Vol. 156, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1988 Pages 457-462

UNREGULATED SECRETION OF AN EXOGENOUS GLYCOTRIPEPTIDE BY RAT ISLETS AND HIT CELLS

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Received August 25, 1988

SUMMARY: Freshly isolated rat islets and cultured hamster insulinoma cells (HIT T15) were incubated with a membrane-permeable octanoyl tripeptide (N-octanoyl-ASN-TYR-THR-NH_2), which contains an acceptor sequence for ASN-linked glycosylation. Labeled octanoyltripeptide (^{125}[I]TYR) was glycosylated by both islets and HIT cells. The carbohydrate moiety of this glycotripeptide was removed by N-glycanase indicating that glycotripeptide was formed in the lumen of endoplasmic reticulum and, subsequently was secreted via the route for secretory protein. Secretion of glycotripeptide began more rapidly than that of insulin newly synthesized from ^{3}[H]leucine. At 30 min glycotripeptide secretion was already significant but, over a 3-h period, it never represented more than 21% of glycotripeptide produced. Glycotripeptide secretion was not affected by compounds shown to regulate insulin secretion (glucose, forskolin, EGTA and streptozotocin). Thus in beta cells, it appears that glycotripeptide secretion is unregulated and that its cellular secretory pathway is different from that for insulin.

INTRODUCTION: In normal rat islets we and others have previously reported that little, if any, newly synthesized insulin was secreted for the first 45 minutes after a ^{3}[H]leucine pulse and, once secretion of newly synthesized insulin began, its release was highly regulated by known secretagogues of pancreatic beta cells (1,2,3). Although unregulated, constitutive release of secretory proteins has been demonstrated in a variety of transformed endocrine cells (4,5), we and others have found that the constitutive pathway represents <1% of insulin released by rat islets (3,6). However, whether or not this stringent secretory regulation in rat islets is unique for insulin or is the case for every protein secreted from islets was unknown.

A simple method to address this problem has recently been described (7). It is based on introduction of an exogenous membrane-permeable peptide,

0006-291X/88 $1.50

which get trapped inside the endoplasmic reticulum after addition of a high mannose chain by the luminal, oligosaccharyl-N-transferase (8). This procedure has been used to trap exogenously added lipophilic peptides inside the endoplasmic reticulum of live cells from which the glycosylated product can only reach the cell surface via the secretory pathway (7). In two transformed cells, HepG2 and CHO, unregulated secretion of glycosylated product began after only 10 minutes and was sufficiently rapid that after 2 h, more glycosylated product was in the media than in the cells (7). We report here that octanoyltripeptide (N-octanoyl-ASN-TYR-THR-NH_2) is also N-glycosylated to glycotripeptide by normal islet cells, that onset of secretion of glycotripeptide occurs more rapidly than of newly synthesized insulin, that secretion rate is unaffected by secretagogues of insulin and that unlike HepG2 and CHO cells, in both rat islets and HIT cells, the majority (~80%) of glycotripeptide remains associated with the cells for at least 3 h.

EXPERIMENTAL PROCEDURE

Preparation of Rat Islets. Islets were prepared from 300-350 g, male Long-Evans rats that had free access to both food and water. After pentobarbital anesthesia, rat pancreases were inflated with Hanks buffer, removed, diced with a scissor and digested at 37°C with collagenase (Boehringer-Mannheim) using a three-step procedure (9,10). Digests were pipetted onto a discontinuous Percoll gradient, centrifuged 10 minutes at 1,000 g and a crude islet preparation was removed from the 38/60% Percoll interface. Islets were manually selected under a dissecting microscope and groups of 100-150 were put in 12 x 75 mm Falcon plastic tubes for labeling experiments.

Streptozotocin Treatment. Freshly isolated islets were exposed to streptozotocin (0.5 to 1.5 mg/ml) for 20 minutes at 37°C in Krebs-Ringer Bicarbonate (KRB), which contained 20 mM glucose and was gassed with 95% air:5% CO_2. Before experiments, streptozotocin-treated islets were washed twice with fresh KRB.

HIT Cells. HIT T15 cells (passage 77-78) were maintained at 37°C in RPMI 1640 + 10% fetal bovine serum + penicillin/streptomycin and were gassed with 95% air:5% CO_2 (11,12). Twenty-four hours before an experiment, 0.3-1.0 x 10^6 HIT cells were plated in the two centermost wells of a 12-well plate and returned to the incubator.

Iodination of Octanoyltripeptide. Five to 50 nmol of octanoyltripeptide in 50 µl acetonitrile was mixed with 100 µl of 0.5 M NaPi (pH 7.5). Between 0.5 to 10 mCi of ^{125}[I]NaI (carrier free, ICN) was added. To this solution, 100 µl of chloramine T (Sigma) (2 mg/ml) in 0.5 M NaPi (pH 7.5) was added. After two minutes at room temperature, the reaction was stopped by introduction of 400 µl of a solution of sodium bisulfite (2.4 mg/ml) in 0.05M NaPi (pH 7.5) followed by 600 µl water. This solution was loaded onto a Sep-Pak C18 cartridge (Waters) and washed sequentially with 20 ml of 0.1% TFA and 20 ml of 5% acetonitrile in 0.1% TFA. Iodinated octanoyltripeptide was eluted with 60% acetonitrile in 0.1% TFA, concentrated in a speed-vac centrifuge and dissolved and stored in DMSO. Iodination yields were between 25 and 50% and specific radioactivities were between 0.1 and 4.0 x 10^8 cpm/nMol (7).

Labeling of Cells with ^{125}[I]Octanoyltripeptide. After medium was removed, cells were preincubated at 37°C in KRB: islets (Dubnoff metabolic shaker bath, 50-60 cycles/minute, 95% O_2:5% CO_2); and HIT cells (incubator, 95% air:5% CO_2). After 45 min of preincubation, medium was removed and replaced with fresh KRB containing ^{125}[I]octanoyltripeptide (1% final concentration of DMSO) and other specified additions. At indicated times, incubation medium and cells were separated. An aliquot of incubation medium was taken for insulin radioimmunoassay and both the cells and the remainder of each incubation medium were rapidly frozen on dry ice.

Extraction and Purification of Glycotripeptides. Cells were thawed and extracted in 440 µl of ice-cold buffer A (10 mM Tris HCl [pH 7.4], 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.5% Triton X-100). Medium was thawed and brought to 0.5% Triton X-100, 1 mM $MnCl_2$ and 1mM $CaCl_2$ by additions from concentrated stock solutions. Extracts were centrifuged for 1 minute in a microfuge and supernatants loaded on Con A-Sepharose (Pharmacia) columns (approximately 250 µl bed volume). Columns were washed with buffer A (5 x 1 ml) then eluted with 0.5 M α-methylmannoside in buffer A (3 x 0.5 ml). Quantitation of eluted ^{125}[I]glycotripeptide was done in a gamma counter (Tracor).

Immunoassay of insulin. Insulin was extracted from islets with ice-cold ethanol:water:HCl, 83.3:14.9:1.8 (v/v/v). Extracted cellular insulin and insulin in the incubation medium were quantitated by radioimmunoassay using tubes coated with guinea pig anti-insulin serum (Linco) and rat insulin as standard (13). Interference by residual ^{125}[I]octanoyltripeptide was eliminated by two additional washing of immunoassay tubes with water (2 x 1 ml).

Statistical Analysis. Numbers represent mean ± SEM for (n) experiments done on separate days. Data were analyzed with student's paired t-test and 1-way ANOVA.

RESULTS AND DISCUSSION

Rat islets were incubated for 3 h with ^{125}[I]-labeled substrate peptides and glycosylated products isolated on Con A-Sepharose columns. Membrane pearmeability of substrate peptides was absolutely necessary for glycosylation; only 57 cpm was recovered on a Con A-Sepharose column with a charged and membrane-impermeable ^{125}[I]hexapeptide while 11,488 cpm was recovered with an equal amount of ^{125}[I]octanoyltripeptide (both had the same glycosylation acceptor sequence).

Synthesis of ^{125}[I]glycotripeptide was saturable, i.e. inhibited 90 to 100% by addition of unlabeled octanoyltripeptide 20 to 40 µg/0.4 ml (data not shown). To determine if carbohydrate moieties were N-linked, glycosylated products were incubated with or without N-glycanase (an enzyme which hydrolyzes only N-linked carbohydrate bonds) and then run on a silica-gel, thin-layer chromatographic system. Figure 1 is a radioautogram from such an experiment. For both untreated (lane 1) and mock incubated (without N-glycanase) (lane 2) controls, there were several slowly migrating bands indicating synthesis and subsequent processing of glycotripeptide had occurred in rat islets. After incubation with N-glycanase (lane 3), slowly migrating bands were converted to broad bands that migrated as

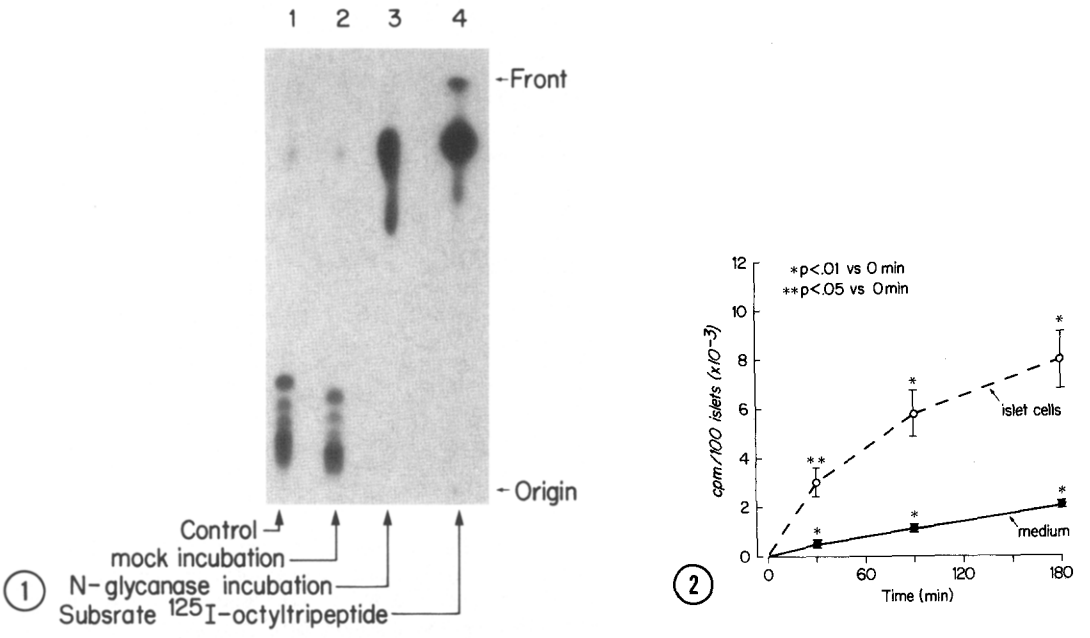

Fig. 1. Characterization of ^{125}[I]glycotripeptide synthesized by rat islets. Glycotripeptide was purified from extracts of rat islets by Con A-Sepharose and aliquots were incubated with or without N-glycanase, an enzyme which hydrolyzes only N-glycosidic linkages. Samples were applied to silica-gel thin-layer plates, chromatographed with butanol:acetic acid:water [5:2:2 (v/v/v)] as the mobile phase and then exposed to X-ray film. Lanes on autoradiograph are: 1. Control, untreated glycotripeptide; 2. Control, mock-digested (without N-glycanase) glycotripeptide; 3. N-glycanase-digested glycotripeptide; and 4. the original substrate octanoyltripeptide.

Fig. 2. Cumulative appearance of ^{125}[I]glycotripeptide in rat islet cells and their medium.

*P <.01 vs. 0 min.
**P <.05 vs. 0 min.

In four experiments, freshly isolated rat islets were continuously incubated in 0.4 ml KRB (20 mM glucose) containing 22.5 ± 1.3 x 10^6 cpm ^{125}[I]octanoyltripeptide. At indicated times, media and cells were separated and glycotripeptide in each fraction was purified (Con A-Sepharose) and quantitated.

rapidly as the substrate ^{125}[I]octanoyltripeptide (lane 4) and only 8% (754 of 9749 cpm) was recovered on a subsequent Con A-Sepharose column; after mock digestion, 95% (9252 of 9749 cpm) was recovered. Thus, octanoyltripeptide must penetrate islet membranes to be glycosylated and the site of glycosylation is the lumen of endoplasmic reticulum (the only organelle known in which N-glycosylation occurs).

With islets continuously incubated with ^{125}[I]octanoyltripeptide, synthesis of glycotripeptide occurred for 3 h (Figure 2) and rate was not affected by glucose concentration (data not shown). Like results reported for HepG2 and CHO cells (7), glycotripeptide was both stored and secreted by

rat islets. However in islets, unlike these other two cell lines, after 3 h secreted glycotripeptide never represented more than 21% of the total glycotripeptide produced. Partitioning of glycotripeptide predominantly in the cellular fraction of islets does not represent an underestimation of extracellular glycotripeptide due to extracellular degradation because when purified ^{125}[I]glycotripeptide was incubated with islets under the same conditions, it was completely recovered (16,092 cpm at 0 min vs. 17,202 cpm at 180 min).

Partioning of glycotripeptide predominantly in the cellular fraction also occurred with transformed hamster beta cells (HIT T15); in three experiments, only 8% of glycotripeptide was secreted after 3 h (secreted, 1080 ± 260 cpm vs. cell, 14,500 ± 3,500 cpm per million cells).

Although islets secreted less than 1/4 of the newly synthesized glycotripeptide in 3 hr, onset of secretion was surprisingly rapid. Glycotripeptide in media was significantly elevated at 30 min, the first measured time point, and for 3 h, it was secreted at a nearly constant rate (Figure 2). Under near identical conditions, we and others previously reported that newly synthesized insulin required more than 30 min to transit from endoplasmic reticulum to secretory granules or plasma membrane (1,3,14,15). Thus, it is likely that glycotripeptide and insulin diverge at some point along the cellular secretory route, suggesting that there is more than one secretory pathway in normal islets.

As a test of whether or not glycotripeptide and insulin were localized in the same secretory granules, islets were first loaded with ^{125}[I]glycotripeptide and then tested for secretory regulation by glucose, forskolin and EGTA (Table 1). Rates of insulin secretion were significantly faster with 20 mM glucose + 10 nM forskolin than with any other condition tested and observed secretory rates spanned a more than 8-fold range from 1.96 to 17.5%/h. In contrast, rates of glycotripeptide secretion were not significantly affected by any of these compounds indicating that, as reported for HepG2 and CHO cells (7), there also is an unregulated secretory pathway in islets.

In summary, this is the strong evidence that: 1. exogenous octanoyltripeptide can be glycosylated and secreted by normal islet cells; 2. there is an unregulated secretory pathway in islets; and 3. both islets and HIT cells differ from HepG2 and CHO cells in that they store, rather than secrete, most of the newly synthesized glycotripeptide.

Table 1
Effects of Glucose, Forskolin and EDTA on Secretion Rates of Insulin and ^{125}[I]Glycotripeptide

Regulators of Insulin Secretion	Secretion Rate of Insulin (% IRI/h)	Secretion Rate of ^{125}[I]Glycotriptide (cpm/h)
20 mM glucose + 10 uM forskolin	17.5 ± 3.1	730 ± 35
20 mM glucose	7.98 ± 1.6*	723 ± 136
20 mM glucose + 3 mM EGTA	3.93 ± 1.4*	613 ± 66
2 mM glucose + 10 uM forskolin	4.82 ± 1.5*	777 ± 109
2 mM glucose	1.96 ± 0.66*	785 ± 57

* p <.01 vs. 20 mM glucose + 10 uM forskolin; all other comparisons are not significant;
Numbers represent mean + SEM for three experiments. One hundred percent immunoreactive insulin was 53.6 ± 14 ng/islet.
Prior to measurement of secretion rates of insulin and ^{125}[I]glycotripeptide, islets were:

1. Incubated 45 min in 0.4 ml Krebs-Ringer bicarbonate buffer containing 20 mM glucose;
2. Incubated 2 hr in 0.4 ml Krebs-Ringer bicarbonate buffer containing 20 mM glucose and 21.0 ± 10 x 10^6 cpm ^{125}[I]-octyltripeptide; and
3. Washed once and returned to incubation for 1 hr with 0.4 ml Krebs-Ringer bicarbonate containing specified regulators.

ACKNOWLEDGMENT We thank Dr. James E. Rothman for his suggestions and critical review of the manuscript.

REFERENCES

1. Jain, K., and Logothetopoulos, J. (1977) Diabetes 26, 650-566.
2. Gold, G., Gishizky, M. L., and Grodsky, G. M. (1982) Science 218, 56-58.
3. Gold, G. Landahl, M. D., Gishizky, M. L., and Grodsky, G. M. (1982) J. Clin. Invest. 69, 554-563.
4. Moore, H-P., Gumbiner, B., and Kelly, R. B. (1983) Nature 302, 434-436.
5. Kelly, R. B. (1985) Science 230, 25-32.
6. Rhodes, C. J., and Halban, P. A. (1987) J. Cell Biol. 105, 145-153.
7. Wieland, F. T., Gleason, M. L., Serafini, T. A., and Rothman, J. E. (1987) Cell 50, 289-300.
8. Welphy, J. K., Shenbagamurthi, P., Lennarz, W. J., and Naider, F. (1983) J. Biol. Chem. 258, 11856-11863.
9. Lacy, P. E., and Kostianovsky, M. (1967) Diabetes 16, 35-39.
10. Gold, G., Pou, J., Gishizky, M. L., Landahl, H. D., and Grodsky, G. M. (1986) Diabetes 35, 6-12.
11. Hill, R. S., and Boyd, A. E. III (1981) Diabetes 34, 115-120.
12. Gold, G., Qian, R. L., and Grodsky, G. M. (1988) Diabetes 37, 160-165.
13. Lundquist, I., Fanska, R., and Grodsky, G. M. (1976) Endocrinology 99,1304-1
14. Orci, L, Lambert, A. E., Kanazawa, Y., Amherdt, M., Rouiller, C., and Renold, A E. (1971) J. Cell Biol. 50, 565-582.
15. Nagamatsu, S., and Grodsky, G. M. (1987) Biochem. Biophys. Res. Commun. 148, 1418-1424.