

Increased Proinsulin/Insulin Ratio in Pancreas Extracts of Hyperglycemic Rats

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The plasma ratio of proinsulin/insulin is raised in people with NIDDM. A relative hypersecretion of proinsulin is thought to be the cause, because pancreas extracts from diabetic rats have a raised proinsulin/insulin ratio. We tested the hypothesis that the pancreatic proinsulin/insulin mismatch results from hyperglycemia-induced β -cell degranulation. Normal rats made hyperglycemic with 48-h glucose infusions had a raised pancreatic percentage of proinsulin. In contrast, rats infused with enough glucose to induce compensatory hyperinsulinemia without changing the plasma glucose level had a normal percentage of proinsulin. The raised percentage of proinsulin in the hyperglycemic rats reflected a reduction in pancreatic insulin content. Administering an inhibitor of insulin release, diazoxide, to hyperglycemic rats blocked the fall in pancreatic insulin content and prevented the rise in the percentage of proinsulin. Normal rats infused with tolbutamide for 3 days and enough glucose to maintain euglycemia had a 50% reduction in pancreatic insulin content. The β -cell degranulation from this nonhyperglycemic mechanism resulted in a raised pancreatic percentage of proinsulin. In summary, chronic hyperglycemia causes β -cell degranulation primarily because of hyperstimulated insulin release. The net result is a rise in the ratio of immature (proinsulin-rich) to mature (insulin-rich) granules, which is reflected as an increased relative proportion of proinsulin. Mobilization of these proinsulin-enriched granules may explain the relative hypersecretion of proinsulin that occurs with diabetes. *Diabetes* 42:22–27, 1993

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NIDDM, non-insulin-dependent diabetes; HPLC, high-performance liquid chromatography; RIA, radioimmunoassay; IRI, immunoreactive insulin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ANOVA, analysis of variance; NS, no significance; IDDM, insulin-dependent diabetes mellitus.

The plasma ratio of proinsulin/insulin is raised in people with NIDDM (1–10). Proinsulin conversion to insulin is a multienzyme cascade with several partially processed intermediates preceding insulin (11,12). Because few studies have measured the intermediates separately from proinsulin, the term proinsulin is used in the context of a raised proinsulin/insulin ratio to generally represent the proinsulinlike peptides. The increased proinsulin/insulin ratio is presumed to reflect a relative hypersecretion of proinsulin, although changes in clearance have not been ruled out.

Studies in humans have suggested that hyperglycemia is a critical feature in the pathogenesis of the raised proinsulin/insulin ratio. Hyperglycemia from NIDDM and cystic fibrosis are both associated with an increased ratio (1–10,13), and cross-sectional data in NIDDM are consistent with the rise occurring after the onset of hyperglycemia (8–10). In contrast, states of increased insulin secretion without hyperglycemia, such as obesity (9,14) and experimental insulin resistance (15), have a normal to reduced proinsulin/insulin ratio. Finally, some (3,6,16) but not all (17) of the limited data available have shown normalization of the proinsulin/insulin ratio in NIDDM when euglycemia is restored by diet or sulfonylureas. Two hypotheses have been proposed to explain how hyperglycemia could cause hypersecretion of proinsulin, but both lack direct experimental testing. The first is that hyperglycemia acts through sustained secretory demand to deplete mature granules so that less mature granules end up being released (3). Alternatively, chronic hyperglycemia could impair proinsulin processing (18).

Animal models may provide insight into the pathogenesis of the raised plasma proinsulin/insulin ratio. It has recently been observed that pancreas extracts and portal vein samples (obtained during *in vitro* pancreas perfusion) from a diabetic rat model have a raised

proinsulin/insulin ratio (19), which suggests that one defect is an enrichment of the β -cell insulin stores with proinsulin such that mobilization of this material causes a relative hypersecretion of proinsulin. This study explores the pathophysiology of the raised pancreatic proinsulin/insulin ratio by testing the first hypothesis stated above, that the relative increase in proinsulin is secondary to a hyperglycemia-driven secretory demand that results in β -cell degranulation and depletion of mature granules. The aims of this study were as follows: 1) to determine whether a link exists between hyperglycemia and a raised pancreas proinsulin/insulin ratio; 2) to determine whether hyperglycemia is acting through stimulation of insulin release and depletion of mature granules by blocking insulin release with diazoxide; and 3) to determine whether hyperstimulation of insulin release leading to β -cell degranulation through a mechanism other than hyperglycemia results in a raised pancreas proinsulin/insulin ratio.

RESEARCH DESIGN AND METHODS

Glucose infusion rat model. Catheters were placed during amobarbital sodium anesthesia (100 mg/kg i.p.) into the right jugular vein of 200-g male Sprague-Dawley rats (Taconic, Germantown, NY; 20). Infusions were started the next day with a syringe pump (Orion, Cambridge, MA) and a swivel assembly mounted above the cage (Emdie, Goochland, VA) so that the rats were unrestrained and had free access to food and water. All infusions were 2 ml/h for 48 h. Protocol 1: 50% glucose (wt/vol) or the diluent 0.45% NaCl. Protocol 2: 20, 30, or 35% glucose or 0.45% NaCl. Protocol 3: 35% glucose (pH 9.5) plus 7.5 mg \cdot kg⁻¹ \cdot h⁻¹ diazoxide (Schering, Kenilworth, NJ) or 0.45% NaCl (pH 9.5) plus 7.5 mg \cdot kg⁻¹ \cdot h⁻¹ diazoxide. The syringes and tubing were wrapped with aluminum foil during the last set of infusions because diazoxide is light sensitive.

Tolbutamide infusion rat model. Indwelling jugular catheters were placed into 200-g rats as described above. Infusions were started the next day at 2 ml/h for 72 h. Protocol: 1 g \cdot kg⁻¹ \cdot day⁻¹ tolbutamide (gift of Upjohn, Kalamazoo, MI) dissolved in 23% glucose on day 1, 18% glucose on day 2, and 23% glucose on day 3. Controls were infused with 0.45% NaCl.

Pancreas extraction, insulin/proinsulin precipitation, and HPLC separation. At the end of the infusions, whole pancreases were excised and homogenized in 6 ml of cold acid ethanol. After standing overnight at 4°C, the extracts were centrifuged, and the supernatants were stored at 4°C. Pellets underwent two more cycles of homogenization and centrifugation. The combined supernatants underwent insulin/proinsulin precipitation with an adaptation of the Davoren method (19,21): pH adjusted to 8 with ammonium hydroxide, precipitate discarded; pH adjusted to 5.3 with 3 M HCl, add 200 μ l of 2 M ammonium acetate, 12.5 ml of absolute ethanol, and 25 ml of ether, 4°C overnight; precipitate resolubilized in 1 ml of 1 M acetic acid. The recovery rate for the insulins and proinsulins was ~50% (19).

The insulins were separated from the proinsulinlike

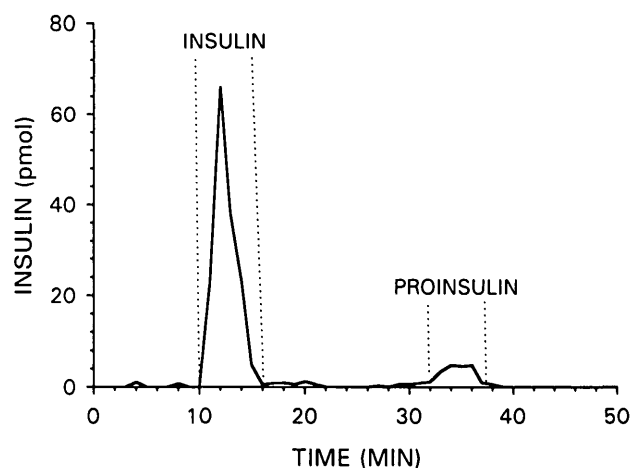


FIG. 1. HPLC profile of pancreas extract from a normal rat infused for 48 h with 0.45% NaCl. Pancreas underwent extraction and insulin/proinsulin precipitation followed by separation of insulins and proinsulins by HPLC. Buffer A: 50 mM phosphoric acid, 20 mM triethylamine, 50 mM sodium perchlorate, adjusted to pH 3.0 with NaOH. Buffer B: 90% acetonitrile, 10% H₂O (vol/vol). Protocol: 66.5:34.5% buffer A:B for 25 min, then a step up to 59:41% A:B for 25 min. Fractions were analyzed by insulin RIA.

peptides by reverse-phase HPLC (22). A 25- μ l aliquot from the total 1-ml pancreas extract was used in all protocols. The HPLC system (model 332, Altex Division, Beckman, San Ramon, CA) consisted of a Model 420 System Controller, two Model 110B pumps, and two Altex Ultrasphere ODS 420 columns (4.6 mm ID, 10 cm pre-column, and 25 cm main column). Buffer A: 50 mM phosphoric acid, 20 mM triethylamine, 50 mM sodium perchlorate, pH 3.0. Buffer B: 90% acetonitrile, 10% H₂O (vol/vol). The program was 65.5:34.5% buffer A:B for 25 min followed by a step up to 59:41% A:B for 25 min. One-milliliter fractions (flow rate 1 ml/min) were collected in polypropylene tubes containing 0.1 ml sodium borate (0.5 M, 10 mg/ml BSA, pH 9.3), lyophilized, and reconstituted in PBS plus 0.1% BSA. Insulin I and II eluted as single or multiple peaks at 8–15 min, and the proinsulins coeluted as a single broad peak at 33–38 min (Fig. 1). The conversion intermediates are presumed to elute with the proinsulins (19). The insulin and proinsulin peaks were quantified by RIA with rat insulin standards (Lilly, Indianapolis, IN). The insulin RIA gives valid proportional measures of proinsulin as shown by parallel serial dilutions curves for the insulin and proinsulin peaks (19).

Analytical methods. Plasma glucose was measured with a Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA). The insulin RIA used charcoal separation (23).

Data presentation and statistical methods. All data are means \pm SE. Insulin and proinsulin values are expressed in units IRI. Extract IRI (combination of insulin and the proinsulinlike peptides) was measured on the extract after insulin/proinsulin precipitation; this value is a relative measure of pancreatic IRI content because of the incomplete recovery of the precipitation technique. IRI insulin, IRI proinsulin, and percentage of proinsulin were determined from the HPLC samples. IRI insulin and IRI proinsulin were calculated as the summed immunoreactivity of

TABLE 1
General characteristics of the rat groups infused with glucose

	<i>n</i>	Weight (g)	Plasma glucose (mM)	Plasma IRI (pM)	Percentage of proinsulin in pancreas extract
Protocol 1					
0.45% NaCl	6	247 ± 12	6.7 ± 0.4	ND	11.4 ± 0.7
50% Glucose	6	243 ± 10	20.1 ± 0.7†	ND	17.6 ± 0.8†
Protocol 2					
0.45% NaCl	5	198 ± 7	7.1 ± 0.2	18 ± 2	11.6 ± 1.0
20% Glucose	5	203 ± 10	7.8 ± 0.5	48 ± 5†	10.9 ± 0.9
30% Glucose	4	203 ± 8	9.4 ± 0.5†	84 ± 19*	16.4 ± 1.6*
35% Glucose	4	194 ± 14	12.1 ± 0.8†	132 ± 9†	17.6 ± 1.2†
Protocol 3					
0.45% NaCl plus diazoxide	6	204 ± 3	8.3 ± 0.6	10 ± 1	12.0 ± 1.4
35% Glucose plus diazoxide	8	213 ± 4	16.4 ± 2.1†	40 ± 6†	10.8 ± 0.7

Data are means ± SE. ND, not done. Statistical significance was assessed against the control group from that protocol.

* $P < 0.05$ vs. control group from that protocol.

† $P < 0.01$ vs. control group from that protocol.

the different peaks. The percentage of proinsulin was calculated as IRI proinsulin/(IRI proinsulin plus IRI insulin). The lack of available rat proinsulin standards means that the cross-reactivity of proinsulin I and II in the insulin assay is not known. Therefore, the proinsulin results are relative, not absolute, measures. The statistical methods were the unpaired Student's *t* test and ANOVA.

RESULTS

Percentage of proinsulin in pancreas extracts from glucose-infused rats. The rats infused with 50% glucose became markedly hyperglycemic. Plasma glucose at the end of the 48-h infusion was 20.1 ± 0.7 versus 6.7 ± 0.4 mM in NaCl-infused rats ($P < 0.0001$). The hyperglycemia was accompanied by substantial β -cell degranulation, with only 30% as much IRI precipitated from the pancreases of the glucose-infused rats as from the NaCl-infused rats (2.1 ± 0.3 nmol of IRI glucose-infused vs. 6.7 ± 1.6 nmol of IRI NaCl-infused, $P < 0.023$).

A typical HPLC profile of a pancreas extract from a NaCl-infused rat after extraction and insulin/proinsulin precipitation is shown in Fig. 1. The elution profile of glucose-infused rats was indistinguishable from the control rats (data not shown). The proinsulin peak in the glucose-infused rats constituted $17.6 \pm 0.8\%$ of total IRI versus $11.4 \pm 0.7\%$ in the NaCl rats ($P < 0.002$).

The 50% glucose caused marked hyperinsulinemia along with the hyperglycemia (20). To separate the effects of hyperstimulated insulin output from those of hyperglycemia on pancreatic percentage of proinsulin, infusions were conducted with lower glucose concentrations. Infusing 20% glucose caused compensatory hyperinsulinemia without any rise in plasma glucose, as opposed to 30 and 35% glucose, both of which caused hyperglycemia and hyperinsulinemia (Table 1). The percentage of proinsulin was unchanged in the normoglycemic 20% glucose rats ($10.9 \pm 0.9\%$ for 20% glucose vs. $11.6 \pm 1.0\%$ for NaCl, NS), as opposed to both hyperglycemic groups, which had a raised pancreatic percentage of proinsulin ($16.4 \pm 1.6\%$ for 30% glucose, $P < 0.03$; $17.6 \pm 1.2\%$ for 35% glucose, $P < 0.007$). The increased percentage of proinsulin in the hyperglycemic

groups represented a reduction in insulin content rather than an increase in proinsulin content (Fig. 2). The 20% glucose did not change IRI insulin or IRI proinsulin, so that the percentage of proinsulin remained normal. In contrast, in the 30% group, IRI proinsulin was equal to the control rats (11 ± 3 pmol for 30% glucose vs. 12 ± 1 pmol for NaCl, NS), but IRI insulin was reduced 25% (70 ± 16 pmol for 30% glucose vs. 95 ± 7 pmol for NaCl, $P < 0.02$), thereby causing the raised percentage of proinsulin. Even more severe degranulation was observed in the 35% glucose rats.

Effect of inhibiting insulin secretion on the percentage of proinsulin in pancreas extracts from hyperglycemic rats. Diazoxide was coinfused with 35% glucose to determine whether the reduced insulin content was an effect of hyperglycemia-induced insulin secretion. Diazoxide inhibits insulin secretion by opening the ATP-sensitive K^+ channel, thereby preventing β -cell depolarization (24,25). Control rats were infused with diazoxide in 0.45% NaCl.

Diazoxide reduced plasma IRI in the NaCl-infused rats nearly 50% compared with the control rats of protocol 2 (Table 1, $P < 0.01$). Diazoxide also lowered plasma IRI in 35% glucose-infused rats (40 ± 6 pM for 35% glucose plus diazoxide vs. 132 ± 9 pM for 35% glucose in protocol 2, $P < 0.0001$) to a level that was now identical to that of rats infused with 20% glucose. This partial suppression of insulin release prevented the fall in pancreatic insulin content noted previously in rats infused with 35% glucose alone, instead resulting in a significantly higher IRI content versus the control rats given diazoxide (4.7 ± 0.5 nmol of total IRI for 35% glucose plus diazoxide vs. 3.0 ± 0.5 nmol of IRI for NaCl plus diazoxide, $P < 0.03$). Diazoxide normalized the percentage of proinsulin in the glucose-infused rats ($10.8 \pm 0.7\%$; Fig. 3). The value in the NaCl plus diazoxide rats ($12.0 \pm 1.4\%$) was unchanged from the controls in the previous protocols.

Percentage of proinsulin in pancreas extracts from tolbutamide-infused rats. To determine if β -cell degranulation from a mechanism other than hyperglycemia would result in a raised pancreas percentage of proinsu-

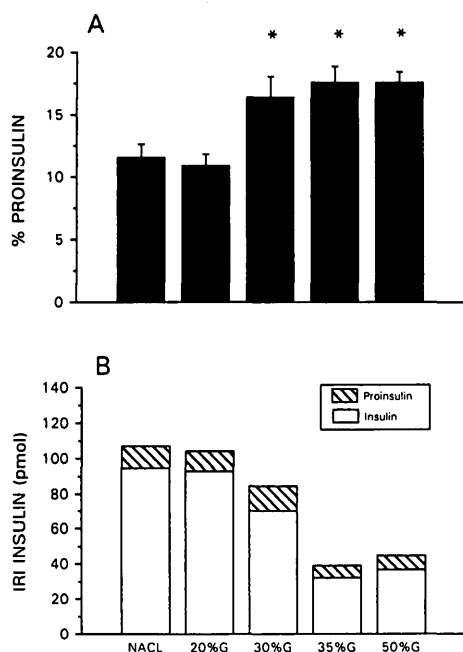


FIG. 2. Relative proportion of proinsulin in pancreas extracts from 48-h glucose-infused rats. Pancreases underwent extraction, insulin/proinsulin precipitation, and HPLC separation of insulins and proinsulins. HPLC sample volumes were 25 μ l from a 1-ml total extract in all groups. IRI insulin and proinsulin are the summed immunoreactivity of different peaks as measured by insulin RIA. Percentage of proinsulin = IRI proinsulin/(IRI insulin + IRI proinsulin) (A). Insulin and proinsulin IRI in HPLC samples (B).

lin, rats were continuously infused for 3 days with tolbutamide. Euglycemia was maintained by adding glucose to the infusate; the plasma glucose values in the tolbutamide-glucose rats at 24, 48, and 72 h were similar to NaCl-infused rats (Table 2).

Tolbutamide reduced the total extract IRI 50% (4.0 ± 0.8 nmol of IRI for tolbutamide vs. 8.1 ± 1.1 nmol of IRI for NaCl rats, $P < 0.02$). This degranulation resulted in a raised percentage of proinsulin ($14.0 \pm 1.4\%$ for tolbutamide vs. $9.8 \pm 0.7\%$ for NaCl rats, $P < 0.02$; Fig. 4).

DISCUSSION

This study examines the pathogenesis of the raised proinsulin/insulin ratio in pancreas extracts of diabetic rats. The HPLC methodology did not distinguish the conversion intermediates from the proinsulins, so that the term proinsulin generally represents the proinsulinlike peptides. Our results demonstrated a raised percentage of proinsulin in pancreas extracts from normal rats made hyperglycemic and hyperinsulinemic with glucose infusions. This finding replicates a similar observation made in a nonhyperinsulinemic diabetic model, 90% pancreatectomized rats (19), which suggests that the raised percentage of proinsulin is a result of the hyperglycemia. This idea was confirmed by finding that 20% glucose caused compensatory hyperinsulinemia without hyperglycemia, and the percentage of proinsulin remained normal. Our results further showed that the raised percentage of proinsulin in the hyperglycemic rats resulted

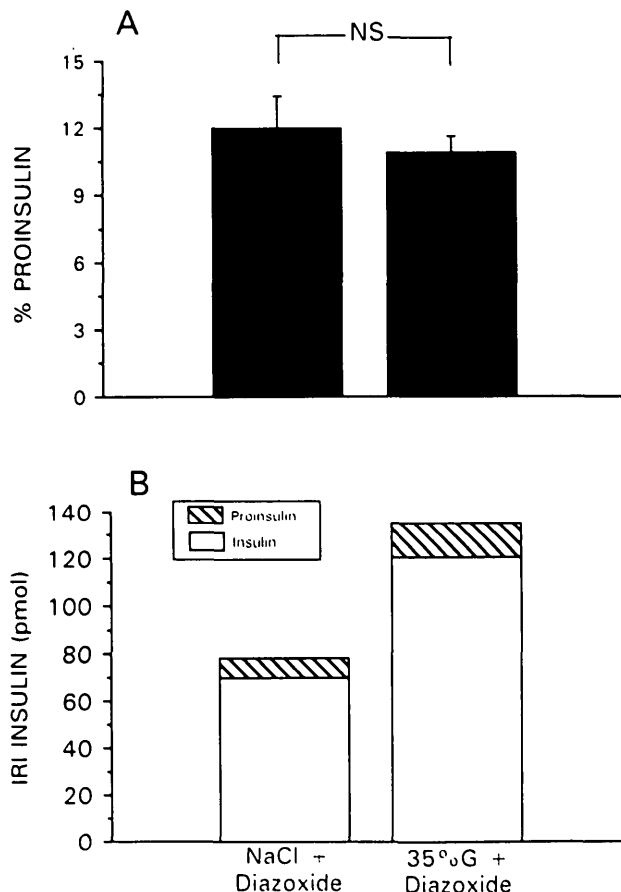


FIG. 3. Normalization of the relative proportion of proinsulin in pancreas extracts from hyperglycemic rats by diazoxide. Rats were infused for 48 h with $7.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ diazoxide in 35% glucose or 0.45% NaCl. Pancreases underwent extraction, insulin/proinsulin precipitation, and HPLC separation of insulins and proinsulins. HPLC sample volumes were 25 μ l from a 1-ml total extract. IRI insulin and proinsulin are the summed immunoreactivity of different peaks as measured by insulin RIA. Percentage of proinsulin = IRI proinsulin/(IRI insulin + IRI proinsulin) (A). Insulin and proinsulin IRI in HPLC samples (B).

from a fall in the amount of stored insulin rather than an increase in proinsulin. Understanding how the insulin content in the normoglycemic 20% group remained intact despite the threefold rise in plasma IRI values might provide clues into how the stored insulin and proinsulin are normally regulated and how hyperglycemia disrupts the system. Dexamethasone treatment in normal rats is another situation where hyperstimulated insulin output occurs without hyperglycemia. The exaggerated output is counterbalanced by an increase in proinsulin synthesis so that pancreatic insulin content remains unchanged (26). Similar events presumably occurred in the 20% glucose rats. Therefore, under normoglycemic conditions, proinsulin synthesis and processing appear to be matched in some fashion to insulin release, so that the stored insulin and proinsulin remain unchanged. The nature of the regulatory signal remains unknown. In contrast, hyperglycemia induces a reduction in the amount of stored insulin in excess of any change in proinsulin, thereby causing the relative proportion of proinsulin to rise.

TABLE 2
General characteristics of the tolbutamide-infused rats

Group	n	Plasma glucose (mM)*			Extract IRI content (nmol)	Percentage of proinsulin in pancreas extract
		24 h	48 h	72 h		
0.45% NaCl	7	7.7 ± 0.4	7.6 ± 0.3	7.8 ± 0.3	8.1 ± 1.1	9.8 ± 0.7
Tolbutamide	6	7.3 ± 0.4	6.9 ± 0.3	7.8 ± 0.2	4.0 ± 0.8†	14.0 ± 1.4†

Data are means ± SE.

*Plasma glucose values obtained during 72-h infusion.

† $P < 0.02$.

How did hyperglycemia reduce the insulin content? Theoretically, hyperglycemia could act through several mechanisms, including a hyperstimulated insulin output that surpasses the capacity for proinsulin synthesis, stimulation of intracellular degradation of insulin (27,28), or an effect of chronic hyperglycemia that impairs proinsulin synthesis (29) or its conversion to insulin (18). The results with the insulin-release inhibitor diazoxide are most consistent with the first possibility. Reducing the plasma IRI level in the 35% glucose rats to that of the normoglycemic 20% glucose rats prevented any fall in insulin content and normalized the percentage of proinsulin. In contrast, the level of hyperglycemia was unaffected by diazoxide so that an effect of hyperglycemia to stimulate intracellular degradation of insulin or suppress proinsulin synthesis and/or processing all seem unlikely given the restoration of a normal percentage of proinsulin. Further support for the conclusion that proinsulin synthesis was not impaired was the observation of a raised pancreatic IRI content in the glucose-infused rats given diazoxide versus the controls given diazoxide, which is what would be expected in the presence of hyperglycemia.

Therefore, our results suggest that the raised proinsulin/insulin ratio in the β -cells of diabetic rats is a result of degranulation from hyperglycemia-induced hyperstimulated insulin release. Additional evidence for degranulation causing the raised pancreatic percentage of

proinsulin was obtained with the insulin secretagogue tolbutamide by showing that degranulation from a mechanism other than hyperglycemia would reproduce a raised percentage of proinsulin. The tolbutamide protocol was an adaption of that reported by Gold et al., which caused marked β -cell degranulation in normal rats by the oral administration of 500 mg/kg tolbutamide twice daily for 3 days (30). In preliminary trials with this protocol, we observed hypoglycemia throughout much of the first 2 days (data not shown) and thus turned to continuous infusion of tolbutamide in a glucose-containing infusate to maintain euglycemia. This approach resulted in a near 50% fall in pancreatic IRI content and a raised percentage of proinsulin. Of interest, the percentage of proinsulin ($14.0 \pm 1.4\%$) was not as high as that of 30% glucose-infused rats ($16.4 \pm 1.6\%$), even though the reduction in insulin content was considerably greater in the tolbutamide rats. Gold et al. (30) observed no change in proinsulin biosynthesis rate in isolated islets from their tolbutamide-infused rats. If that observation applies to the infusion approach, then the elevated pancreatic percentage of proinsulin represents an effect of degranulation alone. The exacerbated proinsulin/insulin mismatch in the glucose-infused rats may represent an additional factor, a hyperglycemia-induced rise in proinsulin synthesis. It should not be inferred from these results that tolbutamide treatment in humans causes a rise in the relative proportion of pancreatic proinsulin. The protocol in this study was designed specifically to cause β -cell degranulation by giving doses of the sulfonylurea that are far in excess of those used for control of hyperglycemia.

In summary, the raised proinsulin/insulin ratio in pancreas extracts of diabetic rats appears to represent an effect of chronic hyperglycemia to cause a preferential reduction in the pancreatic stores of insulin through hyperstimulation of insulin release. The net result is that the stored material becomes enriched with poorly active insulin precursors. Mobilization of this material would then cause a relative hypersecretion of proinsulin. These results support the hypothesis put forth several years ago that depletion of mature granules by hyperglycemia may be an important mechanism for the raised plasma proinsulin/insulin ratio in human NIDDM (3). Confirmation of this extrapolation requires parallel data showing a raised blood proinsulin/insulin ratio in the rats with a raised pancreatic proinsulin/insulin ratio. Unfortunately, the HPLC method is not sufficiently sensitive to accurately

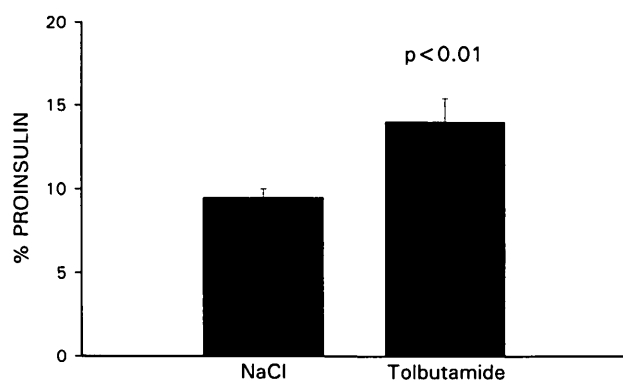


FIG. 4. Raised relative proportion of proinsulin in pancreas extracts from tolbutamide-infused treated normal rats. Rats were infused for 3 days with $1 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ tolbutamide or 0.45% NaCl. Euglycemia was maintained in tolbutamide-infused treated rats by adding glucose to the infusate. Pancreases underwent extraction, insulin/proinsulin precipitation, and HPLC separation of insulins and proinsulins. Percentage of proinsulin = IRI proinsulin/(IRI insulin + IRI proinsulin).

measure blood proinsulin in rats, and these studies must await the development of a rat proinsulin RIA. Also, it is known that the plasma proinsulin/insulin ratio is raised in normoglycemic relatives of people with IDDM (31–33) and in normoglycemic streptozocin-induced diabetic monkeys (34), which implies that additional mechanisms unrelated to hyperglycemia may cause a raised plasma proinsulin/insulin ratio.

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