A Method for the Iodination of Insulin and its Binding to Dissociated Mouse Mammary Cells

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Abstract

The iodination of insulin was accomplished by a modification of the lactoperoxidase method. The use of a low concentration of hydrogen peroxide (1.5 ng/ μ l) followed by Sephadex gel filtration and purification on a cellulose column yielded iodoinsulin with an activity equal to that of native insulin in stimulation of glucose oxidation in rat epididymal fat cells and with high specific binding to collagenase-dissociated mouse mammary cells from pregnant and lactating mice. Other hormones tested did not displace the binding. Analysis of displacement curves and Scatchard plots suggests that both the affinity and the number of sites for insulin binding differ between pregnant and lactating mammary cells.

The mammary gland is one of the possible targets of insulin action (Juergens *et al.*, 1965). Insulin receptors exist in mammary glands from pregnant and lactating mice (O'Keefe and Cuatrecasas, 1974; Apostolova *et al.*, 1976) and in mammary adenocarcinoma from rats (Harmon and Hilf, 1976). However, nonspecific binding of iodoinsulin to mammary cells was abnormally high compared with that to fat cells (Cuatrecasas, 1974), likely as a result of either damage to the insulin by iodination or to the mammary cells during the dissociation process. Availability of iodoinsulin and mammary cells with intact biological activity seemed necessary for further investigation into insulin-receptor interaction in the mammary gland. Several methods have been developed to iodinate insulin (Freychet *et al.*, 1971; Hamlin and Arquilla, 1974; Gliemann *et al.*, 1979). We have been iodinating prolactin under

mild conditions using lactoperoxidase (Sakai *et al.*, 1975). The iodoprolactin showed high specific binding activity to mammary epithelial cells dissociated with collagenase (Enami *et al.*, 1973). These methods were modified for studies on insulin binding to mammary glands, and are described herein along with the bioactivity of the iodoinsulin and its binding to mammary cells.

Materials and Methods

Iodination of insulin

Unless otherwise stated, 0.05 M phosphate buffer (pH 6.9) was used to dissolve reagents mentioned in this subsection. To 1.0 mCi carrier-free Na^{125}I (New England Nuclear Corp.) in 20 μ l 0.1 N NaOH, were added 25 μ l 0.5 M phosphate buffer (pH 6.9), 10 μ l lactoperoxidase (10 μ g, Calbiochem), 8 μ l bovine crystalline insulin (4 μ g, Organon, 23.8 IU/mg), and 10 μ l H_2O_2 (15 ng) in sequence. The same amount of H_2O_2 was added 1, 3 and 6 min after the first addition of H_2O_2 . The mixture was diluted with 500 μ l phosphate buffer 10 min after the first addition of H_2O_2 . The insulin-bound iodine was separated from unbound iodine by gel filtration on a Sephadex

G-50 column (1×18 cm) which had been pretreated with 1.5% bovine serum albumin (BSA, Fraction V) and equilibrated with the elution buffer (0.05 M sodium phosphate). Eluates (1.0-ml) were collected in tubes containing 0.2 ml of 1.5% BSA. The first peak of radioactivity was further purified on a cellulose column (Whatman CF-11, 1×2 cm) as described by Kabuto *et al.* (1977). Iodinated insulin was eluted with 4 ml of 7% BSA in 0.1 M tris-HCl buffer (pH 7.6) at 30°C after the column was washed with 15 ml of 0.1% BSA in 0.02 M phosphate buffer (pH 8.0), and stored at -20°C until used.

Specific activity of iodinated insulin

The specific activity of iodinated insulin after gel filtration was calculated by the method of Greenwood *et al.* (1963). To correct the change in specific activity during purification on a cellulose column by preferential absorption of diiodoinsulin to cellulose, iodinated insulins before and after purification were digested with Pronase P (Kaken Chemicals Ltd., Tokyo) at 37°C for 5 hr, and analyzed for the ratio of diiodotyrosine to monoiodotyrosine by paper chromatography with 1-butanol, acetic acid and water (20:3:7 by volume). Before purification, iodoinsulin contained 7.5% of diiodoinsulin; almost no diiodoinsulin (1.6%) was detected after purification. The specific activity of purified iodinated insulin was estimated to be 90% of that of iodinated insulin before purification, based on the efficiency of iodination (55%) and the radioactivity ratio of diiodotyrosine to monoiodotyrosine.

Acrylamide gel electrophoresis

Mixtures of iodinated insulin (about 8000 cpm) and 10 µg native insulin were subjected to analytical disc electrophoresis on 10% acrylamide gel at pH 9.5. Gels were stained with 1% amidoblack 10B, and cut into 2-mm segments after destaining. The radioactivity of each segment was counted in a well-type scintillation counter (Packard Model 5110).

Bioactivity of iodinated insulin

The epididymal fat pads from two male Wistar rats were incubated in Krebs-Ringer bicarbonate buffer containing 1% dialyzed BSA (KRB-1%BSA) and 0.2% collagenase (type I, Sigma Chemicals) at 37°C for 45 min (Rodbell, 1964; Kasuga *et al.*, 1977). Isolated fat cells (2×10^5 cells) were incubated in 1 ml KRB-0.25%BSA containing 0.2 µCi [$U\text{-}^{14}C$]glucose (334 mCi/mmol, The Radiochemical Centre, U.K.), 300 nmole of unlabelled glucose, and 0.25 to 4 ng iodinated or native insulin. The concentration of iodinated insulin was calculated from the specific activity. After incubation for 2 hr, 0.5 ml 8 N H_2SO_4 was added to the incubation mixture, and CO_2 released was trapped in 0.25 ml Hyamine 10X at 37°C. The radioactivity was counted in a liquid scintilla-

tion counter (Aloka, Model LSC-651, Tokyo) with a counting efficiency of 85%.

Binding activity of iodinated insulin to mammary cell

Two- to three-month-old pregnant and lactating KA mice were used. The days of plug observation and parturition were counted as days 0 of pregnancy and lactation, respectively. Mammary epithelial cells dissociated with collagenase (Enami *et al.*, 1973; Sakai *et al.*, 1978) were incubated in M199-0.25%BSA (3×10^6 cells/0.4 ml) with iodinated insulin (100000 cpm) and native insulin (0 to 64 ng) at room temperature for 45 min and then centrifuged at $12000 \times g$ for 5 sec in an Eppendorf centrifuge (Model 5412). The pellet was rinsed twice with 1 ml 0.01 M phosphosaline-0.25%BSA (pH 7.4). Nonspecific binding was determined by incubation in the presence of 5 µg native insulin. Our observation had shown that the specific binding reached equilibrium in 40 min and remained on a plateau at least up to 60 min. Specific bindings at 10, 20 and 30 min were 61, 89 and 97% of the maximum.

Cross reaction was determined with other hormones: ACTH (Armour), ovine prolactin (NIH-P-S12), glucagon (Novo), pancreozymin (Boots) (4 µg each for the hormones mentioned above), secretin (Boots), EGF and NGF (5 µg each for these three hormones).

Results

Table 1 shows the results of iodinations in which various amounts of insulin (1 to 5 µg) and $Na^{125}I$ (0.5 to 1 mCi) were used. The specific activity of iodinated insulin increased in proportion to the ratio of iodine to insulin. When 1 mCi $Na^{125}I$ and 4 µg insulin were used, the specific activity was $200 \pm 4 \mu Ci/\mu g$ (mean $\pm$ SE) in 5 experiments. Fig. 1 shows the effect of H_2O_2 concentration on the incorporation of radioactivity into insulin. Seventy-four percent of the radioactivity was incorporated into insulin when 1.5 ng/ μl H_2O_2 was used. The iodination mixture was gel-filtered on Sephadex G-50, and separated into two radioactive peaks. The first peak was judged to be iodinated insulin from its R_f value. The second peak was identified as inorganic iodine from the elution pattern

Table 1. Effect of amounts of radioactive iodine and insulin to be labelled on the specific activity and recovery of iodinated insulin before purification.

Na ¹²⁵ I (mCi)	0.5			1	
Insulin (μ g)	1	3	4	4	5
Specific activity (μ Ci/ μ g)	334	120	90	200	110
Recovery of ¹²⁵ I-insulin (%)	1.3	41	48	50	46

The recovery of insulin is a ratio of the eluted to the total protein-incorporated radioactivity.

of Na¹²⁵I on a column of the same size. No aggregation was seen on gel filtration. After purification on a cellulose column, 10% trichloroacetic acid (TCA) precipitated 95% of the radioactivity. Upon acrylamide gel electrophoresis, the radioactivity of purified iodinated insulin formed a single peak (relative mobility, $R_m=0.80$) migrating ahead of native insulin ($R_m=0.75$) toward the anode (Fig. 2). When unpurified iodinated insulin was used, a small amount of radioactivity was seen ahead ($R_m=0.90$) of the main peak.

As shown in Fig. 3, there was no significant difference between iodinated and native insulins in stimulating glucose oxidation in isolated fat cells.

Iodinated insulin competed with native insulin in binding to mouse mammary epithelial cells (Fig. 4). Cells from early pregnant mice (days 0 to 3) show 2680 ± 110 cpm (mean $\pm$ SE) of specific binding and 283 ± 31 cpm of nonspecific binding in 7 experiments. One half of the specific binding was displaced by 10.8 ± 0.6 ng/ml of native insulin. When early lactating mice (days 3 to 4) were used, amounts of specific and nonspecific binding were 3130 ± 102 cpm and 306 ± 50 cpm, respectively, in 5 experiments. Half-displacement was accomplished with 55.7 ± 9.7 ng/ml of insulin.

Insulin degradation during incubation was investigated by TCA precipitation and rebinding to fresh mammary cells. The

Fig. 1. Effect of hydrogen peroxide concentration on the percentage of radioactivity incorporated into insulin. Bovine insulin (4μ g in 8μ l) was added to 1 mCi Na¹²⁵I followed by the addition of 10μ g lactoperoxidase (10μ l). Then 10μ l H₂O₂ at the concentration shown above was added to the mixture four times, totalling 40μ l.

Fig. 2. Distribution of radioactivity on acrylamide gel. Bovine insulin (4μ g) was iodinated with 1 mCi Na¹²⁵I by 10μ g lactoperoxidase and 60 ng H₂O₂. Hydrogen peroxide was added four times (15 ng each). Iodinated insulin was separated on a Sephadex G-50 column (1×18 cm) from inorganic iodine. The eluate was further purified on a cellulose column (Whatman CF-11). An aliquot of purified iodinated insulin (8000 cpm) was subjected to 10% acrylamide gel electrophoresis. The radioactivity of 2-mm gel segments is shown. Note the absence of radioactivity in segments other than the main peak.

Fig. 3. Stimulation of glucose oxidation in fat cells by native and iodinated insulin. Fat cells (2×10^5) were incubated in KRB for 2 hr with ^{14}C -glucose and native (●) or iodinated (○) insulin. Released $^{14}\text{CO}_2$ was trapped in Hyamine-10X and measured. The concentrations of iodinated insulin were calculated from the specific activity. Dose-response curves are identical for both types of insulin.

TCA precipitability in the medium incubated with mammary cells for 30 min was 88% of that in the fresh control medium. After 30-min incubation with mammary cells, the medium was reincubated with fresh mammary cells for another 30 min. The specific binding of insulin in the incubated medium decreased to 89% of that in a fresh medium.

The specific binding of iodinated insulin to cells from pregnant mice in the presence of ACTH, prolactin, glucagon, secretin, EGF and NGF was 94, 95, 99, 93, 77 and 81% of that without these hormones, respectively, and that with cells from a lactating mouse was 96, 98, 100, 98, 97 and 79%, respectively. The specific binding to lactating mammary cells decreased to 90% when pancreozymin was added to the incubation.

Discussion

A characteristic of our method of iodination is slow addition of a low concentration of hydrogen peroxide in contrast to the method of Thorell and Johansson (1971) in which the authors used about 20 times

Fig. 4. Displacement of specific iodinsulin binding by native insulin. Smaller amounts of native insulin displaced the specific binding of iodinsulin to mammary cells from pregnant mice (●) than to those from lactating mice (○).

more concentrated hydrogen peroxide and terminated the reaction in 1–2 or 60 sec. Our method may diminish the damage to insulin, giving the hormone a lower risk of exposure to hydrogen peroxide. Iodinated insulin had high specific activity ($180 \mu\text{Ci}/\mu\text{g}$), and retained biological potency unimpaired as judged by glucose oxidation in fat cells. Paper chromatography showed that the purified iodinated insulin was mostly composed of monoiodoinsulin. Purification of iodinated insulin on a cellulose column (Freychet *et al.*, 1972) requires no special facility nor time-consuming processes, and is, therefore, useful for small amounts of iodinsulin, being easier and faster than DEAE chromatography (Freychet *et al.*, 1971; Hamlin and Arquilla, 1974) or long-gel electrophoresis (Linde and Hansen, 1974). Monoiodoinsulin labelled by the chloramine-T method is reported to have high bioactivity and high binding activity to insulin receptors (Freychet *et al.*, 1971). A14 tyrosine-monoiodoinsulin, which is the major product (75%) of lactoperoxidase iodination (Hamlin and Arquilla, 1974), is known to maintain its entire bioactivity whereas A19- or B-iodinated insulin has lower bioactivity (Gliemann *et al.*, 1979). Linde and Hansen (1974) separated iodinsulins into 5 bands (uniodinated insulin, A19- or B-monoiodoinsulin, A14-monoiodoinsulin, diiodoinsulin and triiodoinsulin) on acrylamide gel electrophoresis using long columns. The small amount of radioactivity observed ahead ($R_m=0.90$) of the main peak on electrophoresis of unpurified iodinated insulin seems to represent diiodoinsulin, evidence to support the inference that diiodoinsulin was removed by purification on a cellulose column.

Our iodinsulin had high binding activity to dissociated mouse mammary cells, which was specific and saturable. The amount of nonspecific binding was less than 10% of that of total binding, probably owing to both good iodinsulin and undamaged

mammary cells. Mammary epithelial cells dissociated by the method used are known to grow *in vitro* on floating collagen gels (Emerman *et al.*, 1977) and to retain specific prolactin receptors (Sakai *et al.*, 1978). The amount of specific binding to cells from lactating mice was higher than to cells from pregnant mice, suggesting a difference in the number of insulin receptors per cell. Low concentrations of native insulin displaced the specific binding of iodinsulin. However, displacement curves for pregnant mice were different from those for lactating mice. The displacement of half the amount of specific binding to pregnant cells with a smaller concentration of native insulin than that needed for lactating cells suggests a difference in the affinity of insulin-receptor binding between pregnancy and lactation. The difference was also suggested by the Scatchard plot (Fig. 5). A greater number

Fig. 5. Scatchard plot for insulin-receptor binding. Mammary cells from pregnant (●) and lactating (○) mice were incubated for 45 min at room temperature in M199 with iodinated insulin purified on a cellulose column. After cells were washed with phosphosaline, the radioactivity bound to cells was counted. The bound-to-free ratio was plotted vs. bound insulin.

of binding sites in lactating mammary cells than in pregnant cells has been reported with no obvious change in affinity (O'Keefe and Cuatrecasas, 1974). The change in the affinity of binding may be resulted from the presence of heterogeneous insulin receptors with different affinity (Krupp and Livingston, 1980), or by a negative cooperative type of site-site interaction as observed by Kahn *et al.* (1978) in liver plasma membranes at different glucocorticoid levels. The property of insulin binding to dissociated cells from mice in various stages of mammary development is now under investigation using the iodinsulin described herein.

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