

BRIEF REPORT: IMPAIRED PROCESSING OF PROHORMONES ASSOCIATED WITH ABNORMALITIES OF GLUCOSE HOMEOSTASIS AND ADRENAL FUNCTION

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INSULIN, the central hormone controlling the utilization of fuel, is initially synthesized in pancreatic beta cells as its relatively inactive precursor, proinsulin. A series of enzymes that process prohormones act on proinsulin to produce mature insulin and C peptide. Abnormalities in the secretory products of beta cells are a rare but well-documented cause of impairment in glucose tolerance and hyperinsulinemia.¹

Two specific syndromes have been defined. First, mutations in the insulin gene that lead to the production of biologically ineffective insulin have been described in several families.²⁻⁴ All cases described to date have involved heterozygotes who produce normal insulin molecules from the unaffected allele. The second syndrome is familial hyperproinsulinemia,^{5,6} in which heterozygous mutations affecting the cleavage of proinsulin lead to the secretion of excessive amounts of proinsulin.

We report a case that represents a third disorder. In the patient we describe here the sequence of the proinsulin gene was normal but plasma concentrations of proinsulin and one of the early products of proinsulin cleavage were high, and plasma insulin concentrations were low. In addition to the abnormality of proinsulin processing, there was evidence of impaired processing of proopiomelanocortin (POMC), secondary hypocortisolism, and hypogonadotropic hypogonadism, suggesting the possibility of a more generalized defect related to impaired processing of prohormones.

CASE HISTORY

The patient, a 43-year-old woman, was referred for the evaluation of symptoms suggestive of postprandial hypoglycemia. These symptoms were long-standing, and the referral was precipitated by a particularly severe episode. The patient's history included severe childhood obesity (weight at the age of three years, 36 kg), for which she had been treated successfully with diet. Her subsequent development, including the development of secondary sexual characteristics, was normal, except that she did not begin menstruating and continued to have moderate obesity. Studies undertaken at the age of 21 detected no cause of her primary amenorrhea. At the age of 30, ovulation

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Dr. O'Rahilly and Dr. Gray are supported by the Wellcome Trust.

was induced with gonadotropins. She became pregnant and delivered healthy quadruplets. The pregnancy was complicated by gestational diabetes mellitus that required treatment with insulin. After this pregnancy, her fasting plasma glucose level was normal, but persistent amenorrhea recurred.

The patient, an only child, weighed 89.2 kg at presentation and was 1.61 m tall. She had a body-mass index (calculated as the weight in kilograms divided by the square of the height in meters) of 34.4, a waist:hip ratio of 0.83, and a blood pressure of 130/70 mm Hg. Her parents had died, and her only living first-degree relatives were her four children, who were clinically normal. In addition to symptoms suggestive of reactive hypoglycemia, she reported fatigue and excessive daytime somnolence. She gave informed consent for all studies.

METHODS

The normal pathways of proinsulin processing are shown in Figure 1. (Details are given in the Discussion section.) Plasma concentrations of insulin, proinsulin, and products of proinsulin cleavage (32,33 split proinsulin and 65,66 split proinsulin) were assayed with two-site immunoradiometric or immunoenzymometric assays as previously described,⁷ and these values are expressed in Système International (SI) units to facilitate the comparison of molar ratios. The assay for 32,33 split proinsulin did not distinguish between 32-33 split proinsulin and des-31,32 proinsulin, the product formed when the dibasic residues of 32-33 split proinsulin are removed. To simplify this presentation, the two molecules detected in this assay are referred to as 32,33 split proinsulin. Similarly, the assay for 65,66 split proinsulin did not distinguish between 65-66 split proinsulin and des-64,65 proinsulin; therefore, the former term is used here for simplicity. Insulin did not cross-react in any of the other assays. However, 65,66 split proinsulin cross-reacted completely in the insulin assay (unpublished data).

For the analysis by high-performance liquid chromatography (HPLC), immunoreactive insulin, proinsulin, and proinsulin-cleavage products were extracted from plasma by immunoaffinity chromatography,⁸ and the resulting material was then separated by reverse-phase HPLC. Immunoreactivity was measured in the insulin peak by an immunoradiometric assay using an insulin standard, and in the peaks corresponding to proinsulin and the intermediate cleavage products by an enzyme-linked immunosorbent assay for proinsulin using a proinsulin standard.⁸

Corticotropin precursors were measured with a two-site immunoradiometric assay.⁹ This assay measures both full-length POMC and pro-corticotropin (the species containing the N terminal of POMC, γ -melanocyte-stimulating hormone, the joining peptide, and corticotropin). Corticotropin was measured by a two-site immunoradiometric assay specific for the mature sequence of corticotropin, with less than 1 percent cross-reactivity with POMC and less than 10 percent cross-reactivity with pro-corticotropin.⁹

Glucagon-like molecules were assayed with a two-site immunoradiometric assay that identified all species containing the core sequence of glucagon.¹⁰ Because plasma concentrations of glucagon-like immunoreactivity in our patient were low, no specific assays for glucagon precursors or mature glucagon were undertaken.

To establish the nucleotide sequence of the coding region of the insulin gene, exons 2 and 3 of the patient's genomic DNA were amplified with use of the polymerase chain reaction.³ One of the primers for each exon was biotinylated, to allow the sequencing of single-stranded DNA with modified T7 polymerase.¹¹ In exon 2 there were a number of ambiguities in the sequence that could not be resolved on repeated direct sequencing. Exon 2 was therefore subcloned into PCR-Script (Stratagene, La Jolla, Calif.), and eight independent clones were sequenced.

RESULTS

Glucose Homeostasis

The patient's fasting plasma glucose concentration was normal (87 mg per deciliter [4.9 mmol per liter]), but the plasma glucose concentration two hours after the oral administration of 75 g of glucose was high (206 mg per deciliter [11.5 mmol per liter]). The plasma

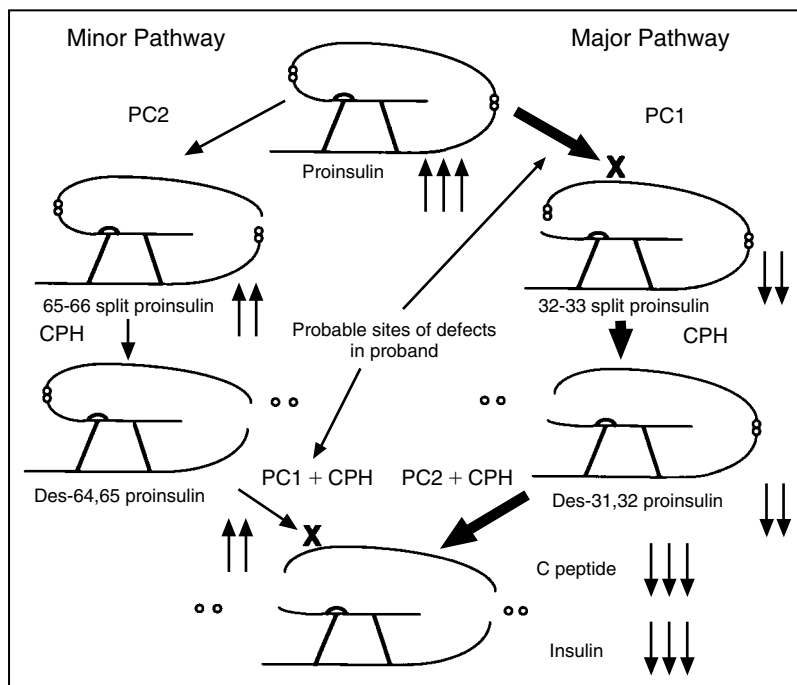


Figure 1. Normal Pathways of Proinsulin Processing and Effects of the Putative Defect in Prohormone Convertase 1 in the Study Patient.

Normally, prohormone convertase 1 (PC1) and prohormone convertase 2 (PC2) both act on proinsulin, but the major pathway involves the initial cleavage of proinsulin by PC1 to form 32-33 split proinsulin, followed by the rapid removal of Arg31 and Arg32 (small circles) by the ubiquitous carboxypeptidase H (CPH) to form des-31,32 proinsulin. This intermediate product is then cleaved by PC2 and CPH to form insulin and C peptide, with the removal of Lys64 and Arg65 (small circles) at the 65-66 site. Ordinarily, PC2 activity at the 65-66 site appears to be facilitated by prior cleavage by PC1 at the 32-33 site. In vitro, PC2 has low but detectable activity at the 32-33 site, but this is not thought to be biologically relevant. A defect in PC1 activity in the patient at the sites marked by an X would explain the high plasma concentrations of proinsulin and 65-66 split proinsulin and the low and undetectable concentrations of 32-33 split proinsulin and insulin, respectively.

concentrations of intact proinsulin and 65,66 split proinsulin two hours after the oral administration of glucose were very high (>5000 pmol per liter and >500 pmol per liter, respectively). Considering the cross-reactivity of 65,66 split proinsulin in the insulin assay, negligible amounts of plasma insulin were detected. In an HPLC analysis of the insulin-containing immunoreactive material in 1.0 ml of plasma, the principal peak was for proinsulin; des-64,65 proinsulin was the next most abundant species, and no insulin was detected (Fig. 2). In a 10-ml sample of plasma, insulin was barely detectable (data not shown). These results contrast sharply with the findings in the plasma of normal subjects or patients with non-insulin-dependent diabetes mellitus, in which the concentrations of 32,33 split proinsulin usually exceeded those of proinsulin and 65,66 split proinsulin and in which des-64,65 proinsulin was undetectable.⁷ The nucleotide sequences of both exons of the insulin gene were normal.

Measurements obtained before and after two standardized meals are shown in Table 1. The patient had borderline hypoglycemia after the first meal (plasma glucose at 5 hours, 50 mg per deciliter [2.8 mmol per

liter]), accompanied by mild light-headedness and somnolence. The fasting plasma concentrations of proinsulin and 65,66 split proinsulin were markedly elevated; they increased after the meals in a normal fashion. Although the absence of a history of fasting hypoglycemia and the normal responsiveness of plasma proinsulin to meals and oral glucose made the diagnosis of an insulinoma unlikely, the patient underwent a 48-hour fast, during which she had no symptoms or hypoglycemia.

Processing of Other Prohormones

Table 2 shows the plasma concentrations of corticotropin and corticotropin precursors, as measured basally and during an insulin-tolerance test. The concentration of corticotropin precursors in the basal sample, obtained at 9 a.m., was high (151 pmol per liter),¹² and precursor molecules were present in a molar excess of 50 to 75 times the number of molecules of mature corticotropin throughout the test. Although this ratio was higher than normal (normal molar excess of precursor molecules at 9 a.m., 1 to 10 times the number of molecules of mature corticotropin),¹³ it was less abnormal than the defect in proinsulin processing. Because corticotropin precursors have some (<10 percent) cross-reactivity in the corticotropin

assay, the concentrations of mature corticotropin were probably lower than the assay values indicate.

The fasting plasma concentration of glucagon-like molecules was 20 pmol per liter (normal range, 20 to 40). Although no assays for specific proglucagon gene products were undertaken, the low-normal overall concentrations of glucagon-like molecules make a major defect in proglucagon processing unlikely.

Investigation of Other Endocrine Systems

Hypothalamic-Pituitary-Adrenal Function

Although the plasma cortisol concentration at 9 a.m. on the day of the cosyntropin test was low (1.6 μ g per deciliter [44 nmol per liter]), the concentration 30 minutes after the intramuscular injection of 0.25 mg of cosyntropin was normal (23.4 μ g per deciliter [646 nmol per liter]), indicating normal adrenal responsiveness. The results of a test of responses to insulin-induced hypoglycemia performed at 9 a.m. are shown in Table 2. Both the plasma cortisol concentration and the growth hormone responses to insulin-induced hypoglycemia showed impairment. In addition, the plasma cortisol values obtained at 9 a.m. on three other days were all

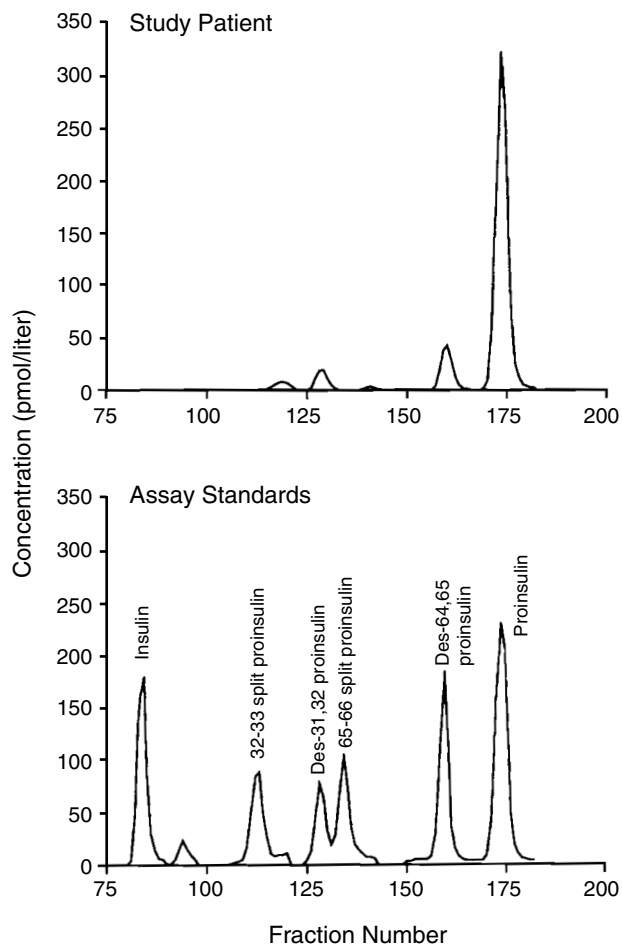


Figure 2. Quantitation of Insulin and Insulin-Precursor Molecules from the Study Patient by High-Performance Liquid Chromatography, and Assay Standards.

The patient's plasma contained no detectable insulin, large amounts of proinsulin, and smaller amounts of des-64,65 proinsulin. Des-31,32 proinsulin and 32-33 split proinsulin were detectable in low amounts. (The relative amounts of proinsulin precursors and insulin shown in the lower panel are not indicative of those in the plasma of normal subjects.)

below 5 μg per deciliter (138 nmol per liter). Plasma concentrations of 17-hydroxyprogesterone, dehydroepiandrosterone, and androstenedione were all in the normal range. The low 9 a.m. plasma cortisol concentrations and slight impairment of the plasma cortisol response to hypoglycemia were interpreted as indicating the presence of hypoadrenalism. Despite this finding, the adrenal responsiveness to 0.25 mg of cosyntropin was normal, a fact that suggested the absence of substantial adrenal atrophy. The patient's fatigue and excessive daytime somnolence lessened with hydrocortisone-replacement therapy.

Other Endocrine Investigations

The patient's linear growth during childhood was normal, and the plasma concentration of insulin-like growth factor I was normal (500 U per liter; normal

range for the patient's age, 300 to 1200). The poor response of plasma growth hormone to hypoglycemia (Table 2) can be explained by the patient's obesity.¹⁴

The serum estradiol concentration was 27 pg per milliliter (99 pmol per liter) (normal range in the follicular phase, 27 to 204 pg per milliliter [99 to 749 pmol per liter]). The serum concentration of follicle-stimulating hormone (FSH) was 1.5 IU per liter (normal follicular-phase range, 2.9 to 8.4), and that of luteinizing hormone (LH) 0.3 IU per liter (normal follicular-phase range, 1.3 to 8.4). The results of a stimulation test with 100 μg of gonadotropin-releasing hormone indicated hypogonadotropic hypogonadism (basal and peak plasma FSH values, 0.7 and 3.5 IU per liter, respectively; basal and peak plasma LH values, 0.3 and 2.1 IU per liter). Plasma concentrations of testosterone, sex hormone-binding globulin, and prolactin were normal, as were the results of magnetic resonance imaging of the hypothalamus. There was no abnormality of thirst, and the osmolality of plasma and urine was consistently normal. Plasma concentrations of renin and aldosterone were normal.

The patient was clinically euthyroid, with no thyroid enlargement. A test for antithyroid microsomal antibodies was strongly positive. The serum thyrotropin concentration was slightly high (5.8 mU per liter; normal range, 0.4 to 4); the concentrations of free thyroxine (0.7 ng per deciliter [9.0 pmol per liter]; normal range, 0.7 to 1.6 ng per deciliter [9.0 to 20.6 pmol per liter]) and free triiodothyronine (0.27 ng per deciliter [4.1 pmol per liter]; normal range, 0.20 to 0.50 ng per deciliter [3.1 to 7.7 pmol per liter]) were at the low end of the normal range. The serum thyrotropin response to the administration of 200 μg of thyrotropin-releasing hormone was normal (basal, 3.1 μU per milliliter; peak [after 40 minutes], 29). The plasma prolactin values before and after the administration of thyrotropin-releasing hormone were 3.7 and 24.1 ng per milliliter, respectively. These results indicated that the patient had intermittent subclinical hypothyroidism caused by chronic autoimmune thyroiditis.

DISCUSSION

This woman with impaired glucose tolerance and reactive hypoglycemia had a barely detectable plasma insulin concentration and high plasma proinsulin concentrations — indications that the primary defect was one of proinsulin processing. There was a coexistent, although less severe, defect in the processing of corticotropin precursors that led to mild hypocortisolemia. Thus, the patient's hyperproinsulinemia was accompanied by features suggestive of a more widespread endocrine disorder.

With regard to the circulating proinsulin-like molecules, the previously reported defects in proinsulin processing were caused by heterozygous mutations in the insulin gene that resulted in the synthesis of abnormal molecules resistant to processing.^{5,6} In another family in which a primary defect in proinsulin processing was proposed,¹⁵ the affected subjects had substan-

Table 1. Plasma Concentrations of Glucose, Insulin, and Insulin Precursors in the Study Patient in Response to Test Meals.*

NO. OF MINUTES AFTER START OF FIRST MEAL	GLUCOSE	INSULIN	PRO- INSULIN	65,66 SPLIT PROINSULIN
	mg/dl	picomoles per liter		
0	96	425	2460	390
120	164	1543	8250	1600
300	50	369	3525	325
420	78	280	2100	250
610	85	582	2180	420
660	184	1946	6895	1455
780	90	1246	8160	1240
Normal fasting values	<108	<100	<15	<5

*After an overnight fast, the patient ate a standardized meal between 8 and 8:15 a.m. and another between 6:10 and 6:25 p.m. Each meal contained 800 kcal, consisting of 50 percent carbohydrate, 35 percent fat, and 15 percent protein. In the insulin assay, 65,66 split proinsulin, which is normally undetectable in human plasma, has 100 percent cross-reactivity. Thus, most, if not all, of the assayed insulin is accounted for by the 65,66 split proinsulin detected. Measurements of 32,33 split proinsulin are unreliable when proinsulin concentrations are very high. Concentrations of insulin and proinsulin-like molecules are expressed in SI units to facilitate the comparison of molar ratios. To convert values for plasma glucose to millimoles per liter, multiply by 0.056; to convert values for plasma insulin to microunits per milliliter, multiply by 0.17.

tial amounts of circulating true insulin; subsequently, linkage to the insulin gene was demonstrated.¹⁶ The results in our patient provide biochemical evidence of primary defects in insulin processing.

Proinsulin has about 5 percent of the biologic activity of insulin.¹⁷ Therefore, at least in the basal state, this patient's beta cells have compensated for the failure of normal processing by secreting large amounts of proinsulin and thus maintaining fasting normoglycemia. The combination of impaired glucose tolerance and reactive hypoglycemia 5 hours after a meal is probably related to the considerably slower clearance of proinsulin (plasma half-life of proinsulin, approximately 20 minutes, vs. approximately 4 minutes for insulin¹⁸).

The normal sequence of the proinsulin gene and the absence of any abnormally migrating peaks in the HPLC analysis of insulin rule out an intrinsic abnormality in the insulin molecule itself. Insulin is normally produced by two prohormone-cleaving enzymes on the proinsulin molecule (Fig. 1).¹⁹ One of these, prohormone convertase 1 (PC1), cleaves proinsulin between the 32 position and the 33 position, and the other, prohormone convertase 2 (PC2), cleaves it between the 65 position and the 66 position, and the resultant intermediate product is trimmed of its terminal lysines or arginines by the action of carboxypeptidase H (also known as carboxypeptidase E). Further details about the normal processing of proinsulin are given in the legend to Figure 1. A defect in PC2 would be expected to lead to an excess of des-31,32 proinsulin over proinsulin, the opposite of the findings in this patient. In addition, PC2 cleaves proglucagon in alpha cells,²⁰ which appeared to be normal in the patient. Thus, the cleavage of proinsulin at the 65,66 site and the absence of an elevation of the proglucagon

concentration make it unlikely that a primary defect in PC2 explains the findings in our patient.

If, on the other hand, the activity of PC1 is defective, one would predict a low concentration of 32,33 split proinsulin, but to some extent proinsulin should be processed by PC2 to 65,66 split proinsulin. However, because PC2 processes 32,33 split proinsulin more efficiently than proinsulin, a large excess of intact proinsulin over 65,66 split proinsulin would also be predicted. Because 65,66 split proinsulin cannot be processed further without PC1, a substantial elevation in plasma concentrations of this molecule would be expected. All the abnormalities of proinsulin processing that would be expected as a result of defective PC1 activity were found in this patient.

In addition to its role in the processing of proinsulin, PC1 may be involved in the production of corticotropin from POMC in the corticotrophs. The precise series of events by which POMC is converted to corticotropin is not known, however. For example, PC2, which is expressed at a low level in corticotrophs, has some activity at various POMC-cleavage sites,²¹ and the widely expressed processing enzyme furin may be capable of processing some POMC.²² Our patient had a moderate elevation of corticotropin precursors in plasma, together with sufficient corticotropin to maintain nearly normal adrenal production of glucocorticoid. This suggests that the underlying defect affects the processing of proinsulin more than that of POMC. An infant with severe hypocortisolemia who had a very high ratio of precursors to corticotropin in plasma despite a normal POMC gene sequence was recently described.²³ In contrast to our patient, that infant had no evidence of an abnormality in proinsulin processing.

Given the probable nature of the defect in the processing of prohormones, a genetic defect appears most likely. Unfortunately, our patient was an only child

Table 2. Hormonal Responses to Insulin-Induced Hypoglycemia.*

NO. OF MINUTES AFTER INSULIN INJECTION	PLASMA GLUCOSE	PLASMA CORTISOL	PLASMA CORTICO- TROPIN PRE- CURSORS	PLASMA CORTICO- TROPIN	PRECURSOR: CORTICO- TROPIN RATIO	PLASMA GROWTH HORMONE
	mg/dl	μg/dl	pmol/liter			μg/liter
0	88	4.3	151	2.0	75	<0.2
30	60	3.1	70	1.3	54	<0.2
45	27	4.7	99	1.3	76	0.7
60	27	1.8	315	4.9	64	0.9
90	70	15.8	653	12.4	53	3.1
Normal 9 a.m. values	—	—	5–40	0.9–11.3	1–10	—
Normal peak values	—	>20	—	—	—	>8

*Insulin was injected intravenously in a dose of 0.15 U per kilogram of body weight at 9 a.m. Plasma concentrations of corticotropin and corticotropin precursors are shown in SI units to facilitate the comparison of molar ratios. To convert values for plasma glucose to millimoles per liter, multiply by 0.056; to convert values for plasma cortisol to nanomoles per liter, multiply by 27.6; and to convert values for plasma corticotropin to picograms per milliliter, multiply by 4.54. Because values for corticotropin precursors represent the sum of the values for various molecular species, no simple conversion factor can be used.

whose parents are no longer living. Plasma proinsulin concentrations in the patient's four children were normal, but the children did have a slight increase in the fasting plasma proinsulin concentration relative to the concentration of 32,33 split proinsulin (data not shown), which would be consistent with a mild defect in PC1 activity. Complementary DNA for PC1 has been isolated,²⁴⁻²⁶ but no information about its genomic structure is available. Because PC1 has restricted expression in tissue,²⁴ this information will be needed before this candidate gene can be readily examined for mutations.

The finding of abnormalities in our patient other than those directly related to the defect of proinsulin processing is unusual, in that such abnormalities were not described previously in patients with disorders of insulin processing. The potential relevance of the early-onset obesity, hypogonadotropic hypogonadism, and defective POMC processing in the patient has been highlighted by the recent discovery of the molecular basis for the fat/fat phenotype in mice.²⁷ This recessive syndrome of obesity, hyperglycemia, and impaired fertility is associated with abnormalities of proinsulin and POMC processing and results from a mutation in the gene for carboxypeptidase H.²⁷ Although the precise nature of our patient's defect in proinsulin cleavage is not identical to that in fat/fat mice, there are similarities in the morphologic and endocrine phenotypes.

In summary, our patient was almost completely unable to process normal proinsulin to insulin. Despite the presumed absence of insulin, she had fasting normoglycemia, although impaired glucose tolerance and mild reactive hypoglycemia were present, presumably because of the altered plasma kinetics of proinsulin as compared with insulin. The associated clinical features of early-onset obesity, hypogonadotropic hypogonadism, and mild secondary hypoadrenalism in association with biochemical evidence of a moderate defect in the processing of POMC to corticotropin suggest, but do not prove, that the expression of this disorder may not be restricted to pancreatic beta cells.

We are indebted to Professor C.N. Hales, Dr. J. Hutton, Dr. J. Creemers, Dr. P. Raggett, Dr. S. Nussey, Mr. C. Montague, Dr. C. Matthews, Dr. K. Chatterjee, and Dr. O.M. Edwards for helpful discussion; to Dr. Cathrin Orskov for the glucagon assays; to Dr. Nick Martensz, Dr. Fatima Zaidi, and Ms. Fiona Tulloch for technical assistance; and to Ms. Louise Sanders for careful reading of the manuscript.

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CORRECTION

Impaired Processing of Prohormones Associated with Abnormalities of Glucose Homeostasis and Adrenal Function

Impaired Processing of Prohormones Associated with Abnormalities of Glucose Homeostasis and Adrenal Function . On page 1387, in Figure 1, in the Minor Pathway, the left side of the proinsulin molecule should be cleaved, and in the Major Pathway, the right side of the molecule should be cleaved — the reverse of what is shown in the figure. The legend is correct as published.