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Inhibition of *p*-Chloromercuribenzoate- and Glucose-Induced Insulin Release *in Vitro* by Methylene Blue, Diamide, and *tert*-Butyl Hydroperoxide

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SUMMARY

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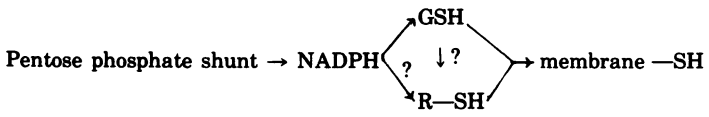
This investigation was designed to explore the possible role of NADPH and reduced glutathione in insulin secretion and their relationship to thiol groups located in the β-cell membrane. We studied the effects of 2 μg/ml of methylene blue (an oxidant of NADPH), 0.1 mM diamide (an oxidant of GSH), and 2 mM *tert*-butyl hydroperoxide (a substrate of GSH peroxidase) on the release of insulin from isolated, perfused rat pancreas and pancreatic islets. *p*-Chloromercuribenzoate (a thiol reagent triggering insulin release supposedly by virtue of its action on superficial thiol groups in β-cell membranes) was used at 0.1 mM to initiate insulin secretion from isolated, perfused rat pancreas, while isolated islets were stimulated to release insulin with 3 mg/ml of glucose. Both phases of *p*-chloromercuribenzoate-triggered insulin release were significantly suppressed by methylene blue, diamide, and *tert*-butyl hydroperoxide. Similarly, these substances inhibited glucose-stimulated insulin release from isolated islets. These results suggest that stimulation of insulin release by *p*-chloromercuribenzoate and glucose depends on the content of NADPH and GSH in the islets. It is postulated that thiol groups in the β-cell membrane, which are thought to be related to insulin release, are kept in the reduced state via the following sequence of events:



INTRODUCTION

We have previously demonstrated that inhibition of the pentose phosphate shunt in the pancreatic islets by 6-aminonicotinamide, an antimetabolite of pyridine nucleotide synthesis, is associated with the inhibition of glucose-stimulated insulin

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release (1–3). 6-Aminonicotinamide has also been shown to decrease NADPH levels in various tissues, including pancreatic islets (4, 5), and to diminish the content of reduced glutathione in certain other tissues (6, 7). It was therefore hypothesized that a possible function of the pentose phosphate shunt in the release of insulin could be to form NADPH, which in turn might be required to keep glutathione in the reduced state (1). However, there is so far little evidence available to show such a possible role of reduced glutathione in the process of insulin secretion.

Recently, sulfhydryl reagents like *p*-chloromercuribenzoate, which only slowly penetrate into cells, have been found to stimulate insulin release rapidly (8, 9), and it has been suggested that these compounds initiate insulin secretion by blocking certain superficial thiol groups in the β-cell membrane (10–12). Since it has been shown that the membrane sulfhydryl groups are modulated by the oxidation-reduction state of the GSH:GSSG ratio in cells (13), we thought it of interest to investigate whether compounds known to decrease intracellular levels of NADPH and GSH would inhibit the insulin-releasing action of *p*-chloromercuribenzoate. Furthermore, we wished to determine whether these substances would inhibit insulin secretion stimulated by α-D-glucose.

The data presented here demonstrate the inhibition of *p*-chloromercuribenzoate- and glucose-stimulated insulin release by methylene blue, diamide, and *tert*-butyl hydroperoxide. These findings suggest that the stimulation of insulin secretion by *p*-chloromercuribenzoate and glucose depends on the NADPH and GSH contents of the pancreatic islets.

MATERIALS AND METHODS

Chemicals

p-Chloromercuribenzoate and *tert*-butyl hydroperoxide were obtained from Merck-Schuchardt. Methylene blue was purchased from Fluka. Dextran (mol wt 60,000–90,000) and α-D-glucose were obtained from Serva Feinbiochemica, Heidelberg. Diamide [diazenedicarboxylic acid bis-(*N*,*N*-dimethylamide)] was prepared and donated by HAG AG, Bremen, through the courtesy of Dr. O. Vitzthum. Bovine serum albumin was purchased from Behringwerke AG, and collagenase (196 units/mg), from Worthington Biochemicals. Rat insulin was obtained from Novo Laboratories. The insulin radioimmunoassay kit (INSIK-I), a product of CIS-SORIN, Italy, was supplied by Isotopen Dienst West, Sprendlingen. All other chemicals and reagents of analytical grade were obtained from E. Merck, Darmstadt.

Animals

Wistar rats of either sex, taken from a local strain reared in our laboratory and weighing between 300 and 350 g, were used for these studies. They were maintained on a standard pellet diet (Altromin) and tap water ad libitum.

Experimental Procedures

Two different preparations were used for studying insulin release: isolated, perfused pancreas and isolated pancreatic islets.

Isolation and perfusion of pancreas. The rat pancreas was isolated by a slight modification of the technique described by Curry *et al.* (14). The animals were anesthetized by intraperitoneal injection of 140 mg/kg of hexobarbital sodium, and the pancreas, along with the proximal parts of the duodenum, spleen, and stomach, was completely separated from adjacent organs. The perfusate was administered into the celiac artery of the prepared pancreas by an open-circuit, nonrecycling perfusion system. The flow rate was maintained at 5 ml/min by making changes in perfusion pressure. The perfusion media were continuously gassed with a mixture of 95% oxygen and 5% carbon dioxide. The perfusion medium contained Krebs-Ringer-bicarbonate (15) with 4% dextran and 0.25% bovine serum albumin. D-Glucose (1 mg/ml) was also added to provide a source of energy. The final pH of the solution was always adjusted to 7.35.

All pancreases were allowed to equilibrate for 15 min, followed by a 5-min basal period, designated in Fig. 1 as −5–0 min.

During this period effluent samples were collected at 1-min intervals for determination of insulin. At zero time the test substance(s), dissolved in the basic medium, was allowed to flow into the isolated pancreas by changing the medium reservoir, and effluent samples were collected every 30 sec for the next 7 min. During the following 23 min, samples were again taken every 1 min. Thus the period from −5 to 0 min represents the prestimulation period, and from 0 to +30 min, the stimulation period.

First the insulin-stimulatory effects of glucose (1 mg/ml) alone and together with *p*-chloromercuribenzoate (0.1 mM) were studied separately by perfusing these substances during the stimulation phase. Then the effects of methylene blue (2 μg/ml), diamide (0.1 mM), and *tert*-butyl hydroperoxide (2 mM) on the insulin secretion induced by *p*-chloromercuribenzoate plus glucose were studied separately by adding each substance both during and before perfusion with *p*-chloromercuribenzoate.

In order to observe any possible damage to β-cells produced by these metabolic inhibitors, separate experiments were carried out in which perfusion with inhibitor was performed only for 15 min before the 30-min perfusion with *p*-chloromercuribenzoate plus glucose; during the latter period, samples were collected at frequent intervals for the determination of insulin. If the inhibitor had caused permanent damage to the β-cells, no significant insulinotrophic effect of *p*-chloromercuribenzoate should have been observable.

Isolation and incubation of pancreatic islets. Islets were isolated by a version of the collagenase procedure (16). Batches of five islets were incubated in 1 ml of medium containing 2% bovine serum albumin and 3 mg/ml of glucose in Krebs-Ringer-bicarbonate buffer, pH 7.4, with and without methylene blue (2 μg/ml), diamide (0.1 mM), or *tert*-butyl hydroperoxide (2 mM). Incubation of islets was carried out at 37° for 90 min under continuous gassing with 95% O_2–5% CO_2, after which samples were collected for the determination of insulin.

Assay of insulin. Insulin concentrations in the effluent from perfused pancreases and that released into the medium by isolated islets were measured in duplicate by a double antibody radioimmunoassay (17), using the insulin reagent kit already described. Rat insulin was used as standard, and the results for the perfused pancreas were expressed as immunoreactive insulin per milliliter. Insulin released by the isolated islets was expressed as microunits of immunoreactive insulin per milliliter (five islets) per 90-min incubation period. Results are given as means ± standard errors; Student's *t*-test was used for statistical analysis.

RESULTS

p-Chloromercuribenzoate-Induced Insulin Secretion by Perfused Pancreas

Glucose alone at a concentration of 1 mg/ml produced no significant effect on the release of insulin (Fig. 1A), but *p*-chloromercuribenzoate (0.1 mM) in the presence of 1 mg/ml of glucose caused a biphasic secretory response (Fig. 1B). The addition of methylene blue (2 μg/ml) to the medium both before and during perfusion with *p*-chloromercuribenzoate (0.1 mM) significantly suppressed the insulin-releasing effect of the latter (Fig. 1C). This apparently specific effect of methylene blue did not involve damage to the β-cells, since insulin secretion resumed (Fig. 1D) when perfusion with the inhibitor was stopped after 15 min and medium contained only *p*-chloromercuribenzoate and glucose (1 mg/ml). At 0.1 mM, diamide significantly inhibited *p*-chloromercuribenzoate-induced insulin release, and at 1 mM, completely abolished it (data not shown). Any permanent damage by diamide also would seem unlikely, because after the 15-min perfusion with this inhibitor was stopped, *p*-chloromercuribenzoate still caused a significant insulin-stimulatory response (compare Fig. 1E and F).

Similarly *tert*-butyl hydroperoxide (2 mM) significantly diminished the insulin-releasing effect of *p*-chloromercuribenzoate. The discontinuation of *tert*-butyl hy-

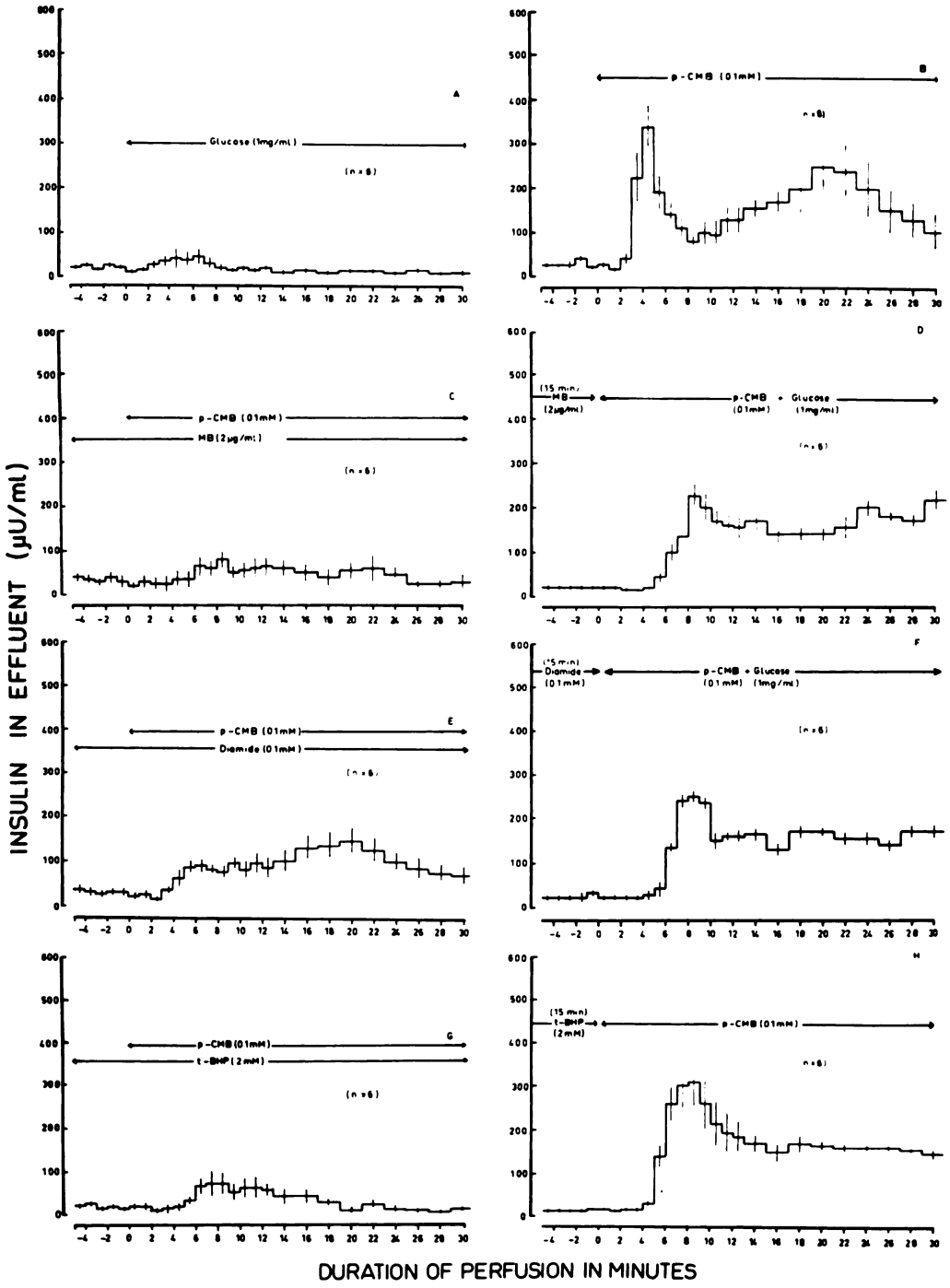

FIG. 1. *Effects of methylene blue (MB), diamide, and tert-butyl hydroperoxide (t-BHP) on p-chloromercuribenzoate-induced insulin release from isolated, perfused rat pancreas*

All perfusions were carried out for 35 min. After the 15-min "equilibration" period, samples were collected for insulin determination at 1-min intervals for 5 min (−5–0 min). Then glucose or *p*-chloromercuribenzoate (p-CMB) was added to the purfusion medium for 30 min. Samples were taken every 30 sec during the first 7 min, and at 1-min intervals for the remaining 23 min. A and B. No inhibitor. C, E, and G. Inhibitor was added both before and during perfusion with *p*-chloromercuribenzoate. D, F, and H. Inhibitor was added only during the 15 min before *p*-chloromercuribenzoate. The number of experiments (*n*) and means ± standard errors are shown for each point.

TABLE 1

Effects of methylene blue, diamide, and tert-butyl hydroperoxide on glucose-induced insulin release by isolated rat pancreatic islets

Values are means ± standard errors of the numbers of experiments shown in parentheses.

Medium glucose concentration	Insulin released			
	No addition (controls)	Methylene blue (2 μg/ml)	Diamide (0.1 mM)	*tert*-Butyl hydroperoxide (2 mM)
mg/ml	*μunits IRI[a]/ml (5 islets)/90 min*			
0	42 ± 7[b] (8)	28 ± 4 (8)	92 ± 16 (12)	69 ± 10 (10)
3	693 ± 33 (16)	325 ± 42[b] (8)	502 ± 64[b] (16)	177 ± 32[b] (8)

[a] Immunoreactive insulin.

[b] $p < 0.001$ compared with the control value obtained with 3 mg/ml of glucose.

droperoxide perfusion after 15 min again allowed *p*-chloromercuribenzoate to trigger insulin release, showing that the inhibitor had not caused any permanent injury to the β-cells (compare Fig. 1G and H). The observation that *p*-chloromercuribenzoate did not stimulate the first phase of insulin release as strongly or as rapidly in pancreases that had first been perfused with any of the inhibitors may have resulted from the experimental design. Since *p*-chloromercuribenzoate was added after 15 min perfusion with inhibitor, it is possible that enough inhibitor remained in the pancreas to prevent full normalization of cellular metabolism, thus retarding the stimulatory effect. This is supported by the finding that there was no significant depression of the second phase of insulin release.

Glucose-Induced Insulin Secretion by Isolated Islets

In the absence of glucose, 42 ± 7 μunits of immunoreactive insulin per milliliter (five islets) were found in the medium at the end of the 90-min incubation period (Table 1). In the presence of 3 mg/ml of glucose, significant stimulation of insulin release occurred (42 ± 7 vs. 693 ± 33). However, when methylene blue (2 μg/ml) was present in the medium in addition to glucose, glucose-induced insulin release was significantly lower (693 ± 33 vs. 325 ± 42). Incubation of islets in the presence of glucose (3 mg/ml) and diamide (0.1 mM) also significantly depressed the glucose-induced insulin release (693 ± 33 vs. 502 ± 64). Similarly, *tert*-butyl hydroperoxide decreased the glucose-stimulated insulin output from islets (693 ± 33 vs. 177 ± 32).

DISCUSSION

A possible role of NADPH, a main product of the pentose phosphate shunt, in insulin release induced by glucose has recently been suggested by us on the basis of the observations that the insular NADPH:NADP ratio increases with glucose concentration[2] and that a decrease in this ratio, caused, for example, by methylene blue, leads to a diminished insulin-secretory response of the β-cell to glucose (18). We further observed that the decreases in NADPH levels in islets produced by 6-aminonicotinamide (3), addition of exogenous insulin *in vitro* (19), or fasting,[3] and as observed during old age (20), are also associated with a decrease in glucose-stimulated secretion of insulin. The reverse has likewise been found, since insulin release in response to leucine accompanied an increased NADPH:NADP ratio in pancreatic islets (21).

Whereas only little is known as to how (or whether) the glucose molecule itself acts as a signal for insulin release, *p*-chloromercuribenzoate, which possesses a poor capacity to penetrate the cell membrane, is thought to stimulate insulin secretion by reacting with free thiol groups in the β-cell membrane, forming a readily reversible mercaptide (8). This requires the availability of free thiol groups on the β-cell

[2] H. P. T. Ammon, E. Verspohl, and D. Wagner-Teschner, manuscript in preparation.

[3] H. P. T. Ammon and D. Wagner-Teschner, unpublished observations.

membrane. Since NADPH in the extramitochondrial cytoplasm has strong reducing power, it is natural to speculate on its role in the oxidation-reduction state of thiol groups, which is believed to be related to insulin release. Thus, by employing methylene blue (2 μg/ml), which has proved to be a safe and certain tool for selectively reducing insular NADPH content (3), we could determine whether the insulin-triggering effect of *p*-chloromercuribenzoate is dependent on the islet NADPH. Figure 1C shows that methylene blue significantly inhibited the insulinotrophic effect of *p*-chloromercuribenzoate, suggesting that the secretory response depends on insular NADPH levels.

It has been suggested that the membrane thiol groups are at least partly in equilibrium with intracellular GSH (13), and it has been speculated that they are kept reduced by the latter (22). The reduction of glutathione in turn is mediated by glutathione reductase, with NADPH as the specific H^+ donor (23). In view of our finding that the *p*-chloromercuribenzoate-induced insulin-secretory response depends on insular NADPH, it is conceivable, as we (1) and others (24) had earlier suspected, that NADPH may play an important role in pancreatic islets by keeping glutathione in the reduced state, because GSH is a potent reductant of protein disulfides. If this theory is true, the insulinotrophic effect of *p*-chloromercuribenzoate should also be inhibited by agents that are capable of directly lowering the GSH content in cells. For this purpose, we selected diamide, a compound known to lower intracellular GHS content in many tissues (22, 25, 26) by the following reaction:

$$(CH_3)_2NCON{=}NCON(CH_3)_2 + 2\ GSH \rightarrow (CH_3)_2NCONHNHCON(CH_3)_2 + GSSG$$

In contrast to *p*-chloromercuribenzoate, diamide can easily penetrate the membranes and oxidize GSH to its disulfide. In perfused pancreas in the presence of 0.1 mM diamide (Fig. 1E and F), both phases of *p*-chloromercuribenzoate-stimulated insulin secretion were significantly inhibited in a reversible manner. This was also observed for glucose, since, as is evident from Table 1, diamide inhibited the insulin-releasing effect of glucose, suggesting that GSH is involved in the insulin-releasing mechanism not only of *p*-chloromercuribenzoate but also of glucose. This conclusion, however, is not fully justified, since although diamide has been shown to be highly specific for the oxidation of GSH (25, 26), there is evidence that oxidation of NADPH also occurs (27). We therefore decided to include in our studies *tert*-butyl hydroperoxide, which has been shown to lower intracellular GSH (28, 29) by a mechanism entirely different from that of diamide, as is shown in the following equation:

$$\begin{array}{l} CH_3 \\ \quad\diagdown \\ CH_3{-}C{-}O{-}OH + 2\ GSH \xrightarrow[\text{peroxidase}]{\text{glutathione}} \\ \quad\diagup \\ CH_3 \end{array}$$

$$\begin{array}{l} CH_3 \\ \quad\diagdown \\ CH_3{-}C{-}OH + 2\ GSSG + H_2O \\ \quad\diagup \\ CH_3 \end{array}$$

Figure 1G shows that in the presence of *tert*-butyl hydroperoxide *p*-chloromercuribenzoate also failed to stimulate either phase of insulin release from the perfused pancreas. In addition, the secretory response of isolated islets to glucose was inhibited by the same substance (Table 1). Assuming that diamide and *tert*-butyl hydroperoxide decrease the GSH level in islets as they do in other tissues, these data further support our hypothesis that GSH is essential for the insulin-releasing effects of *p*-chloromercuribenzoate and glucose. However, since the use of metabolic inhibitors as tools does not always reveal full information concerning their specifity, and because so far no direct data are available to show the diamide and *tert*-butyl hydroperoxide really lower insular GSH levels, this hypothesis is quite speculative at present.

Nevertheless, it is our contention that GSH, by virtue of its general reducing power (12), may also serve to reduce those sulfhydryl groups in β-cell membranes which are believed to be related to *p*-

chloromercuribenzoate-induced insulin release. Glucose and thiol reagents have been suggested to affect insulin release by acting on sulfhydryl groups which are in some way related to the receptor sites in the β-cell membrane (11). Our data obtained with glucose (Table 1) seem to be consistent with this hypothesis. It is possible, however, that some other intracellular thiol groups, also concerned with the insulin secretion triggered by *p*-chloromercuribenzoate and glucose, are decreased in the presence of diamide and *tert*-butyl hydroperoxide. In addition, our data support the view that the oxidation-reduction equilibrium between sulfhydryl and disulfide bridges in the β-cell membrane is of more general significance for ion permeability (24).

The present studies support our earlier speculation (1), and it now seems reasonable to propose the following working hypothesis. Glucose metabolism via the pentose phosphate shunt is necessary to produce NADPH, which keeps oxidized glutathione or some other intracellular thiol groups in the reduced form. These perhaps are further required to keep the thiol groups in β-cell membranes, linked with insulin release, in the reduced form. This hypothesis would imply that the oxidation-reduction state of membrane thiol groups may be responsible for the sensitivity of the β-cells to a given signal for insulin secretion. In this connection, thiol groups have already been suggested to play a role in the function of receptors responsible for adrenergic stimulus recognition (30–32).

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