% NaCl (*control*), 20%-glucose (*20%*), and 50%-glucose (*50%*) were immunoblot analyzed, as previously described (see Methods), using specific antibodies for PC3, PC2, and CP-H. An example blot is shown as representative of 3–4 separate experiments. The migration of molecular size standards is indicated.

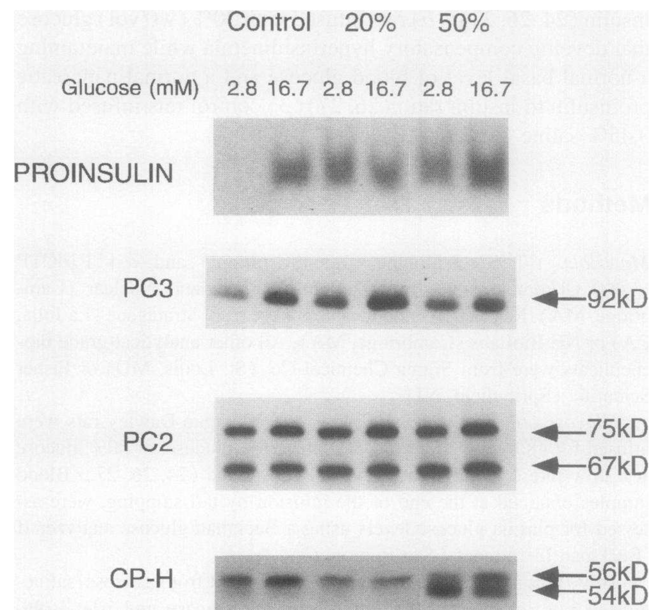

Figure 3. Glucose regulation of the biosynthesis of proinsulin and proinsulin-processing enzymes. Isolated islets from rats infused for 48 hours with 0.45% NaCl (*control*), 20%-glucose (*20%*), and 50%-glucose (*50%*) were incubated for 70 min in modified Krebs-Ringer bicarbonate buffer containing 2.8 or 16.7 mM glucose, the final 30 min were in the additional presence of [^{35}S]methionine. Proinsulin, PC2, PC3, and CP-H were sequentially immunoprecipitated from islet lysates and analyzed by polyacrylamide gel electrophoresis followed by fluorography. An example fluorogram is shown as representative of 2–4 separate experiments.

finding likely reflects the localization of these proteins, since PC3 is predominately expressed in β cells (26-fold higher PC3 protein expression in islet β cells verses non–β cells) (34), as opposed to PC2 and CP-H that are also abundant in the non–β cells of the islet (34, 35). As such, a reduction of the β cell content of these proteins would appear to cause a greater fall in the islet level of PC3. Consistent with this idea, pancreatic glucagon content is normal in the 50% glucose-infused rats in contrast to the marked depletion of insulin (27).

Islet biosynthesis of proinsulin, PC2, PC3, and CP-H. To determine whether a defect in the production of PC2, PC3, CP-H, and proinsulin caused lowered islet content in 50% glucose-infused rat islets, the gene expression of these proteins was examined. Northern blot analysis showed no reduction of the PC2, PC3, CP-H, and preproinsulin-II total mRNA content in isolated islets from hyperglycemic rats versus control and 20%-glucose infused rats (Fig. 2). On the contrary, in islets from both the 20% and 50% glucose-infusions PC3 and preproinsulin mRNAs were increased, whereas no measurable change in mRNA levels of PC2 or CP-H with increasing hyperglycemia was observed (Fig. 2).

The biosynthesis of proinsulin, PC3, PC2, and CP-H, as determined by 30-min [^{35}S]methionine-radiolabeling of isolated islets, was not reduced in 50%-glucose infused hyperglycemic rats versus control and 20% glucose-infused rats (Fig. 3). [^{35}S]methionine incorporation into total (TCA-precipitable) islet proteins was equivalent in the three groups of islets. In islets from saline-infused control rats the biosynthesis of proinsulin and PC3, but not that of PC2 or CP-H, was increased by short term exposure to stimulatory 16.7 mM glucose versus that at basal 2.8 mM glucose concentrations (Fig. 3), in agreement with previous observations (28, 29). However, in islets from both 20 and 50% glucose-infused rats the basal rate of proinsulin and PC3 biosynthesis at 2.8 mM glucose was increased to that of 16.7 mM glucose control stimulatory levels (Fig. 3). Nevertheless, a degree of glucose regulation was still present in 50% glucose-infused islets, since an incubation at a stimulatory 16.7 mM glucose further stimulated proinsulin biosynthesis above the already increased basal rate at 2.8 mM glucose. There was no effect of the 20 or 50% glucose-infusions on islet PC2 and CP-H biosynthesis other than a slight increase in the processing of the precursor 56-kD form of CP-H to the mature 54-kD form in the 50% glucose-infused islets. In summary, the biosynthesis of proinsulin and the conversion enzymes was not negatively impaired in the hyperglycemic glucose-infused islets that would account for a reduction in the islet content of these proteins (Fig. 1).

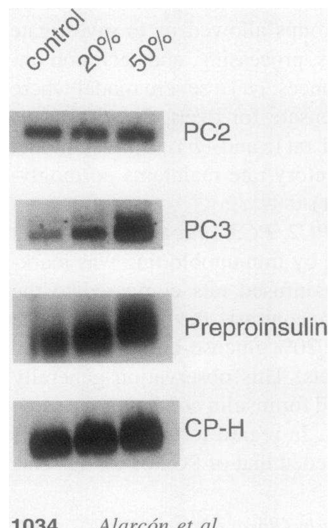

Figure 2. Northern blot analysis of the proinsulin-processing enzymes. Total RNA (5 μg/lane) from isolated islets of rats infused for 48 h with 0.45% NaCl (*control*), 20%-glucose (*20%*), and 50%-glucose (*50%*) was fractionated and transferred to a nylon membrane. The blot was prehybridized for 3 h at 42°C and hybridized for 24–48 h at 42°C successively with [^{32}P]-labeled PC2, PC3, CP-H, and insulin cDNA probes.

Islet processing and secretion of proinsulin. Proinsulin processing was assessed by a [^{35}S]methionine-radiolabeling pulse–chase experiment, where the pulse was at 16.7 mM glucose to stimulate proinsulin biosynthesis and the chase at a basal 2.8

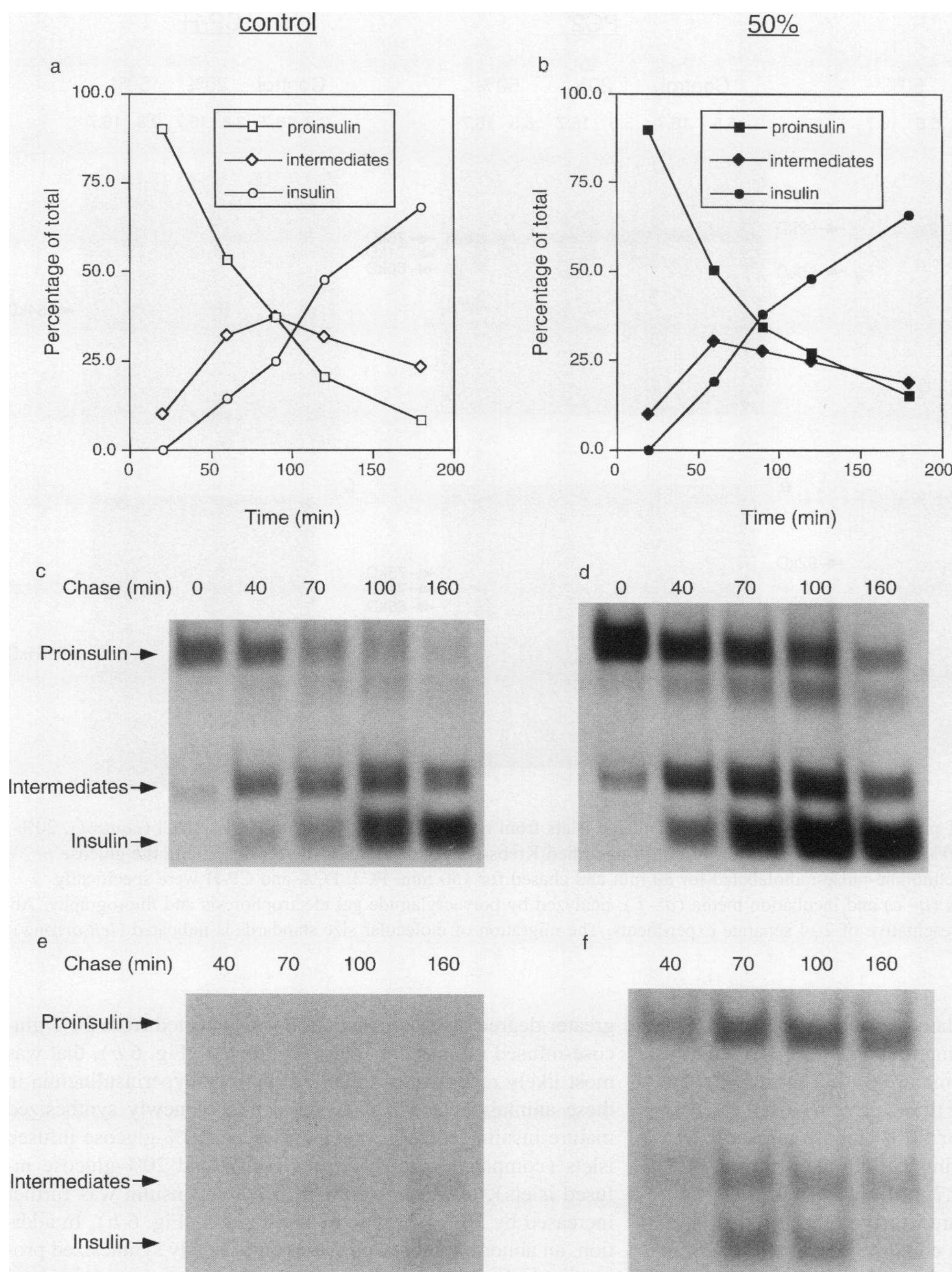

Figure 4. Proinsulin processing in hyperglycemic rat isolated islets. Isolated islets from rats infused for 48 h with 0.45% NaCl (*control*) and 50%-glucose (*50%*) were preincubated for 30 min in modified Krebs-Ringer bicarbonate buffer containing 16.7 mM glucose, then [^{35}S]methionine-pulse-radiolabeled for 20 min at 16.7 mM glucose and chased at 2.8 mM glucose for the indicated times. Insulin-related proteins were specifically immunoprecipitated from islet lysates (*c* and *d*) and incubation media (*e* and *f*), analyzed by polyacrylamide gel electrophoresis and fluorography. The migration of insulin-related proteins is indicated. *a* and *b* show the densitometric scanner analysis of the islet fluorograms, expressed as percentage of total radioactivity into insulin-related proteins.

mM glucose to minimize (pro)insulin release (16). The rate of proinsulin processing, in terms of insulin production, was identical in the 50% glucose and the saline control islets through 180 min (Fig. 4, *a* and *b*). However, by 180 min the amount of newly synthesized proinsulin was twice as high in hyperglycemic rats compared to controls (Fig. 4, *c* and *d*), that was comparable to previous measurements of the islet content proinsulin to insulin ratio (24). Nevertheless, because of the increased biosynthesis of proinsulin in the 50% glucose-infused rats at 16.7 mM glucose (Fig. 3; Fig. 4, *c* vs. *d*), in absolute terms, the amount of newly synthesized proinsulin being converted to insulin by 180 min was almost twice that of the saline islets (Figs. 4, *c* vs. *d*). Analysis of the incubation media was used to determine secretion of newly synthesized (pro)insulin (Fig. 4 *e*). As would be predicted (16), there was minimal secretion of (pro)insulin from the saline-infused control islets during the chase at basal 2.8 mM glucose. In contrast, exaggerated secretion of proinsulin, conversion intermediates and insulin from 50% glucose-infused rat islets was observed throughout the chase period at 2.8 mM glucose (Fig. 4 *f*), with proinsulin being detectable at 60 min and the intermediates and insulin appearing somewhat later (Fig. 4 *f*).

Islet processing and secretion of PC2, PC3, and CP-H. In further experiments, islets were [^{35}S]methionine pulse-radiolabeled for 30 min and chased for an additional 150 min at either basal glucose (2.8 mM and 5.5 mM) or stimulatory 16.7 mM glucose (Fig. 5). During the chase, the 92-kD precursor form of PC3 was processed normally to a mature 66-kD form (29,

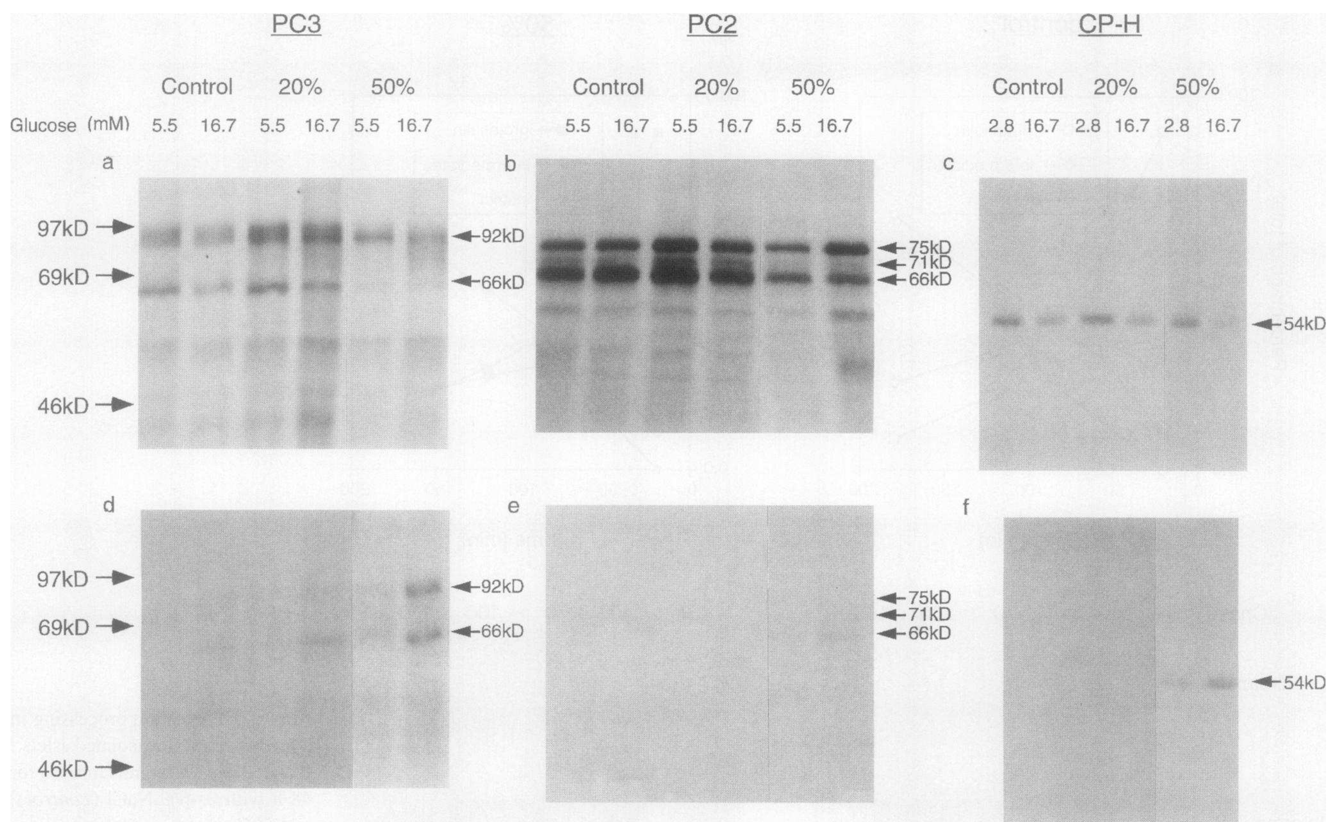

Figure 5. Biosynthesis and release of proinsulin-processing enzymes. Isolated islets from rats infused for 48 h with 0.45% NaCl (*control*), 20%-glucose (*20%*), and 50%-glucose (*50%*) were preincubated for 40 min in modified Krebs-Ringer bicarbonate buffer containing the glucose concentration indicated, then [^{35}S]methionine-pulse-radiolabeled for 30 min and chased for 150 min. PC3, PC2, and CP-H were specifically immunoprecipitated from islet lysates (*a–c*) and incubation media (*d–f*), analyzed by polyacrylamide gel electrophoresis and fluorography. An example fluorogram is shown as representative of 2–4 separate experiments. The migration of molecular size standards is indicated (*left arrows*).

36), as was the 75-kD glycosylated precursor of PC2 to its mature 66-kD form via a 71-kD intermediate (29, 37), and the 56-kD precursor of CP-H to its mature 54-kD form (30). No deficiency in the processing of these proteins was noted in the 20% glucose-infused islets or 50% glucose-infused islets. However, there was an observed intracellular depletion of PC2, PC3, and CP-H (especially at 16.7 mM glucose) in islets from 50%-glucose infused rats compared to the other groups (Fig. 5, *a–c*). However, this depletion could be accounted for by an increased release of newly synthesized PC2, PC3, and CP-H at both basal glucose and stimulatory 16.7 mM glucose concentrations in 50%-glucose infused islets (Fig. 5, *d–f*). It has previously been shown that PC2 and CP-H are co-secreted with insulin from pancreatic islets (35, 37), and this study demonstrated that PC3 was also co-secreted in a parallel manner.

In the same experiment, > 92% of newly synthesized proinsulin was processed to insulin in the control and 20% glucose-infused islets within the 150 min chase (Fig. 6 *a*), whereas in the 50% glucose-infused islets the ratio of newly synthesized proinsulin to insulin suggested slightly less conversion (~ 85% proinsulin converted; Fig. 6 *a*) comparable with previous observations (Fig. 4). The control and 20% glucose-infused islets secreted only newly synthesized mature insulin at 5.5 and 16.7 mM glucose as opposed to substantial amounts of the secreted peptides in the 50% glucose islets (even at 2.8 mM glucose) being proinsulin and the intermediates (Figs. 4 *f* and 6 *b*). A greater degree of insulin secretion was detected from 20% glucose-infused rat islets at 16.7 mM glucose (Fig. 6 *b*), that was most likely reflective of the compensatory hyperinsulinemia in these animals (26, 27). The basal rate of newly synthesized mature insulin secretion was increased in 50%-glucose infused islets (compared with that from saline- and 20%-glucose infused islets), however secretion of mature insulin was further increased by 16.7 mM glucose stimulation (Fig. 6 *b*). In addition, an abnormal increased secretion of newly synthesized proinsulin and proinsulin conversion intermediates was observed from 50% glucose-infused islets compared to saline- and 20%-glucose-infused islets (Fig. 6 *b*). In contrast to secretion of mature insulin from the same 50% glucose-infused islets, the release of proinsulin and proinsulin conversion intermediates was not stimulated by 16.7 mM glucose (Fig. 6 *b*).

Discussion

Hyperproinsulinemia in NIDDM is thought to reside at the level of β cell dysfunction (3, 19, 20). Such a dysfunction is likely a consequence of the increased secretory demand placed on the β cell by chronic hyperglycemia and/or a defect in the β cell's proinsulin processing machinery (3). In this study we examined the β cell's handling of the proinsulin processing enzymes under hyperglycemic conditions in pancreatic islets isolated from 48-h glucose-infused rats. In normoglycemic 20% glucose-infused

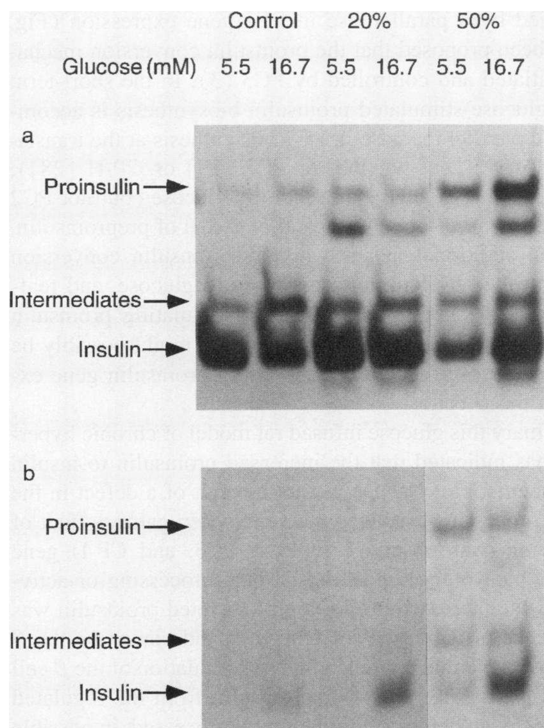

Figure 6. Biosynthesis and release of proinsulin. Isolated islets from rats infused for 48 hours with 0.45% NaCl (*control*), 20%-glucose (*20%*), and 50%-glucose (*50%*) were preincubated for 40 min in modified Krebs-Ringer bicarbonate buffer containing 5.5 or 16.7 mM glucose, then [^{35}S]methionine-pulse-radiolabeled for 30 min and chased for 150 min. Insulin-related proteins were specifically immunoprecipitated from islet lysates (*a*) and incubation media (*b*), analyzed by polyacrylamide gel electrophoresis and fluorography. The migration of insulin-related proteins is indicated.

rat islets, there was no derogatory change in PC2, PC3, and CP-H content compared with saline-infused control islets. In contrast, in the hyperglycemic 50% glucose-infused rats the islet content of PC2, PC3, and CP-H was depleted, similar to a reduction in β cell insulin content previously observed (27). However, depletion of islet PC2, PC3, CP-H, or (pro)insulin content in hyperglycemic rats could not be correlated with any reduction in gene expression, biosynthesis or post translational processing of these proteins. Rather, decreased islet content of PC2, PC3, CP-H, and (pro)insulin was attributable to an increased rate of secretion of these proteins induced by hyperglycemia.

We have previously documented in normal rat islets that > 99% of (pro)insulin is secreted via a regulated secretory pathway (16, 38). Newly synthesized proinsulin has been observed to be released from the β cell 45–60 minutes after initial synthesis (16). This lag period reflects the fastest time required from synthesis on the endoplasmic reticulum to passage through the Golgi apparatus, packaging into secretory vesicles and transit to the plasma membrane for exocytosis (1, 16). From 60 min onwards newly synthesized (pro)insulin is secreted in a glucose-regulated manner indicating that it is derived from a secretory granule compartment and released via the β cell's regulated secretory pathway (16, 38). Passage of newly synthesized proinsulin through the early stages of the β cell's secretory pathway to an immature secretory granule compartment takes around 30 minutes (1). Thus, the minimum transit time for a newly formed secretory granule to be transported to the plasma membrane for exocytosis under glucose stimulatory conditions can be estimated to be ≥ 30 min (16). However, the immature secretory granule compartment is the major site of proinsulin in the β cell (1, 2, 39), and it has been established that 3 h after proinsulin synthesis is the time period necessary for 90% of newly synthesized proinsulin to be converted to insulin within the normal β cell (1, 16, 17, 40). It has also been shown that there is preferential secretion of newly synthesized (pro)insulin, so that up to 50% of the initial proinsulin synthesized can be released within the same 3 h period at maximal glucose stimulatory conditions (16, 41). It follows therefore, that under maximal glucose stimulatory conditions newly synthesized (pro)insulin released between 60–120 min (post proinsulin synthesis) would be reflective of the content of immature secretory granules in which proinsulin conversion is only partially completed, and thus a higher proportion of newly synthesized proinsulin can be released (16). In normal islets glucose stimulated insulin release is mostly reflective of exocytosis from the mature insulin secretory granule storage pool, and the proportion of newly synthesized (pro)insulin contributed from immature secretory granules is relatively minor (41, 16). However, this may not be the situation in islets exposed to severe chronic hyperglycemia, such as the 50% glucose-infused islets described in this study. The increased secretory demand placed on the β cell by the hyperglycemia results in a 75% depletion in β cell secretory granule content (26, 27). Furthermore, the persistent elevation in blood glucose in the 50% glucose-infused rats also induced increased proinsulin biosynthesis (Figs. 3, 4, and 6), and presumably secretory granule biogenesis (28). Thus, in 50% glucose-infused islets there is likely to be a much higher proportion of immature secretory granules present, which are preferentially and rapidly transported to the plasma membrane for exocytosis before the newly synthesized proinsulin has been fully converted to insulin. As a result, there is an increased proportion of proinsulin (and conversion intermediates) released from the β cell's regulated secretory pathway under hyperglycemic conditions, the consequences of which is a raised proinsulin to insulin ratio in the circulation.

This study indicated there was also a proportion of abnormal (pro)insulin secretion arising from chronic exposure to severe hyperglycemia. Several lines of evidence indicated an increased proportion of (pro)insulin was released in an unregulated manner from the 50% glucose-infused islets compared to saline-infused control islets. First, a rapid secretion of newly synthesized proinsulin from 50% glucose-infused islets was observed under non-stimulated conditions (2.8 mM glucose in the chase medium) 20–60 min after initial proinsulin synthesis, followed by an unregulated release of proinsulin, conversion intermediates and insulin in the subsequent 60–120-min period (Fig. 4 *f*). Negligible release of (pro)insulin was observed in the saline-infused control islets incubated at a basal 2.8 mM glucose (Fig. 4 *e*). Second, under normal conditions, secretion of PC2, PC3, and CP-H was only observed under glucose stimulation implying that these proteins were co-released with insulin via the β cell's regulated secretory pathway (35, 37, 38). However, in 50% glucose-infused islets the basal rate (at 2.8 or 5.5 mM glucose) of PC2, PC3, and CP-H secretion was increased in parallel to that of (pro)insulin (Figs. 5 and 6) compared with that from 20%- and saline-infused rat islets, implying a increased proportion of unregulated secretion in islets exposed to

hyperglycemia. Third, a most intriguing observation was that from the very same 50% glucose-infused islets release of newly synthesized proinsulin and proinsulin conversion intermediates was not regulated by glucose whereas, in contrast, secretion of newly synthesized mature insulin was clearly stimulated by glucose (Fig. 6 *b*). This raises the possibility that in the 50% glucose-infused islets a proportion of newly synthesized proinsulin (and proinsulin conversion intermediates) were independently released from a different, nonregulatable, intracellular pool compared to the majority of newly synthesized mature insulin which was secreted in a regulated manner. It should be noted that in saline-infused control and 20% glucose-infused islets there was negligible release of proinsulin or proinsulin conversion intermediates (Fig. 6 *b*). The abnormal (pro)insulin secretion from 50% glucose-infused islets may be symptomatic of the severe chronic hyperglycemia increasing the proportion of immature secretory granules and their transit time to the plasma membrane for exocytosis. However, other possible explanations should be considered such as functional β cell heterogeneity within hyperglycemic islets (42), or abnormal and/or loss of glucose sensing by islets exposed to chronic hyperglycemia (3, 18, 26, 27). In addition, a partial factor could be an increased contribution of (pro)insulin secreted in an unregulated manner, perhaps via a constitutive-like pathway (38, 43). In normal rat islets a small amount of rapidly released newly synthesized proinsulin ($< 1\%$ of the total proinsulin synthesized secreted from islets between 30 and 60 min) is not subjected to regulation by glucose, and may therefore be representative of a minor degree of proinsulin released via a constitutive pathway (16, 38, 43). However, in islets exposed to severe chronic hyperglycemia (where there is larger quantities of newly synthesized proinsulin traversing the secretory pathway due to corresponding increased proinsulin biosynthesis caused by the increased secretory demand placed on the β cell), the contribution of (pro)insulin missorted for secretion via an unregulated constitutive pathway may be increased. Nevertheless, it is clear that further studies are required to more thoroughly characterize this abnormal (pro)insulin secretion from islets exposed to severe chronic hyperglycemia, to better implicate the aforementioned hypotheses.

Aside from the derogatory effect of chronic hyperglycemia on the β cell's secretory pathway, there were some other interesting aspects of the results concerning glucose regulation of PC3 and proinsulin biosynthesis that warrant discussion. In islets from 20% and 50% glucose-infused rats it was found that the basal rate of proinsulin and PC3 biosynthesis was raised (Fig. 3). This abnormality may well be associated with the loss of glucose-sensitivity for regulated insulin secretion in the 50% glucose-infused rat model (18, 26, 27), supporting the idea that there is likely to be certain shared elements in the glucose sensing stimulus-coupling mechanisms for insulin secretion and biosynthesis (44). Alternatively, the "off-rate" for glucose-stimulated proinsulin biosynthesis is relatively slow (45), and the increased basal rate of proinsulin/PC3 biosynthesis in hyperglycemic islets could result from a slowed shut down process as a "memory" of the prior hyperglycemia. Another explanation might be that the amount of preproinsulin/PC3 mRNA available for translation increased in 50% glucose-infused islets (Fig. 2) which, in turn, was reflected as enhanced basal proinsulin/PC3 biosynthesis. Under longer term situations, such as prolonged hyperglycemia, an increase in preproinsulin mRNA has been previously observed (46), that this study indicated is accompanied by a parallel rise in PC3 gene expression (Fig. 2). It has been proposed that the proinsulin conversion mechanism is initiated and controlled by PC3 (3). In the short-term (< 2 h), glucose-stimulated proinsulin biosynthesis is accompanied by a parallel increase in PC3 biosynthesis at the translational level (29) (but not that of PC2 [29] or CP-H [28]). Thus, increased PC3 gene expression by glucose (but not PC2 or CP-H gene transcription), in parallel to that of preproinsulin, provides an additional means whereby proinsulin conversion can be controlled by long term exposure to glucose, and reaffirms PC3 as the key endopeptidase in regulating proinsulin processing (3). Similar transcription factors might possibly be involved in glucose-regulated PC3 and preproinsulin gene expression in the β cell.

In summary this glucose infused rat model of chronic hyperglycemia has indicated that the increased proinsulin to insulin ratio characteristic of NIDDM is not a result of a defect in the proinsulin processing enzymes per se. No derogatory effect of hyperglycemia was observed on PC2, PC3, and CP-H gene expression, biosynthesis, posttranslational processing or activity. Rather, the increased proportion of secreted proinsulin was a consequence of increased secretory demand placed on the β cell by hyperglycemia. Chronic glucose stimulation of the β cell resulted in premature release of proinsulin from the regulated secretory pathway before it could be fully processed, in possible combination with a defective increase in unregulated (pro)insulin release. Thus a functional, rather than microanatomical, defect may account for hyperproinsulinemia in this model of chronic severe hyperglycemia. However, it should be noted that the glucose-infused rat is only one experimental model of NIDDM that is characterized by marked chronic hyperglycemia. Thus, questions will persist as to relevance of these findings to less hyperinsulinemic states with hyperglycemia, more common to NIDDM. Since NIDDM likely encompasses a family of disorders, it will be important to pursue similar studies in other animal models of NIDDM. Thus, hyperproinsulinemia as a result from a direct dysfunction in proinsulin processing enzyme activity in the β cell rather than a secretory defect (3) should not yet be ruled out in other experimental animal models or various forms of human NIDDM.

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