Biochemical Pharmacology, Vol. 21, pp. 517–523. Pergamon Press, 1972. Printed in Great Britain

EFFECTS OF THIOLS AND DISULFIDES ON GLUCOSE UTILIZATION AND INSULIN ACTION IN THE ISOLATED RAT DIAPHRAGM*

ELLA S. HAUGAARD, MARGARET J. SMITH† and NIELS HAUGAARD

Department of Pharmacology, School of Medicine, University of Pennsylvania, Philadelphia, Pa. 19104, U.S.A.

(*Received* 13 *April* 1971; *accepted* 13 *August* 1971)

Abstract—The actions of 2,2′-dithiodipyridine (DTP) and dithiothreitol (DTT) on glucose utilization and lactate formation by rat diaphragm were studied *in vitro*.

The disulfide DTP had no significant effect on glucose utilization in the absence of insulin but inhibited the effect of insulin on glucose utilization and increased lactate formation and breakdown of glycogen by the tissue. The interference by DTP with the action of insulin could be reversed by the sulfhydryl compound DTT.

DTT stimulated glucose utilization and net lactate formation by rat diaphragm. This effect of the sulfhydryl compound was different from that of insulin in that it commenced not immediately but only after about 30 min of incubation. In contrast to insulin, DTT decreased rather than increased glycogen synthesis.

When the muscle was under the influence of both insulin and a high concentration of DTT, the initial insulin effect was not abolished. However, after about 30 min of incubation, the rate of glucose utilization was the same with DTT alone as with DTT plus insulin, and the muscle appeared to be under the influence mainly of DTT.

AMONG the chemical characteristics of the insulin molecule is the presence of three disulfide bridges. There has been considerable speculation that the biological activity of insulin, the combination of the hormone with the cell or its subsequent action, is in some unknown manner associated with reactions involving these special chemical groups. Recently, Lavis and Williams[1] reported that various sulfhydryl compounds have an insulin-like action on adipose tissue *in vitro* and Chiba[2] observed that various sulfhydryl compounds increased the rate of entrance into the cell of non-metabolizable sugars in adipose tissue and diaphragm. Haugaard and Haugaard[3] found that a disulfide, thioctic acid, stimulated glucose utilization by rat diaphragm *in vitro*. This increase in glucose metabolism occurred after a period of incubation and was not immediate like that of insulin. The experiments to be presented here are concerned with the effect of 2,2′-dithiodipyridine (DTP) and the sulfhydryl compound dithiothreitol (DTT) on glucose utilization of the rat diaphragm in the presence and absence of insulin.

METHODS

Animals. Male Wistar strain rats weighing between 125 and 150 g and fed *ad lib.* were killed by decapitation. Hemidiaphragms were removed and collected in cold solution of the composition described below except for the omission of glucose. The

* The studies reported here were supported by grants from the American Diabetes Association and from the Heart and Lung Institute of the National Institutes of Health (HE-01813).

† Predoctoral trainee supported by USPHS Training Grant 5 TO1 GM 00474.

pieces of diaphragm were blotted lightly on filter paper and weighed on a torsion balance before the experiment.

Incubation. The incubation medium contained 0·040 M sodium phosphate (pH 7·2), 0·005 M KCl, 0·004 M $MgCl_2$, 0·006 M glucose and 0·080 M NaCl. Each hemidiaphragm was placed in a 25-ml Erlenmeyer flask containing 2 ml of the medium. The flasks were oxygenated for 1 min with 100 per cent oxygen and incubated in a Dubnoff shaker at 37° for different periods of time. The incubations were done in the presence and absence of insulin at a concentration of 0·2 units/ml. DTP and DTT were added as indicated.

Analytical determinations. The initial and final glucose content of an aliquot of the medium was determined by the glucose oxidase method after deproteination with $ZnSO_4$ and $Ba(OH)_2$. In the experiments in which DTT or DTP were present in the incubation medium, *N*-ethylmaleimide (NEM) was added to the filtrate before analysis. The final concentration of NEM in each tube during the glucose oxidase assay was 0·4 mM. NEM was found to have no influence on glucose determination by itself but it effectively combines with sulfhydryl compounds and prevents the interference of these substances with the glucose oxidase method. Lactate was determined by an enzymatic method as described by Hohorst.[4] Glycogen was measured by the method of Montgomery[5] after digestion of the tissue in 60% KOH and precipitation with alcohol.

Chemicals. The insulin preparation used was Lilly amorphous insulin (lot No. 192-235B-188); 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and 2,2′-dithiodipyridine (DTP) were obtained from Aldrich Chemical Co., *N*-ethylmaleimide (NEM) from Nutritional Biochemicals, and dithiothreitol (DTT) from CalBiochem.

RESULTS

Action of 2,2′-dithiodipyridine. 2,2′-Dithiodipyridine (DTP) is a disulfide which reacts with sulfhydryl groups to form mixed disulfides. The compound has been studied extensively by Grassetti *et al.*[6] who found that it reacted with tissue sulfhydryl groups and inhibited glycolysis and respiration in Ehrlich ascites tumor cells.[7] DTP is also a potent inhibitor of oxidative phosphorylation and calcium uptake by mitochondria.[8] The action of this substance on glucose utilization and lactate production by rat diaphragm and its effect on the action of insulin are shown in the experiments reported in Table 1.

The first group of experiments demonstrates the expected effect of insulin on glucose utilization and also a significant stimulation of lactate production by insulin. A second group of experiments showed that DTP had no effect on glucose disappearance from the medium but increased lactate accumulation. In the presence of 0·2 mM DTP, the action of insulin was still present but the effect of the hormone was very much smaller than in the absence of the inhibitor. Lactate production in the presence of DTP was not further increased by insulin.

The last series of experiments reported in Table 1 show that the inhibitory effect of DTP on glucose utilization can be overcome by the sulfhydryl compound dithiothreitol (DTT). This compound was first studied by Cleland[9] and found to be very effective in reducing disulfide bonds. DTT alone had a stimulatory effect on glucose metabolism as shown in experiments to be presented subsequently.

We also carried out experiments with the disulfide 5,5′-dithio-bis-(2-nitrobenzoic

acid) (Ellman's reagent or DTNB). This substance like DTP combines with sulfhydryl groups[10] and inhibits oxidative phosphorylation.[11,12] It is, however, strongly electronegative and cannot be expected to penetrate easily biological membranes. In experiments in which diaphragms were incubated in media containing 0·4 mM DTNB, there was little or no inhibition of the action of insulin even when the insulin was added 10 min after the sulfhydryl reagent. At the high concentration of 2·4 mM, DTNB caused only a small decrease in the insulin effect on glucose utilization.

TABLE 1. EFFECT OF 2,2′-DITHIODIPYRIDINE (DTP) ON THE ACTION OF INSULIN ON GLUCOSE UTILIZATION AND LACTATE PRODUCTION BY RAT DIAPHRAGM *in vitro**

Addition to medium	N	Glucose utilization (μmoles/g/2 hr ± S.E.M.)	Lactate production (μmoles/g/2 hr ± S.E.M.)
None	14	14·82 ± 1·01	18·98 ± 1·24
Insulin (0·2 units/ml)	14	31·25 ± 1·24	26·78 ± 1·70
Insulin effect†		+16·43 (P < 0·001)†	+7·80 (P < 0·001)†
None	6	15·09 ± 0·88	21·49 ± 1·01
DTP (0·2 mM)	6	14·44 ± 0·60	26·83 ± 1·85
DTP effect†		−0·65 (N.S.)†	+5·34 (P < 0·01)†
DTP (0·2 mM)	9	13·63 ± 1·10	27·06 ± 1·80
DTP (0·2 mM)+ insulin (0·2 units/ml)	9	16·87 ± 1·07	26·42 ± 1·14
Insulin effect†		+3·24 (P < 0·01)†	−0·64 (N.S.)†
DTP (0·2 mM)+ insulin (0·2 units/ml)	7	13·45 ± 1·95	29·26 ± 1·47
DTP (0·2 mM)+ insulin (0·2 units/ml)+ DTT (1·0 mM)	7	25·79 ± 1·51	31·31 ± 2·56
DTT effect†		+12·34 (P < 0·001)†	+2·05 (N.S.)†

* Rat hemidiaphragms were incubated at 37° as described in Methods. DTP and insulin were present in the medium before the tissue was added. DTT was added 2 min after the hemidiaphragm was placed in the medium.

† Statistical significance of the effects of insulin, DTP or DTT are based on paired differences. N.S. = not significant.

Action of insulin in the presence and absence of dithiothreitol. If the action of insulin involves the formation of a disulfide linkage with the cell membrane, one would expect that a compound such as dithiothreitol (DTT) that reduces S–S bonds would be extraordinarily effective in inhibiting the action of insulin. In liver mitochondria, DTT was observed to reverse the inhibition of oxidative phosphorylation produced by DTNB.[12] We, therefore, studied the effect of this compound alone and in the presence of insulin on glucose utilization by the rat diaphragm. Hemidiaphragms were incubated for 30, 60, 90 and 120 min, and glucose disappearance from the medium determined. Insulin, when present, was added initially and DTT after 2 min of incubation. The experiments are reported in Fig. 1. The first panel shows the usual large insulin effect on glucose uptake by the tissue. The action of DTT was studied at two different concentrations of the compound, 1·0 and 2·5 mM. It is seen that DTT itself stimulated glucose utilization and that this effect was not immediate but became apparent only after about 1 hr of incubation. The higher concentration of DTT had a somewhat larger effect than DTT at 1 mM. When insulin and DTT were present together, utilization appeared to be stimulated by both substances but the effects of insulin and

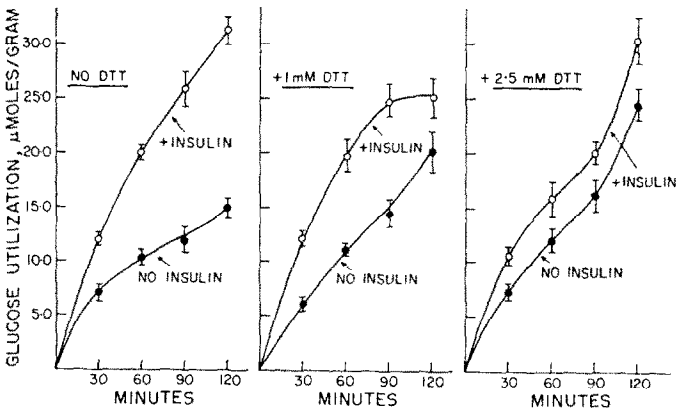

FIG. 1. Rat hemidiaphragms incubated at 37° as described in Methods. Insulin (0·2 units/ml) was added at zero time, DTT after 2 min of incubation. Each point represents the mean of six or nine separate experiments. Bars indicate ± S. E. M.

DTT were not additive. It is important to note that DTT was unable to abolish the immediate effect of insulin seen during the first 30 min of incubation. However, subsequently the effect of DTT became more pronounced and the diaphragm appeared to be mainly under the influence of the stimulatory action of DTT. This is particularly evident in the experiments with 2·5 mM DTT. After the first 30 min during which an insulin effect occurred, the curves of glucose uptake with time became almost parallel.

Action of insulin when added after DTT. In the experiments recorded in Fig. 2, insulin when present was added 2 min after DTT. For comparison the control curves from Fig. 1 (results in the absence of DTT) are also shown. It is clear that the action of DTT appears slowly; the compound has little effect in the first hour of incubation but

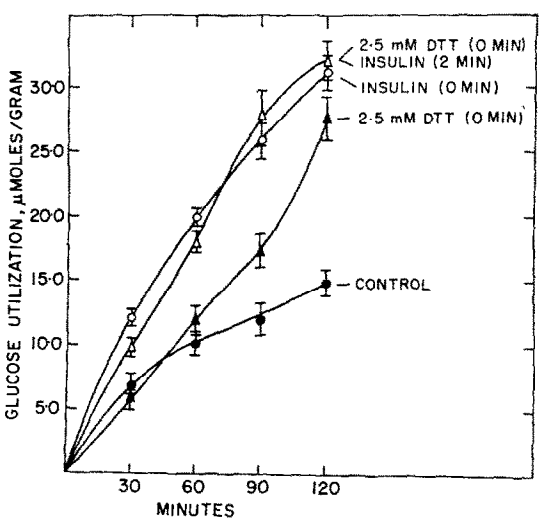

FIG. 2. Rat hemidiaphragms incubated at 37° as described in Methods. DTT (2·5 mM) was added at zero time, insulin (0·2 units/ml) after 2 min of incubation. N = 4 for experiments with DTT. N = 9 for experiments without DTT. Bars indicate ± S. E. M.

TABLE 2. EFFECT OF DITHIOTHREITOL AND INSULIN ON LACTATE ACCUMULATION BY RAT DIAPHRAGM *in vitro**

Addition to medium	N	Lactate accumulation (μmoles/g/2 hr ± S. E. M.)	Significance of insulin effect	Significance of DTT effect†
None	9	16·90 ± 1·33		
Insulin (0·2 units/ml)	9	22·00 ± 1·97	P < 0·05	
DTT (1 mM)	6	21·66 ± 1·54		P < 0·05
DTT (1 mM)+ insulin (0·2 units/ml)	9	22·31 ± 0·70	N.S.‡	
DTT (2·5 mM)	6	28·56 ± 2·97		P < 0·01
DTT (2·5 mM)+ insulin (0·2 units/ml)	6	28·85 ± 3·25	N.S.‡	

* Rat hemidiaphragms were incubated at 37° as described in Methods, and lactate accumulation in the medium was determined after 2 hr of incubation.
† Significance of differences is based on comparisons of means.
‡ N.S. = not significant.

TABLE 3. EFFECT OF DTP ON THE SYNTHESIS OF GLYCOGEN

Addition to medium	N	Final glycogen* (μmoles glucose equivalents/g ± S. E. M.)
None	12	20·64 ± 1·68
DTP (0·2 mM)	12	11·35 ± 1·30
DTP effect		−9·29 (P < 0·001)
Insulin (0·2 units/ml)	6	28·28 ± 2·84
Insulin (0·2 units/ml)+ DTP (0·2 mM)	6	15·15 ± 1·49
DTP effect		−13·13 (P < 0·005)

* After 2 hr of incubation at 37°.

TABLE 4. EFFECT OF DTT ON CARBOHYDRATE METABOLISM IN RAT DIAPHRAGM

Addition to medium	N	Glucose utilization (μmoles/g/3 hr)	Lactate formation (μmoles/g/3 hr)	Final glycogen (μmoles glucose equivalents/g)
None	10	24·57 ± 1·04	27·11 ± 1·47	14·53 ± 1·88
DTT (2·5 mM)	10	32·85 ± 1·29	35·42 ± 2·37	10·93 ± 1·49
DTT effect		+8·28 ± 1·58	+8·31 ± 2·64	−3·60 ± 2·27
P*		< 0·001	< 0·05	< 0·05
+Insulin	10	47·35 ± 1·03	36·18 ± 1·56	25·65 ± 1·55
Insulin + DTT (2·5 mM)	10	40·77 ± 1·47	39·56 ± 1·55	17·62 ± 1·97
DTT effect		−6·58 ± 1·70	+3·38 ± 2·09	−8·04 ± 2·37
P*		< 0·005	N.S.	< 0·001

* Based on paired differences. N.S. = not significant.

has a very large effect on glucose utilization in the second hour of incubation. Where both insulin and DTT are present the utilization of glucose during the 2-hr incubation is not significantly different from that seen with insulin alone. These experiments and the results shown in Fig. 1 demonstrate that DTT, whether added before or after insulin, does not inhibit the initial action of the hormone on glucose utilization.

Effect of DTT and insulin on lactate accumulation by the rat diaphragm. In all experiments reported in Figs. 1 and 2, the lactate concentration in the incubation medium was also determined. There was in all cases a rapid accumulation of lactate during the first 30 min of incubation. During the subsequent period of incubation, the formation of lactate continued but at a much lower rate than during the first 30 min. There was no significant effects of insulin or DTT during the first hour of incubation. The results for the entire 2-hr incubation are given in Table 2. It is seen that both insulin and DTT caused a significant increase in lactate production. One mM DTT increased lactate output to about the same extent as insulin, and at 2·5 mM the effect of DTT was greater than that of insulin. When lactate formation was stimulated by DTT, insulin caused no additional accumulation of lactate in the incubation medium.

Effects of DTP and DTT on glycogen metabolism. The observations that DTP as well as DTT increased lactate formation made it of interest to determine whether these compounds influenced the metabolism of glycogen in the diaphragm.

The results of experiments in which the final concentration of glycogen in the incubated diaphragm was determined are recorded in Table 3. DTP at a concentration of 0·2 mM was found to cause a marked depression of glycogen synthesis and also to inhibit the stimulation of glycogen formation produced by insulin. The increased lactate formation observed in the presence of DTP appears, therefore, to be the result of an increased rate of glycogenolysis.

DTT also interfered with glycogen synthesis as indicated by the results reported in Table 4. In order to obtain large effects of DTT, diaphragms were incubated for 3 hr and glucose utilization, lactate formation and final glycogen tissue content were determined. It is seen that DTT increased glucose uptake and lactate production but produced a significant decrease in glycogen content of the tissue. Insulin, like DTT, stimulated glucose uptake and lactate output but caused a marked increase in the synthesis of glycogen. When DTT was added together with insulin, the action of insulin on glucose utilisation and glycogen synthesis was depressed but still highly significant. The experiments demonstrate the difference between the action of a sulfhydryl compound like DTT and insulin on carbohydrate metabolism in muscle. Insulin increases glucose utilization resulting in stimulation of both lactate formation and glycogen synthesis. DTT increases glucose uptake and a very large fraction of the extra glucose metabolized appears as lactate in the incubation medium.

DISCUSSION

The results of the experiments reported here confirm that a sulfhydryl compound can exert an insulin-like action on tissue metabolism. However, the action of DTT on glucose uptake by rat diaphragm was quite different from that of insulin. The effect of the thiol compound commenced not immediately but consisted of maintaining a high rate of glucose utilization over a protracted period of time. In addition, DTT caused a breakdown rather than an increased synthesis of glycogen.

The effects of two disulfides, DTNB and DTP, were studied. Both compounds react rapidly with thiols; however, the former is strongly electronegative while the latter is not. In their action on mitochondrial metabolism, DTNB inhibits mainly oxidative phosphorylation and phosphate transport,[11,12] while DTP is less specific and also inhibits respiration in the absence of phosphate acceptor.[8] With the diaphragm neither compound interfered with glucose utilization in the absence of insulin. The insulin effect, however, was markedly depressed by the more lipid soluble DTP but not greatly influenced by DTNB. The results strongly indicate that free sulfhydryl groups are involved at some stage in the metabolic action of insulin. This is further supported by the observation that DTT reversed the inhibitory action of DTP on the insulin effect.

The action of insulin can reasonably be considered to take place in two stages, an initial attachment to a receptor probably situated on the cell surface followed by a metabolic effect of the hormone consisting of a change in permeability of the cell membrane or in the activity of an enzyme.

Our experiments do not indicate that sulfhydryl groups on the cell surface are involved in the initial binding of insulin since if that were so one would have expected that the polar disulfide, DTNB, would have inhibited insulin action strongly. If insulin combined with cells through formation of disulfide bridges, these should be vulnerable to reduction by DTT leading to reversal of the initial action of insulin. We feel, however, that our results give considerable support to the view that cellular sulfhydryl groups are of importance in the regulation of tissue glucose utilization and in some stage of the action of insulin on this process. DTT markedly stimulates glucose uptake by the diaphragm and DTP, a disulfide which combines with SH-groups and can be expected to penetrate cell membranes, has a strong inhibitory effect of insulin action.

Acknowledgement—The authors would like to thank Miss Elaine Serlick for her expert technical assistance in these experiments.

REFERENCES

1. V. R. Lavis and R. H. Williams, *J. biol. Chem.* **245,** 23 (1970).
2. T. Chiba, *J. pharm. Soc. Japan* **89,** 248 (1969).
3. N. Haugaard and E. S. Haugaard, *Biochim. biophys. Acta* **222,** 583 (1970).
4. H-J. Hohorst, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), p. 267. Academic Press, New York (1963).
5. R. Montgomery, *Archs Biochem. Biophys.* **67,** 378 (1957).
6. D. R. Grassetti and J. F. Murray, Jr., *Archs Biochem. Biophys.* **119,** 41 (1967).
7. D. R. Grassetti, J. F. Murray, Jr., M. E. Brokke and A. D. Gutman, *J. med. chem.* **13,** 273 (1970).
8. N. Haugaard, N. H. Lee, P. Chudapongse, C. D. Williams and E. S. Haugaard, *Biochem. Pharmac.* **19,** 2669 (1970).
9. W. W. Cleland, *Biochemistry, N.Y.* **3,** 480 (1970).
10. G. L. Ellman, *Archs Biochem. Biophys.* **82,** 70 (1959).
11. N. Haugaard, N. H. Lee, R. Kostrzewa, R. S. Horn and E. S. Haugaard, *Biochim. biophys. Acta* **172,** 198 (1969).
12. N. Haugaard, N. H. Lee, R. Kostrzewa and E. S. Haugaard, *Biochem. Pharmac.* **18,** 2385 (1969).