CHARACTERIZATION OF SUPER-ACTIVE INSULIN, PROLACTIN AND PLACENTAL LACTOGEN

Y. J. Topper, T. Oka, B. K. Vonderhaar and M. Wilchek*

National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014.

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SUMMARY: Complexes between insulin, prolactin or placental lactogen and cyanogen bromide-activated Sepharose release hormone-like materials when treated with bovine serum albumin (BSA) (1). These materials have enhanced biological activities, and are, presumably, N_1N_2-disubstituted guanidines in which the hormones and BSA are the substituents. The present studies show that ammonium bicarbonate can substitute for BSA in the generation of the super-active hormones. Super-activity of the released, guanidinated hormones, therefore, can be manifested in the absence of the BSA substituent. The key derivatized amino acid residues have not yet been identified, but it appears that guanidination of lysine is either unnecessary or insufficient. Several operational considerations which are important for the demonstration of these enhanced activities are discussed.

INTRODUCTION

It has been shown (1,2) that a super-active insulin can be released from insulin-Sepharose in the presence of bovine serum albumin (BSA). Similarly, a super-active prolactin and super-active placental lactogen can be released from the corresponding Sepharose complexes (3). It has also been reported (4) that these forms of insulin (I) prolactin (P) and placental lactogen (L) are presumably N_1N_2-disubstituted guanidines, in which the hormone and BSA are the substituents. This report establishes that the BSA-substituent is not essential for the super-activity of these hormone derivatives. In addition, several operational considerations relating to the demonstration of these activities are discussed.

MATERIALS AND METHODS

Ammonium bicarbonate was purchased from Fisher Scientific Company. ^{14}C-1-D-glucose (sp. act. 8.0 mC per mMole) was obtained from New England Nuclear. ^{14}C-1-α-amino-isobutyric acid (sp. act. 2.4 mC per mMole) was purchased from New England Nuclear.

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* On leave from The Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel.

Porcine zinc-insulin was a gift from the Eli Lilly Company. Human placental lactogen was purchased from ICN Pharmaceuticals, Inc. Bovine prolactin was a gift from the National Institutes of Health.

In the present studies, insulin-like material (ILM), prolactin-like material (PLM) and placental lactogen-like material (LLM) were generated by treating the corresponding hormone-Sepharose complexes with ammonium bicarbonate (NH_4HCO_3), rather than with BSA (1,3). In a standardized procedure, conducted under sterile conditions, the hormone Sepharose complexes (360-440 µg per ml) suspended in freshly prepared 0.1 M NH_4HCO_3 solution (0.15 ml complex per 2 ml NH_4HCO_3) were placed in plastic culture dishes and incubated in closed (to minimize change in pH due to loss of CO_2) plastic boxes at 37° for 24 hours. Then the Sepharose was removed by centrifugation (500 x g for 5 min), and the supernatants were lyophilized. Amino acid analyses of the dry residues indicated that 8-10 per cent of the Sepharose-linked proteins had been solubilized under these conditions. These released materials, by analogy with the materials released in the presence of BSA (4), are probably guanidines with a hormone substituent, i.e., monosubstituted guanidines. This conclusion is supported by the observation that treatment of ornithine-Sepharose with NH_4HCO_3 generates arginine (4).

RESULTS AND DISCUSSION

The data in Table I demonstrate that the ILM released from insulin-Sepharose in the presence of NH_4HCO_3 is similar in its enhanced biological activity to that of ILM released in the presence of BSA (1,2). It stimulates the oxidation of glucose by diaphragm from the obese mice, but native insulin does not exert this effect. Also, the ILM stimulates the accumulation of α-aminoisobutyric acid (AIB) by insulin-insensitive epithelial cells in mammary explants from virgin mice. Native insulin which had been treated similarly with NH_4HCO_3 did not have greater biological activity than untreated insulin.

Table II shows that the placental lactogen-like material (LLM) and prolactin-like material (PLM), released from the corresponding Sepharose complexes in the presence of NH_4HCO_3 are similar to the materials released in the presence of BSA (3), i.e., they have greater biological activity than the native hormones. The stimulation of α-lactalbumin activity elicited by these hormones during culture of mammary explants from pregnant mice is greater than the effects produced by the unmodified hormones.

The results indicate that a BSA-substituent on the guanidinated hormones is not essential for the super-activity of these hormone derivatives. In addition to its intrinsic interest, this observation provides certain operational advantages. It eliminates the necessity for using a foreign protein, such as BSA, for the generation of the super-active hormones. Moreover, since the nature of commercial

TABLE I

STIMULATORY EFFECTS OF ILM

ON INSULIN-INSENSITIVE TISSUES

Incubation System	Diaphragm[1)]			Mammary Gland[2)]
	Relative CO_2 Production[2]			Relative AIB Accumulation
No hormone	100	100	-	100
Insulin	-	103±5	100	96±2
ILM	148±1	-	133±8	149±10

1) Hemidiaphragms from male C57Bl/6J $\overset{++}{ob}/\overset{++}{ob}$ mice (2-3 months of age) were incubated for 90 min at 37° in Erlenmeyer flasks containing 2 ml of Medium 199 (GIBCO), 0.5 µC of ^{14}C-1-glucose, and the indicated addition of 0.5 µg insulin or insulin-like material (ILM: 0.5 µg based on amino acid analyses). The extent of $^{14}CO_2$ production was determined as described previously (2). The control values (expressed as 100) were in the range of 270-360 cpm/mg wet tissue/90 min. Each value represents the mean ±S.E. of three separate experiments.

2) Mammary explants from mature virgin C3H/HeN mice (3-4 months old) were cultured as described previously (9). The accumulation of ^{14}C-α-aminoisobutyric acid (^{14}C-AIB) was determined as described previously (1). The concentration of insulin was 5 µg/ml, and that of ILM was 0.2 µg/ml (based on amino acid analyses). The no-hormone control values (expressed as 100) were in the range of 32-40 cpm/mg wet tissue/3 hr. Each value represents the mean ±S.E. of four separate determinations.

BSA is known to vary from one preparation to another, the elimination of BSA from the experimental protocols yields more reproducible results.

The following considerations are important for demonstrating these effects. It was reported previously (2) that super-activity of the ILM is manifested on insulin-resistant diaphragm and adipose tissue from C57Bl/6J $\overset{++}{ob}/\overset{++}{ob}$ mice, but that ILM has the same activity as native insulin on the corresponding tissues from lean litter-mates. Appropriate tissue must, therefore, be used for detection of the super-activity. Moreover, when the hormone-Sepharose complexes, prepared as described previously (1,3), are stored at 4° in sterile isotonic saline containing

TABLE II

EFFECT OF LLM AND PLM ON

LACTOSE SYNTHETASE

Culture Conditions	Lactose formed pmoles per mg wet tissue per 30 min			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
0 hr	3	26	22	10
6 hr				
IFL	30	37	-	-
IFLLM	40	57	-	-
24 hr				
IFL	86	-	-	-
IFLLM	131	-	-	-
24 hr				
IFP	-	-	64	44
IFPLM	-	-	89	85

Mammary gland explants from mid-pregnant C3H/HeN mice were incubated in Medium 199 [as described previously (9)] containing insulin (I) and hydrocortisone (F) at 5 μg/ml each, and one of the following: human placental lactogen (L; 1.0 μg/ml), placental lactogen-like material (LLM; 1.5 μg/ml), bovine prolactin (P; 5.0 μg/ml) or prolactin-like material (PLM; <1.5 μg/ml). All media were sterilized by passage through Millipore filters (0.45 μm pore size). The concentrations of L and P used produce maximal effects of these hormones (3). The indicated concentrations of LLM and PLM were based on amino acid analyses. LLM was generated as described in the text. PLM was generated similarly, except that 0.005 M, rather than 0.1 M NH_4HCO_3 was used. LLM and PLM were dissolved at 37° in Medium 199 immediately before the onset of culture. The cultures in experiments 3 and 4 were supplemented with 1% BSA. At the time of explantation (0 hr), and after 6 or 24 hours, lactose synthetase activity, a measure of the accumulation of α-lactalbumin, was determined as described previously (3).

0.02% NaN_3, they retain their ability to give rise to the soluble super-active hormones for about three months. Furthermore, the dry residues obtained by lyophilization of the supernatants prepared after treatment of the hormone-Sepharose complexes with NH_4HCO_3 retain their super-activity for only a few weeks when stored at -20°. It is clearly desirable to assay promptly the super-active hormone derivatives.

As suggested above, the super-active hormones released by NH_4HCO_3 from the hormone-Sepharose complexes are probably monosubstituted guanidines. It should also be recalled that these released materials represent only about 10% of the Sepharose-linked hormones (Materials and Methods). In attempts to determine the amino acid residue(s) whose guanidination leads to enhanced biological activity, we first focused on lysine, which has been considered to be the amino acid largely involved in the linkage between insulin and Sepharose (5). Accordingly, both insulin and placental lactogen were guanidinated with the Habeeb reagent (6). Amino acid analyses revealed that the one lysine residue in insulin had been completely converted to homoarginine. This form of insulin had no stimulatory activity on AIB accumulation by mammary cells from insulin-insensitive virgin mice, or on glucose oxidation by insulin-resistant diaphragm from the obese mice. It did, however, have the same stimulatory activity as insulin (7) on AIB accumulation by insulin-sensitive mammary cells from pregnant mice. Human placental lactogen has 9 residues of lysine (8). The Habeeb reagent converted more than 6 of these residues into homoarginine, but the derivatized lactogen had no greater effect than the native hormone on α-lactalbumin activity. Therefore, it appeared that guanidination of the lysines is either insufficient or unnecessary for generation of the super-active hormones. This is supported by amino acid analyses of the hormones released from the Sepharose complexes by NH_4HCO_3. The results showed that the super-active insulin contained 0.9 residue of lysine, and only 0.1 residue of homoarginine. Also, the super-active lactogen retained all of its lysine, and contained no detectable homoarginine. The evidence strongly suggests that the super-activity of these modified hormones, particularly that of LLM, is independent of the guanidination of lysine.

The critical derivatized residue(s) remains to be identified. Various possibilities are the N-terminal amino acids, and histidine or tyrosine. N-acetyl-histidine can couple to activated Sepharose through the imidazole nitrogen and N-acetyl-tyrosine ethyl-ester can also couple (unpublished). If tyrosine is the key residue, the super-active hormones would be substituted isoureas, rather than substituted guanidines.

We have demonstrated that certain derivatives of three polypeptide hormones are more active biologically than the corresponding native hormones. The super-effects have been observed on three different mouse tissues, and on such diverse biological activities as AIB accumulation, glucose oxidation and α-lactalbumin activity. Additional hormones, animal species and metabolic responses warrant further study in this context.

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