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STUDIES RELATED TO THE CONCEPT THAT A THIOL DISULFIDE INTERCHANGE IS INVOLVED IN THE ACTION OF INSULIN ON MUSCLE CELLS

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SUMMARY

The accumulation and retention of [^{131}I]insulin were studied in isolated frog sartorius muscles that were first incubated with [^{131}I]insulin and then washed with Ringer's solution. Most of the radioactive material that was retained in the tissue after thorough washing with ice-cold Ringer's solution could be extracted later with 8 M urea. A portion of the labeled material that remained in the tissue after extraction with 8 M urea could be removed by additional washing with solutions that contained cysteine. These findings agree with the earlier results of other investigators who concluded that the action of insulin may involve formation of a disulfide bond linking the hormone to the tissue. However, the present extension of these studies has revealed that the amount of radioactive material (derived from labeled insulin) that could be extracted from frog sartorius muscles with cysteine solutions was not directly related to the magnitude of the effect of insulin on permeability to sugar. Therefore, demonstration of a release of radioactive material in the presence of cysteine does not necessarily indicate that the biological action of insulin in skeletal muscle involves the formation of a disulfide bond between insulin and a specific receptor site in the tissue.

INTRODUCTION

It has been suggested[1–4] that the action of insulin is associated with a thiol disulfide interchange reaction that forms a disulfide bond which attaches insulin to its receptor site in the tissue. This theory is of great interest because it attempts to correlate the chemical structure of a hormone with its action. FONG *et al.*[2] incubated rat epididymal fat pads with [^{131}I]insulin, heated the tissue to inactivate enzymes, and then washed it with 8 M urea and then with 8 M urea *plus* 0.1 M cysteine. EDELMAN, ROSENTHAL AND SCHWARTZ[3] incubated rat femoral muscles with [^{131}I]insulin and then used *N*-ethylmaleimide to cover the remaining free sulfhydryl groups of the tissue. The muscles were crushed and then washed with 8 M urea; 0.1 M cysteine

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was also included in the later washes. In both of these sets of experiments cysteine caused the release of an additional amount of radioactive material, and it was concluded that the reagent had split disulfide bonds that had served to bind [^{131}I]insulin to its site of action in the tissue.

In the present investigation the relationship between the biological effect of insulin and the amount of labeled material that could be extracted from the tissue with cysteine solution has been examined. Frog sartorius muscles were chosen for these studies because they are thin tissues that respond well to bovine and porcine insulin and can withstand long periods of incubation at temperatures that range down to 0°. Insulin gradually increases the permeability to sugar when incubation is conducted at 19°; 2.5 to 3.5 h are required to attain a full hormonal effect[5]. Increasing concentrations of insulin exert an effect that rises to a maximum at 500 to 1000 μunits per ml (ref. 6). The augmentation of permeability that occurs when frog sartorius muscles are exposed to insulin can be reversed later by washing the muscles with Ringer's solution at 19°.

If insulin were specifically bound to its biological site of action by a disulfide bond, and if cysteine selectively released this moiety of tissue insulin by cleaving the bond, then the following results might be expected: (i) After exposure of muscles to different concentrations of insulin, the amount of insulin extracted by cysteine solutions should be proportional to the biological effect exerted by insulin on the tissue. (ii) The amount of insulin extractable with cysteine should decrease significantly after washing of insulinized muscles with Ringer's solution at 19° for a period of time sufficient to cause a reversal of the biological effect of insulin. These postulates have been tested in the present investigation.

METHODS

Crystalline beef insulin was a gift from Dr. OTTO K. BEHRENS of Eli Lilly and Company. [^{131}I]Insulin was obtained from Abbott Laboratories; the average iodine content was 1 atom per molecule of insulin (mol. wt. 6000) and the specific radioactivity was 8 μC/munit. The [^{131}I]insulin was purified further by passing it through a small column of DEAE-cellulose prior to use[6]; at least 98 % of the radioactivity was precipitable with trichloroacetic acid and no significant impairment of biological activity was detectable when the ability to enhance the permeability of frog muscles to sugar was tested.

Female Rana pipiens obtained from J. R. Schettle Biologicals (Stillwater, Minn.) in the month of August were kept in water at 4° for 2–8 weeks before they were used. Prior to an experiment, sartorius muscles weighing approx. 70 to 110 mg were dissected out and were soaked overnight at 4° (*cf.* ref. 7) in frog Ringer's solution[8] that had been equilibrated with a gas phase of O_2–CO_2 (95:5, v/v). The initial rate of penetration of 3-*O*-[^{3}H]methylglucose was measured in the manner described previously[5] at a substrate concentration of 5 mM.

For studies of insulin binding, muscles were incubated in 2 ml of Ringer's solution containing [^{131}I]insulin and 2 mg of crystalline bovine plasma albumin per ml. A concentration of [^{131}I]insulin was used that gave a counting rate of approx. 40000 counts/ml, and crystalline beef insulin was included to provide the total concentration of insulin that was desired for a given medium. After incubation,

muscles were washed with Ringer's solution at 0° to remove extracellular fluid that contained unbound [^{131}I]insulin. Then the muscles were incubated with 0.01 M *N*-ethylmaleimide in order to diminish the opportunity for nonspecific thiol disulfide interchange reactions or enzymatic degradation of [^{131}I]insulin (*cf.* refs. 3 and 8). These incubations and washes were performed in stoppered 25-ml erlenmeyer flasks in a Dubnoff shaker that oscillated 100 times/min. The muscles were then crushed on a glass plate under a glass roller. The crushed tissue was transferred to a test tube and washed with 2 ml of 8 M urea in 0.1 M phosphate buffer (pH 7.4) at 37° with occasional swirling. The tissue was sedimented by centrifugation at the end of each wash period and resuspended in fresh wash fluid. Preliminary experiments revealed that when urea solutions were made up in 0.015 M HCl or in 0.1 M sodium bicarbonate solutions[2,3], the tissue gradually dissolved so that the mass of sedimentable material decreased perceptibly. This complication was minimized by employing 0.1 M sodium phosphate buffer at pH 7.4 for the urea solutions. After thorough extraction of the crushed tissue with 8 M urea, 0.1 M cysteine was included in subsequent urea solutions and extraction was continued; solutions that contained cysteine were equilibrated with 100% N_2 gas. The washed tissue residues were dissolved with 3% KOH and the volume of all tissue and wash samples was adjusted to 5 ml for counting of ^{131}I in a well-type scintillation γ counter. Background was approx. 140 counts/min, counting efficiency was 20%, and a minimum of 2000 counts was measured on each sample.

Trichloroacetic acid precipitable radioactive material present in muscles after incubation with [^{131}I]insulin will be referred to as [^{131}I]insulin. Since the exact chemical nature of the labeled material has not been established, the amount will be expressed as μunit equivalents of insulin per g of muscle instead of μunits/g.

RESULTS

Effect of different concentrations of insulin on permeability of muscles to sugar

Permeability to sugar was definitely augmented after exposure of muscles to insulin at a concentration of 20 μunits/ml; the effect increased in proportion to the logarithm of the concentration of hormone until a maximal enhancement was attained at approx. 500 μunits/ml (Table I). Sensitivity to insulin undergoes moderate seasonal fluctuations; concentrations as low as 2 μunits/ml can significantly stimulate the entry of sugar into muscles of winter frogs (H. J. WOHLTMANN, unpublished observations).

Extraction of retained ^{131}I-labeled material from muscle with 8 M urea after washing the muscles with Ringer's solution at 0°

It was shown previously[6] that when muscles that had been incubated with [^{131}I]insulin were washed with Ringer's solution at 0°, the [^{131}I]insulin in the extracellular fluid was removed whereas the excess [^{131}I]insulin that had accumulated in the tissue was firmly retained. The present results confirm this observation (Tables II and III). The amount of radioactive material that was removed from the muscles during four washes with Ringer's solution at 0° (8 h of washing) corresponded to the amount of [^{131}I]insulin dissolved in 0.20 to 0.25 ml of incubation medium per g of tissue; these values are within the range for the extracellular fluid content of frog

sartorius muscle as determined with raffinose[7] or [^{14}C]mannitol (ref. 5). A fifth wash with Ringer's solution for 5 h at 0° (Wash E) removed little additional radioactive material from the muscles.

TABLE I

PERMEABILITY OF MUSCLES TO 3-*O*-METHYLGLUCOSE AFTER EXPOSURE TO DIFFERENT CONCENTRATIONS OF INSULIN

Muscles were incubated with various concentrations of insulin for 2.5 h at 19°. Then v, the initial rate of penetration of 3-*O*-methylglucose, was measured and is expressed as μmoles/ml of cell water per h. Each value represents six muscles, and the standard error of the mean is given.

Insulin (μ*units/ml*)	v (μ*moles/ml per h*)
0	0.60 ± 0.08
20	1.39 ± 0.12
50	2.28 ± 0.16
100	2.88 ± 0.24
500	3.69 ± 0.21
2 000	3.98 ± 0.30
6 000	3.92 ± 0.23
30 000	3.68 ± 0.20

A major portion of the material that was retained after washing 5 times with Ringer's solution at 0° could be extracted by crushing the muscles and washing them with 8 M urea at 37° (Tables II and III, Washes G–K). The amount of radioactive material that was extracted from a given muscle declined progressively in successive washes and only a small fraction of the residual radioactivity was removed by the last two washes with 8 M urea.

Extraction of ^{131}I-labeled material from muscles with cysteine solutions after incubation with various concentrations of [^{131}I]insulin

When tissue that had been thoroughly washed in the presence of urea was subsequently washed with urea solutions that contained 0.1 M cysteine, there was in each instance a sharp increase in the amount of radioactive material released per wash (Tables II and III). The quantity of radioactive substance extracted in two washes with 0.1 M cysteine was approx. 11 % of the radioactivity that had been retained in the muscle after washing with Ringer's solution at 0° and was approx. 1/3 of the residual radioactivity that was left in the muscle after extraction with 8 M urea. The amount of radioactive material that was selectively extractable with cysteine rose progressively as the concentration of [^{131}I]insulin was raised from 48 to 6000 μunits/ml (Tables II and III, results of experiments in which Wash E was performed at 0°).

Effect of degradation of retained [^{131}I]insulin at 19° on the amount of radioactive material extractable with cysteine

In the experiments of Tables II and III, one of each pair of muscles was washed for 5 h at 19° instead of 0° during the fifth wash with Ringer's solution (Wash E). In accord with earlier results[6], washing at the higher temperature invariably caused

TABLE II

TIME COURSE OF EXTRACTION OF ^{131}I-LABELED MATERIAL FROM MUSCLE WITH UREA AND CYSTEINE SOLUTIONS

Muscles were incubated with 48 μunits of [^{131}I]insulin per ml for 2.5 h at 19°, blotted, and then washed four times at 0° with 5 ml of Ringer's solution for 2 h each (Washes A–D). The muscles were then washed with 2 ml of Ringer's solution for 5 h at either 0° or 19° (Wash E), comparing paired muscles at the two temperatures. The muscles were next treated (Wash F) with 5 ml of 0.01 M *N*-ethylmaleimide in Ringer's solution for 30 min at 19°, crushed, and then washed five times with 2 ml of 8 M urea in 0.1 M phosphate buffer (pH 7.4) at 37°, without shaking (Washes G–K). Wash H was for 5.5 h, and the other urea washes were for 2 h. Next, the muscles were washed twice (Washes L and M) for 2 h each time, anaerobically, with 2 ml of 8 M urea containing 0.1 M cysteine. Radioactive material in Wash E was separated into trichloroacetic acid precipitate and trichloroacetic acid supernatant fractions prior to counting. Each value is an average for eight muscles. The amount of radioactive material in each fraction has been expressed as μunit equiv/g of whole muscle.

Fraction	*Radioactivity in fraction (μunit equiv/g)*	
	Series with 0° Wash E	*Series with 19° Wash E*
Ringer's solution at 0°		
Wash A	9.36 ± 0.45	9.19 ± 0.54
Wash B	1.39 ± 0.03	1.33 ± 0.02
Wash C	0.68 ± 0.01	0.66 ± 0.02
Wash D	0.53 ± 0.02	0.52 ± 0.02
Ringer's solution, 0° or 19°		
Wash E, TCA-ppt.	0.50 ± 0.01	1.08 ± 0.05
Wash E, TCA-sup.	0.18 ± 0.01	1.83 ± 0.07
N-Ethylmaleimide, 19°		
Wash F	0.46 ± 0.01	0.22 ± 0.01
Urea, 37°		
Wash G	2.42 ± 0.10	1.30 ± 0.06
Wash H	1.02 ± 0.05	0.57 ± 0.03
Wash I	0.33 ± 0.01	0.19 ± 0.01
Wash J	0.14 ± 0.003	0.10 ± 0.003
Wash K	0.10 ± 0.01	0.06 ± 0.003
Urea + cysteine, 37°		
Wash L	0.45 ± 0.03	0.34 ± 0.03
Wash M	0.18 ± 0.01	0.12 ± 0.01
Tissue residue	1.21 ± 0.03	1.00 ± 0.04
Sum	18.95	18.51

Abbreviations: TCA-ppt., trichloroacetic acid precipitate; TCA-sup., trichloroacetic acid supernatant.

a marked increase in the amount of trichloroacetic acid soluble radioactive material that appeared in the wash fluid. Washing of muscles with Ringer's solution at 19° (Tables II and III, Wash E) caused only a slight and inconsistently demonstrable decrease in the amount of radioactive material that was subsequently extractable with cysteine (Washes L and M).

TABLE III

EXTRACTION OF MUSCLES WITH UREA AND CYSTEINE SOLUTIONS AFTER EXPOSURE TO DIFFERENT CONCENTRATIONS OF [^{131}I]INSULIN

Experiments were performed in the manner described for Table II, except that different concentrations of [^{131}I]insulin were used for incubation. Since the general pattern of the time course of appearance of radioactivity in the washes was similar to that seen in Table II, the data of some wash fluids have been combined. Each value is a mean for four muscles.

Insulin in incubation medium (μunits/ml)	*Fraction*	*Radioactivity in fraction (μunit equiv/g)*	
		Series with 0° Wash E	*Series with 19° Wash E*
70	Washes A–D (Ringer's, 0°)	16.10 ± 1.52	17.31 ± 2.99
	Wash E (Ringer's), TCA-ppt.	0.78 ± 0.22	1.49 ± 0.28
	Wash E, TCA-sup.	0.22 ± 0.03	2.12 ± 0.12
	Wash F (*N*-ethylmaleimide, 19°)	0.46 ± 0.06	0.26 ± 0.07
	Washes G–K (urea, 37°)	4.32 ± 0.71	2.58 ± 0.32
	Washes L–M (urea + cysteine, 37°)	0.76 ± 0.12	0.78 ± 0.17
	Tissue residue	1.48 ± 0.10	1.34 ± 0.10
	Sum	24.12	25.88
200	Washes A–D (Ringer's, 0°)	44.4 ± 2.6	47.4 ± 1.4
	Wash E (Ringer's), TCA-ppt.	1.62 ± 0.07	3.50 ± 0.18
	Wash E, TCA-sup.	0.54 ± 0.03	5.58 ± 0.57
	Wash F (*N*-ethylmaleimide, 19°)	1.26 ± 0.04	0.72 ± 0.06
	Washes G–K (urea, 37°)	11.63 ± 0.66	6.83 ± 0.40
	Washes L–M (urea + cysteine, 37°)	2.02 ± 0.12	1.71 ± 0.06
	Tissue residue	4.11 ± 0.07	3.64 ± 0.07
	Sum	65.58	69.38
1000	Washes A–D (Ringer's, 0°)	204 ± 10	226 ± 10
	Wash E (Ringer's), TCA-ppt.	7.15 ± 0.07	18.7 ± 2.1
	Wash E, TCA-sup.	1.66 ± 0.06	15.7 ± 1.0
	Wash F (*N*-ethylmaleimide, 19°)	4.72 ± 0.22	2.86 ± 0.07
	Washes G–K (urea, 37°)	41.3 ± 0.4	29.5 ± 1.8
	Washes L–M (urea + cysteine, 37°)	8.04 ± 0.58	7.81 ± 1.14
	Tissue residue	18.6 ± 0.6	19.0 ± 1.2
	Sum	285.5	319.6
6000	Washes A–D (Ringer's, 0°)	1220 ± 21	1290 ± 73
	Wash E (Ringer's), TCA-ppt.	38.3 ± 3.1	90.8 ± 9.1
	Wash E, TCA-sup.	9.4 ± 0.6	56.9 ± 6.3
	Wash F (*N*-ethylmaleimide, 19°)	28.0 ± 1.5	15.0 ± 1.3
	Washes G–K (urea, 37°)	218 ± 12	149 ± 7.4
	Washes L–M (urea + cysteine, 37°)	64.0 ± 5.9	58.8 ± 7.2
	Tissue residue	122 ± 5	116 ± 4
	Sum	1699.7	1776.5

Abbreviations: TCA-ppt., trichloroacetic acid precipitate; TCA-sup., trichloroacetic acid supernatant.

DISCUSSION

Treatment of rat heart[1] or diaphragm[4] muscle with *N*-ethylmaleimide interferes with the subsequent development of an insulin effect on sugar uptake. These findings

suggest that tissue sulfhydryl groups may be important for the binding and action of insulin in cells. However, the observations do not indicate whether the hormone is bound covalently or noncovalently. Moreover, other studies of this type[9,10] have led to conflicting results.

FONG *et al.*[2] and EDELMAN, ROSENTHAL AND SCHWARTZ[3] studied the ability of cysteine to release radioactive material from tissues that had been incubated with [^{131}I]insulin and concluded that the biological action of insulin might be mediated by a thiol disulfide interchange reaction that bonded the hormone to the cell covalently. In their studies [^{131}I]insulin was employed at concentrations of the order of 100000 μunits/ml. In the present investigation the relationship between the presence of cysteine-releasable radioactive material in the tissue and the biological effects of insulin has been explored further.

The binding of insulin to frog skeletal muscle has been studied with the use of [^{131}I]insulin that contains an average of 1 atom of iodine per molecule of insulin[6]. Although more definitive analyses on fully characterized iodinated species of insulin would be desirable, the assays that are available indicate that biological activity is preserved essentially intact when the degree of iodination does not exceed an average of one iodine atom per molecule of hormone[11,12,6]. During incubation of frog sartorius muscles with [^{131}I]insulin, much more [^{131}I]insulin accumulates in the muscles than can be accounted for by the extracellular fluid content of the tissue. This extra material is firmly retained ('bound') when the muscles are subsequently washed with Ringer's solution at 0°. The binding of [^{131}I]insulin by frog skeletal muscle exhibits a component that has been called 'specific' (*cf.* ref. 6) because it becomes saturated at approximately the concentration of extracellular insulin that produces a maximal effect on permeability to sugar. In addition, there is a nonspecific component of binding that is not directly related to the extent of cellular response; this moiety of bound [^{131}I]insulin continues to increase in magnitude even when the concentration in the medium is raised to levels far above the physiological maximum[6] (*cf.* ref. 13).

When the concentration of [^{131}I]insulin in the incubation medium is at the lower end of the range shown in Tables II and III (*i.e.*, 48–200 μunits/ml), approximately half of the [^{131}I]insulin that is retained in the tissue after washing with ice-cold Ringer's solution appears to be specifically accumulated[6]. Most of this retained material is extractable by crushing the tissue and washing it successively with solutions containing urea and cysteine (Tables II and III). Of the material that can be extracted in this manner, approx. 85 % is obtained by washing with 8 M urea alone, in the absence of cysteine. Since the urea washes, therefore, remove much of the specifically bound [^{131}I]insulin, a major portion of the specifically bound [^{131}I]insulin seems to be attached to the cells noncovalently.

Insulin produces a graded, reversible increase in permeability of frog muscles to sugar when different concentrations of hormone are used within the physiological range of approx. 10 to 500 μunits/ml (ref. 6). If the biological action of insulin were to involve the formation of a disulfide bond between the hormone and a receptor site in the tissue, the extent of this specific interaction might be expected to parallel the magnitude of the biological effect. Thus, raising the concentration of insulin above the physiologically maximal level should not lead to any appreciable increase in the number of these specific disulfide bonds.

In the batch of frogs used for the present experiments a maximal effect on permeability to sugar was obtained at an insulin concentration of approx. 500 μunits per ml (Table I). Nevertheless, when the concentration of [^{131}I]insulin to which muscles were exposed was raised from 1000 to 6000 μunits/ml, there was a marked increase in the amount of radioactive material that was selectively extractable from the tissue with cysteine solutions (Tables II and III, Washes L–M in the series where Wash E was performed at 0°). These results suggest that, at least at high concentrations of [^{131}I]insulin, most of the radioactive material that is extracted in the presence of cysteine is derived from nonspecifically bound [^{131}I]insulin.

Muscle homogenates can inactivate insulin[14]. Incubation of [^{131}I]insulin with homogenates of rat skeletal muscle yields radioactive material that is soluble in trichloroacetic acid; this process is not a deiodination but appears to represent enzymatic degradation of the hormone to trichloroacetic acid soluble fragments[15]. Intact isolated frog sartorius muscles can also degrade [^{131}I]insulin during incubation; in this case, [^{131}I]insulin that has been taken up by the cells is degraded but no appreciable destruction of the hormone occurs in the medium itself[8].

When frog muscles that have been incubated with [^{131}I]insulin are washed with Ringer's solution at 0°, some [^{131}I]insulin is firmly retained by the tissue in a trichloroacetic acid precipitable form and permeability to sugar also remains at its initial high level during the washing procedure[6]. Subsequent washing of the muscles with Ringer's solution at 19° causes degradation of the retained [^{131}I]insulin to a trichloroacetic acid soluble form that escapes from the cells; degradation appears to include both the specifically and nonspecifically bound moieties of [^{131}I]insulin. In Tables II and III an approx. 10-fold increase in the amount of trichloroacetic acid soluble radioactive material appears in the wash fluid when Wash E is performed at 19° rather than at 0°. Degradation of retained insulin has been shown to be accompanied by a reversal of the effect of insulin on permeability to sugar; during 5 h of washing at 19° permeability falls approx. 2/3 of the way towards the basal value[6]. In contrast, washing of muscles with Ringer's solution for 5 h at 19° (Wash E, Tables II and III) did not cause a consistent decrease in the amount of radioactive material that was selectively extractable from the tissue with cysteine in 8 M urea (Tables II and III; *cf.* Washes L–M for muscles that were washed at either 0° or 19° during Wash E). These findings are compatible with the concept that most of the radioactive material that is released from the tissue by cysteine is derived from nonspecifically bound [^{131}I]insulin.

Evidence for a thiol disulfide interchange between a peptide hormone and its target tissue was first obtained with vasopressin by the technique of cleaving radioactively labeled hormone from the tissue with cysteine[16,17]. This finding encouraged the search for a similar phenomenon in the case of insulin. However, it has been found more recently[18,19] that analogues of neurohypophyseal hormones that lack a disulfide bridge possess biological activity, and it has been concluded that a thiol disulfide interchange may not be essential for the action of these hormones[20]. In accord with the latter interpretation, the results of the present studies suggest that insulin can enhance the permeability of skeletal muscle to sugar without being covalently bound to the muscle cells. Further studies with suitable analogues of insulin would be desirable to test this concept more definitively.

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