Participation of cellular thiol/disulphide groups in the uptake, degradation and bioactivity of insulin in primary cultures of rat hepatocytes*

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The effects on the uptake (cell-associated ^{125}I) and degradation (^{125}I -labelled products released into the medium) of ^{125}I -insulin and bioactivity (protein, glycogen and lipid synthesis) of insulin caused by altering the cellular thiol/disulphide status in primary cultures of rat hepatocytes were studied. Incubation of hepatocyte cultures with various exogenous thiol compounds (reduced glutathione, 2-mercaptoethanol, cysteamine, dithiothreitol) resulted in increased insulin binding, but markedly decreased degradation and bioactivity. These effects could be reversed by washing or by the addition of oxidized glutathione, which alone had no effect. When cultures were exposed to certain thiol-modifying reagents (*N*-ethylmaleimide, *p*-chloromercuribenzoate, *p*-chloromercuribenzenesulphonate, iodoacetamide, iodoacetate), some decreases in bioactivity were evident, but the pronounced decrease in insulin degradation observed with the thiol-containing compounds was not observed with this class of compounds. None of the thiol-containing or -modifying agents tested had any significant effect on cellular ATP concentrations, indicating that the effects observed were due to perturbation of the thiol/disulphide status. Depletion of intracellular glutathione by DL-buthionine SR-sulphoximine (a specific inhibitor of glutathione biosynthesis) decreased the syntheses of glycogen and lipid by about one-half, while having essentially no effect on protein synthesis, ATP concentrations or on the binding and degradation of insulin. The data presented here indicate that although intracellular thiol (glutathione) concentrations may be important for the maintenance of full expression of certain biological activities (glycogen and lipid synthesis), the thiol/disulphide groups on the cell surface and those immediately inside the cell membrane may be more critical in the mediation of insulin action, including the degradation and bioactivity of insulin in primary cultures of rat hepatocytes.

Although the physiological effects elicited by insulin have been known for many years, the mechanism by which these effects are triggered is

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione.

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still poorly understood. Many theories have been advanced to explain these phenomena, yet none has been able to explain the many observations made regarding the mechanism by which the insulin signal is transmitted. Among the many possible mechanisms currently postulated are phosphorylation of membrane proteins (Benjamin & Clayton, 1978; Seals *et al.*, 1978), generation of an intracellular chemical messenger (Larner *et al.*, 1979; Kiechle *et al.*, 1980; Saltiel *et al.*, 1982), direct hormone–receptor interaction (Roth *et al.*,

1976), hormone-receptor internalization and processing (Schlessinger *et al.*, 1978), oxidation of membrane thiol moieties (Czech, 1977; Czech *et al.*, 1977; Schweitzer *et al.*, 1980), kinase activity of insulin receptors (e.g. Rosen *et al.*, 1983) and control of intracellular enzyme activities by modulation of the cellular thiol/disulphide balance (Varandani, 1977, 1980; Phelps & Varandani, 1977; Kozka & Gould, 1984).

In an effort to gain more insight into the mechanism by which insulin exerts its effects, we have studied the binding, degradation and bioactivity of insulin in primary cultures of rat hepatocytes maintained in a serum-free defined medium and have demonstrated that this cultured preparation provides a useful model system for the study of receptor-mediated mechanisms of insulin action (Varandani *et al.*, 1982a). We have also studied the effects of lectin, lysosomotropic, anti-microtubule and anti-microfilament agents on these processes and have observed that certain medium-/long-term bioactivities of insulin (glycogen, lipid and protein syntheses) appear to be accomplished by a continuous lysosomal processing of a small fraction of the hormone via a receptor-mediated endocytotic process, whereas the degradation of insulin occurs via non-lysosomal mechanisms, including the sequential degradative pathway (Varandani *et al.*, 1982b). Using this same system, we now report the effects on insulin binding, degradation and the hormone-mediated syntheses of glycogen, lipid and protein (hereafter referred to as 'bioactivity' for brevity) induced by altering the cellular thiol status by using various thiol-containing compounds, agents that modify thiol groups and a drug that depletes intracellular glutathione. This approach has allowed us to evaluate the relative roles of thiol and disulphide groups on the cell surface, in the environment immediately inside the plasma membrane and inside the cell in the processes of insulin binding, degradation and bioactivity. Insulin binding, which was measured here as cell-associated ^{125}I radioactivity after a 24 h incubation of monolayers with ^{125}I -insulin, is referred to as 'uptake' in order to avoid any confusion with cell-surface-receptor binding.

Materials and methods

GSH, GSSG, 2-mercaptoethanol, cysteamine, dithiothreitol, *N*-ethylmaleimide, iodoacetate, iodoacetamide, *p*-chloromercuribenzenesulphonate, *p*-chloromercuribenzoate, NADPH and glutathione reductase were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 5,5'-Dithiobis-(2-nitrobenzoic acid) was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

Firefly lantern extract for use in ATP determinations was given by Dr. M. Reporter of C. F. Kettering Research Laboratories (Yellow Springs, OH, U.S.A.). DL-Buthionine and DL-buthionine SR-sulphoximine were synthesized and characterized by melting-point determinations and by their elution from a Durrum D-300 amino acid analyser as described by Griffith (1981). All other chemicals were reagent-grade or better or were procured as previously described (Varandani *et al.*, 1982a).

Cultures of rat hepatocytes were prepared and maintained and assays were performed as previously reported (Varandani *et al.*, 1982a) and as briefly outlined below. Hepatocytes were isolated by collagenase digestion from starved male Sprague-Dawley rats. Approx. 2.5×10^6 isolated cells in a volume of 3 ml of basal serum-free defined culture medium (pH 7.4) supplemented with 50 nM-glucagon were plated in 60 mm $\times$ 15 mm plastic culture dishes. The medium was changed 4 h after the initial plating and every 24 h thereafter, the glucagon-supplemented medium being used. At 48 h after the initial plating, the medium was replaced (zero time) with basal medium (without glucagon) supplemented with unlabelled insulin (1 nM) with or without a tracer amount of ^{125}I -insulin (approx. 30000 c.p.m.) together with the appropriate test reagent in parallel sets of dishes. After an additional 24 h, the set of cells that had received ^{125}I tracer was utilized for the determination of insulin uptake (cell-associated ^{125}I) and degradation (the products of ^{125}I -insulin breakdown released into the medium) and glycogen content. Non-enzymic degradation of insulin was assessed by measuring the breakdown of ^{125}I -insulin in parallel sets of plates with medium containing insulin and the appropriate test reagent in the absence of cells. All degradation data presented here have been corrected for this non-enzymic degradation. One parallel set of plates that received native insulin but no tracer was utilized for the measurement of cellular protein content. Protein and lipid syntheses were studied by determining the incorporation of [4,5- ^3H]leucine into proteins or the conversion of [3- ^3H]glucose into toluene-extractable [^3H]lipids on parallel sets of plates (Varandani *et al.*, 1982a,b). Total soluble GSH and ATP were determined on an additional parallel set of plates. The cells on these plates were washed with 3×2 ml of 0.1 M-potassium phosphate/5 mM-EDTA, pH 7.5, and were scraped into 0.9 ml of this buffer. Then 0.1 ml of 100% trichloroacetic acid was added and the samples were frozen immediately with solid CO_2 . Total soluble GSH was measured in the acid supernatant essentially by the method of Bannai & Tsukeda (1979). ATP in the acid supernatant was monitored after diethyl ether extraction, by the luciferin/luciferase method of Lundin *et al.* (1976).

Results

Effects of several thiol-containing compounds

The effects of the exogenously added monothiol compounds GSH, 2-mercaptoethanol and cysteamine (Fig. 1) and of the dithiol compound dithiothreitol (Fig. 2) on the uptake (cell-associated ^{125}I), degradation (^{125}I -labelled products released into the medium) and bioactivity of 1 nM-insulin in cultured hepatocytes were tested.

Although the addition of GSH had little effect on ^{125}I -insulin uptake over the range of concentra-

tions tested (Fig. 1a), the cell-mediated degradation of insulin and syntheses of glycogen and protein were all depressed in a concentration-dependent manner, with glycogen synthesis being virtually eliminated at 5 mM-GSH. Conversely, addition of GSSG along with 1 nM-insulin had essentially no effect on insulin uptake, degradation or bioactivity at concentrations up to 5 mM (see Table 1).

The addition of various concentrations of 2-mercaptoethanol along with 1 nM-insulin resulted, in contrast with GSH, in markedly increased insulin uptake at concentrations as low as 1 mM (Fig. 1b). As with GSH, mercaptoethanol addition resulted in a concentration-dependent decrease in insulin degradation and bioactivity, with this reagent being a more potent inhibitor of each activity than was GSH.

Like mercaptoethanol, addition of cysteamine resulted in a concentration-dependent enhancement of uptake and inhibition of degradation and bioactivity (Fig. 1c). Of all the thiol compounds tested, cysteamine (e.g. at 2 mM) was the most potent inhibitor of cell-mediated insulin degradation, resulting in an inhibition of over 90% of that observed in parallel control plates.

The effects of a dithiol compound, dithiothreitol, are shown in Fig. 2. Its addition had little effect on uptake, while depressing degradation and the insulin-mediated syntheses of glycogen and protein in a concentration-dependent manner similar to that observed for the monothiol compounds tested. Concentrations of dithiothreitol above 1 mM resulted in little additional effect.

Previous studies (Varandani *et al.*, 1982b) have demonstrated that no extracellular degradation of insulin occurs in our cultured hepatocyte preparations, since there is no leakage of insulin-degrading enzymes into the medium. Also, as noted in the

Fig. 1. Effects of various monothiol compounds on the uptake, degradation and bioactivity of insulin in cultured rat hepatocytes

Cells were cultured for 48 h in basal medium supplemented with 50 nM-glucagon and then washed. These washed cultured cells (zero time) were then continued in culture for 24 h in basal medium supplemented with 1 nM-insulin along with various concentrations of GSH (a), 2-mercaptoethanol (b) or cysteamine (c). The cell-mediated uptake (B) and degradation (D) of ^{125}I -insulin and the insulin-stimulated syntheses of glycogen (G) and protein (P) were monitored as described in the text. Values are expressed as % of control (1 nM-insulin alone); see Table 1 for examples of actual control values. Degradation values have been corrected for non-enzymic degradation, which was determined by running parallel plates without cells. Each point represents the mean $\pm$ S.E.M. of three or more determinations.

Fig. 2. Effects of dithiothreitol on the uptake, degradation and bioactivity of insulin in cultured rat hepatocytes. Details are as given in the legend to Fig. 1.

Table 1. *Effects of adding dithiothreitol (DTT) and GSSG together on the uptake, degradation and bioactivity in cultured rat hepatocytes*

(a) Cells cultured for 48 h and washed as described in Fig. 1 were continued (zero time) in culture for 24 h in basal medium alone or basal medium supplemented with 1 nM-insulin, 1 nM-insulin + 0.75 mM-DTT, 1 nM-insulin + 5 mM-GSSG or 1 nM-insulin + 0.75 mM-DTT + 5 mM-GSSG. After 24 h, the uptake and degradation of insulin and the synthesis of glycogen and protein were determined. (b) Cells were cultured for 24 h in the glucagon-containing medium, followed by 24 h in this medium supplemented with 0.75 mM-DTT. After this pretreatment, the cells were washed and cultured for 24 h in the basal medium supplemented with 1 nM-insulin alone or along with 5 mM-GSSG. Values shown represent the average of two determinations or the mean $\pm$ S.E.M. of three or more determinations.

	¹²⁵ I-insulin		Bioactivity	
	Uptake (%/mg of protein)	Degradation (%/mg of protein)	Glycogen (μ g of glucose/ mg of protein)	[³ H]Leucine incorporated (d.p.m./mg of protein)
(a) No pretreatment				
Basal	—	—	45 $\pm$ 3.1	2223
1 nM-Insulin	1.45 $\pm$ 0.08	20.43 $\pm$ 0.30	376 $\pm$ 32.6	4744
1 nM-Insulin + 0.75 mM-DTT	2.65 $\pm$ 0.03	10.63 $\pm$ 0.12	74 $\pm$ 8.6	3293
1 nM-Insulin + 5 mM-GSSG	1.38 $\pm$ 0.04	22.60 $\pm$ 0.52	389 $\pm$ 40.2	5465
1 nM-Insulin + 0.75 mM-DTT + 5 mM-GSSG	1.41 $\pm$ 0.11	20.06 $\pm$ 0.38	277 $\pm$ 24.9	4315
(b) Cells pretreated with dithiothreitol				
1 nM-Insulin	1.21 $\pm$ 0.02	18.99 $\pm$ 0.62	488 $\pm$ 31.3	6029
1 nM-Insulin + 5 mM-GSSG	1.04 $\pm$ 0.02	18.98 $\pm$ 0.35	390 $\pm$ 15.0	5970

Materials and methods section, all degradation data presented here have been corrected for non-enzymic insulin breakdown by determining the degradation of ¹²⁵I-insulin in a parallel set of plates containing insulin, thiol and medium in the absence of cells. Hence the degradation data reported here reflect actual cell-mediated degradation of insulin. It should be noted that the changes in bioactivity observed in the presence of thiol are not the result of decreased insulin availability, since, as demonstrated previously (Varandani *et al.*, 1982b) and again in separate experiments (results not shown), insulin concentration in the range of 0.1–1 nM has a minimal dose-dependent effect on the syntheses of glycogen and protein. In fact, calculations showed that the degree of inhibition of bioactivity observed in the presence of the thiols was far greater than can be accounted for by the decreased availability of insulin caused by its non-enzymic degradation.

Since incubation with compounds containing reduced thiol moieties was observed to enhance insulin uptake and decrease cell-mediated insulin degradation and bioactivity (Figs. 1 and 2), whereas the compound containing the oxidized disulphide moiety (i.e. GSSG) had no effect on these processes (Table 1), it was decided to determine the effects of adding both an oxidized and a reduced thiol compound together. When cell monolayers were incubated with both dithiothreitol and GSSG simultaneously, the insulin uptake and degradation more closely corresponded to the control values than did the uptake and

degradation by cells that had been incubated with dithiothreitol alone (Table 1a). The inhibition of insulin-stimulated glycogen and protein syntheses observed when cultures were exposed to dithiothreitol alone was also relieved by the simultaneous addition of 5 mM-GSSG (Table 1a). The effects of the monothiol compounds could also be reversed by the addition of GSSG (results not shown). As shown in Table 1(a), incubation of the cells with 5 mM-GSSG along with 1 nM-insulin had little or no effect on any of the activities tested. The reversal of the dithiothreitol effects exhibited by the addition of GSSG could be the result of antagonistic effects produced by the presence of both the oxidized and reduced thiol groups on the cell surfaces, or it could be due to simple 'neutralization' (oxidation of the exogenous dithiothreitol) occurring as the result of the high concentration of GSSG. To test these possibilities, cells were pretreated with dithiothreitol before their exposure to insulin with or without GSSG. After plating, these cultures were incubated for 24 h in the glucagon-supplemented medium and then for 24 h in the glucagon-containing medium supplemented with 0.75 mM-dithiothreitol. After this incubation, monolayers were washed to remove dithiothreitol, and (at zero time) basal medium containing 1 nM-insulin alone or along with 5 mM-GSSG was added, and incubations were continued as usual. The results of these experiments (Table 1b) show that preincubation with 0.75 mM-dithiothreitol appears to have no effect on the uptake or degradation of insulin. Addition of 5 mM-GSSG to cells that had

been preincubated with dithiothreitol also had essentially no effect on either insulin uptake or degradation. In contrast with the inhibition of insulin-stimulated synthesis of glycogen and protein observed when cells were incubated with dithiothreitol, and to a lesser extent with dithiothreitol and GSSG together (Table 1a), preincubation with dithiothreitol resulted in some enhancement of bioactivity (Table 1b). These data together (Table 1) indicate that the inhibition of the bioactivity of insulin in the presence of dithiothreitol is the result of some event associated with the external surface of the cells (e.g. reduction of membrane thiols) and can be diminished by removal of the reducing agent or by the addition of an oxidant.

Effects of various thiol-modifying reagents

The role of thiol groups in insulin metabolism and action was further probed by utilizing a variety of thiol-modifying reagents, including *N*-ethylmaleimide, *p*-chloromercuribenzoate, *p*-chloromercuribenzenesulphonate, iodoacetamide and iodoacetate.

Incubation of hepatocytes in the presence of insulin and the thiol-blocking agent *N*-ethylmaleimide resulted in a concentration-dependent increase in cell-associated radioactivity and insulin degradation (Table 2). Insulin-mediated bioactivity was somewhat depressed by the addition of this reagent.

The effects of the thiol-reactive mercurials *p*-chloromercuribenzoate and *p*-chloromercuribenzenesulphonate were also studied (Table 2).

Both compounds increased uptake substantially at concentrations of 50 μM . Although degradation and glycogen synthesis were inhibited by both reagents, the effects were greater with the more lipophilic mercurial *p*-chloromercuribenzoate, which also inhibited [^3H]leucine incorporation into proteins.

Unlike *N*-ethylmaleimide and the mercurials, the alkylating agents iodoacetamide and iodoacetate produced a significant decrease in the insulin bound by the cells (Table 2). Low concentrations (10 μM) of both agents resulted in a moderate increase (10–15%) in protein synthesis. At higher concentrations (50 μM) both showed significant inhibition of bioactivity, with iodoacetamide producing the more dramatic effect. As with the mercurials, the more lipophilic of the analogues produced the more profound effects.

There are reports (Korbl *et al.*, 1977; Kozka & Gould, 1982, 1984) that *N*-ethylmaleimide, *p*-chloromercuribenzenesulphonate and iodoacetate can induce a dramatic decrease in ATP in rat soleus muscle. In the light of these observations, we monitored the ATP contents of cultured hepatocytes treated with each of the thiol blockers at concentrations that caused decreases of about 50% in glycogenesis, to determine if the effects on the insulin-mediated process that we were observing were actually the result of decreased cellular ATP. The ATP contents in cells exposed to *N*-ethylmaleimide and *p*-chloromercuribenzenesulphonate were lowered to approximately two-thirds of the control (1 nM-insulin only) value (results not shown). ATP contents in cells that had been

Table 2. Effects of thiol-modifying reagents on binding, degradation and bioactivity of insulin in cultured hepatocytes. Cells cultured for 48 h and washed as described in Fig. 1 were continued (zero time) in culture in basal medium supplemented with 1 nM-insulin along with the indicated concentration of the thiol-modifying reagent. After 24 h the cell-mediated binding and degradation of insulin as well as the syntheses of glycogen and protein were monitored as described in the text and are expressed as % of control (1 nM-insulin alone); see Fig. 3 for actual control values. Values shown represent either the average of two determinations or the mean $\pm$ S.E.M. of three to four determinations.

Agent	Concn. (μM)	^{125}I -insulin		Bioactivity	
		Binding	Degradation	Glycogen	Protein
Control (1 nM-insulin alone)	–	100	100	100	100
<i>N</i> -Ethylmaleimide	50	93 $\pm$ 4	114 $\pm$ 8	63 $\pm$ 8	77
	100	149 $\pm$ 15	153 $\pm$ 10	43 $\pm$ 13	91
<i>p</i> -Chloromercuribenzoate	10	124 $\pm$ 9	80 $\pm$ 3	44 $\pm$ 5	122
	50	152 $\pm$ 9	76 $\pm$ 3	22 $\pm$ 2	86
<i>p</i> -Chloromercuribenzenesulphonate	10	112 $\pm$ 4	88 $\pm$ 4	101 $\pm$ 7	98
	50	116 $\pm$ 9	75 $\pm$ 3	66 $\pm$ 9	106
	100	166 $\pm$ 6	59 $\pm$ 0	24 $\pm$ 7	99
Iodoacetamide	10	66 $\pm$ 9	114 $\pm$ 34	88 $\pm$ 14	113
	50	101 $\pm$ 9	182 $\pm$ 13	20 $\pm$ 9	37
Iodoacetate	10	77 $\pm$ 3	80 $\pm$ 2	126 $\pm$ 6	110
	50	68 $\pm$ 10	100 $\pm$ 32	45 $\pm$ 11	81

exposed to iodoacetate, iodoacetamide, *p*-chloromercuribenzoate or any of the thiol compounds and showed 50% decreases in glycogenesis all remained at >65% of the control values (results not shown). Incubation of cells with 5mM-GSSG, however, also gave an ATP content that was 72% of the control. Since GSSG had no effect on any of the activities tested, these data indicate that the effects observed with these agents are not due to a decrease in cellular ATP, but rather are probably the result of perturbations of the cellular thiols resulting from exposure to these agents.

Effects of inhibiting cellular glutathione biosynthesis

DL-Buthionine SR-sulphoximine has been reported to lower endogenous GSH concentrations by specifically and reversibly inhibiting the enzyme responsible for catalysing the final step in GSH biosynthesis, γ -glutamylcysteine synthetase (Griffith, 1981). Since the addition of exogenous GSH resulted in a decrease in insulin degradation and bioactivity (Fig. 1), it was decided to determine what effect lowering the intracellular concentrations of this compound might have on these insulin-mediated cellular processes.

When DL-buthionine SR-sulphoximine was added to rat hepatocytes cultured in the presence of

1 nM-insulin, a dramatic decrease in cellular GSH was observed, to <10% of that of the control at a concentration of 25 μ M (Fig. 3). GSH depletion in these cells was accompanied by a decrease of 50% in glycogen synthesis and of 60% in lipid synthesis. Interestingly, these changes paralleled the concentration-dependent decrease in GSH at concentrations of buthionine sulphoximine up to 25 μ M, but were not changed by concentrations of the drug above this value, presumably since the effects observed here were due to the complete depletion of intracellular GSH. No significant effects on the uptake or degradation of insulin or on protein synthesis or the concentration of cellular ATP were observed over the range of buthionine sulphoximine concentrations tested (Fig. 3). When cells that had been incubated for 24 h in the presence of the drug were washed and then incubated for an additional 24 h in its absence, all the observed effects on GSH content, glycogenesis and lipogenesis were completely reversed (results not shown). Additionally, control experiments using the structural analogue DL-buthionine showed no effect on any of the parameters studied at any of the concentrations tested.

Discussion

Addition of a variety of compounds containing a reduced thiol moiety to rat hepatocytes cultured in the presence of insulin results in a marked inhibition of cell-mediated insulin degradation and bioactivity. Insulin binding is not decreased by the addition of these compounds and is, in some cases, increased.

Of the monothiol compounds tested, cysteamine (i.e. 2-mercaptoethylamine) was the most potent inhibitor of insulin degradation, whereas its structural analogue 2-mercaptoethanol was a more potent inhibitor of bioactivity. GSH and the dithiol compound dithiothreitol were also capable of suppressing insulin degradation and bioactivity. These observations may suggest that an extracellular environment containing a high concentration of reduced thiol may cause the reduction of some membrane-associated disulphide group(s) which must be present in an oxidized state for the expression of the bioactivity of insulin. This postulation is supported by the demonstration that removal of the exogenous thiol compounds reversed or decreased its effects and by the fact that GSSG addition, while alone producing no effect on the insulin-mediated process studied here, does indeed reverse the effects of added thiols. Similar relationships between insulin degradation and bioactivity suggest that insulin degradation is closely associated with the expression of the insulin-stimulated bioactivities studied here.

Fig. 3. *Effects of DL-buthionine SR-sulphoximine on cultured rat hepatocytes*

Cells were cultured for 48 h in basal medium supplemented with 50 nM-glucagon, and then washed. Experiments were then initiated (zero time) by continuing cultures for another 24 h in basal medium supplemented with 1 nM-insulin (125 I-insulin) and various indicated concentrations of DL-buthionine SR-sulphoximine. Cellular GSH and ATP contents as well as the cell-mediated uptake (B) and degradation (D) of 125 I-insulin and the insulin-stimulated syntheses of glycogen (G), protein (P) and lipid (L) were monitored as described in the text and legend to Fig. 1. Results are expressed as % of control (1 nM-insulin alone). Each point represents the mean $\pm$ S.E.M. of three or more determinations.

Unlike the thiol compounds investigated, the thiol-modifying reagents were not potent inhibitors of insulin degradation. Indeed, two of these thiol blockers, *N*-ethylmaleimide and iodoacetamide, actually enhanced degradation, suggesting that in some conditions insulin degradation and insulin bioactivity can be dissociated. Although all these agents induced decreases in glycogen synthesis, the effects were most pronounced for *p*-chloromercuribenzoate and iodoacetamide. Unlike the thiol-containing compounds, which always showed concentration-dependent decreases in synthesis of both glycogen and protein of the thiol-blocking agents tested, only *p*-chloromercuribenzoate and iodoacetamide showed significant decreases in protein synthesis. Significantly, these are the more lipophilic of their respective analogues. Presumably this increased lipophilicity renders these compounds more able to enter the cell and produce their effects. Additionally, our experiments showed that changes in the cellular ATP concentration caused by these agents were not responsible for the observed effects. These data together may indicate that some intracellular thiol-containing proteins (enzymes) in the environment immediately inside the plasma membrane and/or thiol compound (possibly GSH) may be necessary for the insulin-stimulated synthesis of glycogen and protein, since these activities were most affected by the thiol blockers that penetrated the cells. The fact that protein synthesis exhibited little or no inhibition by the agents that probably are less likely to enter the cell (*N*-ethylmaleimide, *p*-chloromercuribenzenesulphonate, iodoacetate) is also consistent with the possibility that some externally associated cellular disulphide group(s) as well as some thiol-containing protein immediately inside the plasma membrane may mediate the insulin-induced synthesis of protein, as was suggested by the observations with the thiol compounds.

Culturing of rat hepatocytes in the presence of various concentrations of DL-buthionine SR-sulphoximine resulted in decreases of approx. 50% in glycogenesis and of 60% in lipid synthesis, but no effect on protein synthesis or on insulin uptake or degradation was observed. This compound, which is reported to be a specific inhibitor of γ -glutamylcysteine synthetase (Griffith, 1981), caused a complete depletion of cellular GSH at a concentration of $\geq 25 \mu\text{M}$ while having no appreciable effect on cellular ATP content. Hence the observed effects of buthionine sulfoximine on lipid and protein synthesis are presumably due to the decrease of GSH within the cell caused by this drug. The fact that glycogen and lipid syntheses are decreased as the concentration of intracellular GSH decreases indicates that the maintenance of

Scheme 1. Diagram depicting three populations of thiol/disulphide groups involved in insulin action in its medium/long-term biological effects

1, Disulphide groups on the cell surface; 2, thiol-containing compounds (proteins) immediately inside the plasma membrane; 3, GSH located intracellularly.

these bioactivities is sensitive to the intracellular GSH/GSSG pool, whereas insulin degradation and protein synthesis are more sensitive to the cell-surface membrane-associated thiol/disulphide balance.

As shown in Scheme 1, the data presented here are consistent with the postulate that the expression and maintenance of the medium/long-term biological effects of insulin require the participation of three distinct types of cellular thiol/disulphide groups: disulphide groups on the cell surface, thiol-containing compounds (proteins) immediately inside the plasma membrane, and GSH within the cell.

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