Inhibition of Mitochondrial Complex I May Account for IDDM Induced by Intoxication With the Rodenticide Vacor

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Human intoxication with the rodenticide Vacor [*N*-3-pyridylmethyl-*N'*-p-nitrophenyl urea or 1-(4-nitrophenyl)-3-(3-pyridylmethyl) urea] induces acute IDDM. We report here that Vacor specifically inhibits the NADH:ubiquinone reductase activity of complex I in mammalian mitochondria. The activity of other respiratory enzymes of mitochondria is unaffected by Vacor at concentrations that completely inhibit the redox and energetic function of complex I. Vacor inhibition of complex I activity quantitatively correlates with the inhibition of insulin release in insulinoma cells and pancreatic islets and is also consistent with the doses reported in cases of human poisoning. These results indicate that the toxic and diabetogenic action of Vacor primarily derives from the inhibition of mitochondrial respiration of NAD-linked substrates in the high-energy demanding cells of the pancreatic islets. This newly identified mechanism of the pathological effects resulting from Vacor intoxication could constitute a paradigm in which to understand environmental or metabolic causes of IDDM. *Diabetes* 45:1531–1534, 1996

The pathogenesis of IDDM involves both genetic and environmental factors (1,2). The role of environmental factors is underlined by the conditions of drug-induced diabetes, occurring in humans from intoxication with a small number of chemicals (2,3) and in laboratory animals from administration of toxins such as streptozotocin (4,5). Intoxication with the rodenticide Vacor is probably the best documented condition of environmentally induced IDDM in humans (3,6–8).

The active principle of the rodenticide Vacor is the urea derivative R-787 usually defined as *N*-3-pyridylmethyl-*N'*-p-nitrophenyl urea or PNU (6–8), even though the precise chemical denomination is 1-(4-nitrophenyl)-3-(3-pyridylmethyl) urea (Fig. 1). Vacor was commercialized in the 1970s as a single-dose rodenticide, but it was later withdrawn from the market because numerous clinical reports demonstrated its high toxicity to humans (7–9). Vacor intoxication was often lethal, and the survivors invariably presented with an acute condition of IDDM, frequently complicated by neurological disturbances of various types and severity (6–9). The diabetogenic action of Vacor derived from a specific destruction of the β-cells in the pancreatic islets (9) and required continuous treatment with insulin as in spontaneous IDDM (6–10). In some documented cases, this destruction of the insulin-producing cells was accompanied by serological reactivity toward islet cell antibodies (6), similar to the immunological response in autoimmune IDDM (1,11).

Although Vacor was commonly assumed to act as a nicotinamide antagonist (6–8,12), the precise biochemical effect underlying its diabetogenic action remains unexplained (2,3,6–8). The similarity of the active ingredient of Vacor with some mitochondrial inhibitors has led us to discover that it specifically inhibits the function of respiratory complex I at the concentrations inducing diabetes. We thus propose that direct inhibition of complex I in the pancreatic β-cells is primarily responsible for the diabetic conditions induced by Vacor intoxication.

RESEARCH DESIGN AND METHODS

Mitochondria and submitochondrial particles from beef and rat heart were prepared by established procedures (13–15). Coupled rat liver mitochondria were prepared and assayed polarographically with glutamate and other substrates as described (14) (see Fig. 3 legend for experimental details). The electron transport activities of complex I (NADH oxidase, NADH:ferricyanide reductase, and NADH:Q reductase) were determined at 30°C in both coupled and uncoupled (i.e., frozen and thawed) preparations (13–17). The electron transport activity of complex III (ubiquinol:cytochrome *c* reductase) was assayed using reduced decyl-Q (DBH2) (15) with either 0.02 mg/ml (biuret) of mitochondria or 0.004 mg/ml of submitochondrial particles treated with 0.2% deoxycholate. The electron transport activity of complex II [succinate:Q:dichlorophenolindophenol (DCIP) reductase] was assayed with either Q-*1* or DB as the substrate in submitochondrial particles treated with rotenone and antimycin (16). Oxonol VI was used for measuring the membrane potential generation in coupled submitochondrial particles (17).

The hamster insulinoma cell line HIT-T15 (18) was obtained from American Type Culture Collection. The cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (wt/vol) of horse serum and 2.5% (wt/vol) of fetal calf serum and kept at a density of 9.0×10^4 cells in 0.80-cm^2 Lab-Tak chambers. Various concentrations of Vacor were added in the same medium containing 0.1% DMSO and incubated with the cells for 22 h. Aliquots of 0.05–0.1 ml of the culture medium were then collected and assayed for insulin content using the ^{125}I RIA ImmuChem Insulin CT diagnostic kit (ICN Biomed, Sydney, Australia).

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DB, decyl-ubiquinone analog; DBH2, reduced decyl-ubiquinone; DCIP, dichlorophenolindophenol; DMEM, Dulbecco's modified Eagle's medium; I_{50}, concentration yielding 50% inhibition; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PNU, *N*-3-pyridylmethyl-*N'*-p-nitrophenyl urea (Vacor); Q, ubiquinone.

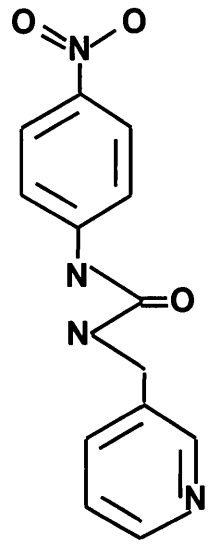

FIG. 1. Structure and chemical names of Vacor: 1-(4-nitrophenyl)-3-(3-pyridylmethyl) urea (Vacor) or *N*-3-pyridylmethyl-*N'*-p-nitrophenyl urea (R-787).

Vacor was obtained from Sigma-Aldrich (Milwaukee, WI) and dissolved in DMSO. To avoid solubility problems, Vacor was never used at final concentrations higher than 0.25 mmol/l in the assays, because nonspecific turbidity at 700 nm increased at concentrations larger than 0.3 mmol/l in the media, indicating aggregation of the compound. Q-*1* was a gift from Eisai (Tokyo, Japan). Methoxy-acrylate stilbene was kindly provided by Dr. P. Rich, Glynn Research Foundation, Bodmin, U.K.

RESULTS

The active principle of Vacor is constituted of a substituted urea group and a nitrophenyl group (Fig. 1). The presence of a urea group in both Vacor and streptozotocin has led to previous speculations of a possible common mechanism for the diabetogenic action of these two agents (6,8). However, various phenylurea derivatives are known to inhibit respiratory enzymes, for instance diuron [1,1-dimethyl-3(3,4-dichlorophenyl) urea] inhibits the ubiquinol:cytochrome *c* reductase activity in mitochondria (15,19). More recently, nitrophenyl compounds have been found to inhibit the electron transport activity of the respiratory complexes I, II, and III in submitochondrial particles (20). It was suggested earlier that Vacor may also interfere with mitochondrial respiration (12), but no study has yet been published regarding its effect on the respiratory enzymes.

We have found that Vacor inhibits completely the NADH:Q reductase activity of complex I but does not significantly affect the enzymatic activity of complex III in submitochondrial particles (Fig. 2*A*). Vacor inhibits with similar potency the electron transport activities of NADH oxidase and NADH:Q reductase with either Q-*1* or decyl-Q (DB) as the substrate (Fig. 2). The average I_{50} of Vacor inhibition of these complex I activities is 0.053 ± 0.008 mmol/l (in nine independent titrations) in different mitochondrial preparations. In contrast, the NADH:ferricyanide reductase activity of complex I, which does not involve electron transport to Q and is characteristically insensitive to the potent inhibitor rotenone (14), is not significantly affected by Vacor concentrations that abolish the activity of NADH:Q reductase (Fig. 2*B*). The activity of complex II is also insensitive to Vacor concentrations that inhibit complex I activity (Fig. 3). Moreover, the coupled respiration of NAD-linked substrates in intact mitochondria and the NADH oxidase activity in broken mitochondria are inhibited by Vacor with similar potency in preparations from either rat or beef (Fig. 3), thereby excluding a marked species specificity in the in vitro effects of the rodenticide.

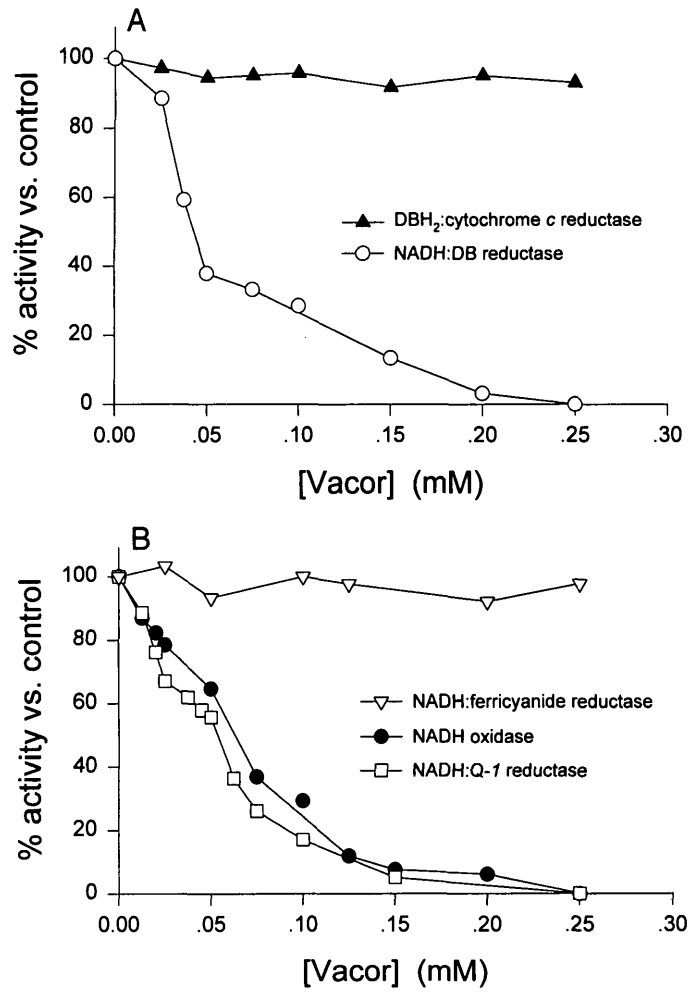

FIG. 2. Effect of Vacor on the activity of respiratory complexes. Vacor was added to the medium within its solubility limit and incubated 1–2 min with submitochondrial particles prepared from beef heart. *A*: the DBH_2 (20 µmol/l):cytochrome *c* (10 µmol/l) reductase activity was assayed with 0.004 mg/ml (biuret) of particles previously treated with 0.2% deoxycholate. The control activity was 4.5 µmol/min per mg of protein. The NADH (103 µmol/l):DB (30 µmol/l) reductase activity was assayed with 0.03 mg/ml of the same particle preparation treated with antimycin and methoxy-acrylate stilbene (13,17). The control activity was 0.3 µmol/min per mg of protein. *B*: complex I activities in another preparation of submitochondrial particles (0.03 mg/ml, with 103 µmol/l NADH in all cases). NADH oxidase was measured in the particles and assay medium without any inhibitors (13,14); the control activity was 0.62 µmol/min per mg of protein. The activities of NADH:Q-*1* (50 µmol/l) reductase and NADH:ferricyanide (1 mmol/l) reductase were assayed in the presence of potassium cyanide (KCN) and with particles treated with antimycin and methoxy-acrylate stilbene (17). A further treatment with 2 nmol of rotenone per mg of protein was used in the ferricyanide reductase assay (14,17).

The data in Fig. 3 also show that Vacor has a comparable potency in mitochondria and submitochondrial particles, which indicates that the hydrophobicity of the

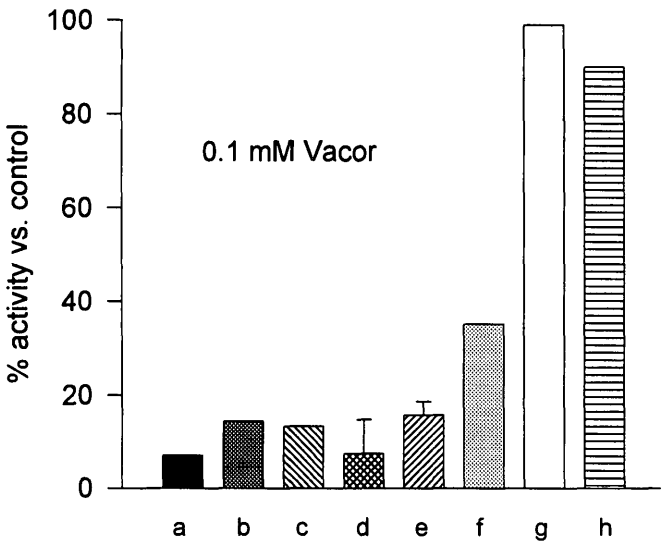

FIG. 3. Comparison of the inhibitory effects of 0.1 mmol/l Vacor on various activities. The experimental conditions for the measurements of insulin release in insulinoma cells were similar to those described in cultured pancreatic islets by Wilson and Gaines (26). The cells were incubated with Vacor dissolved in DMSO for 22 h, and the amount of insulin in the medium was determined by radioimmunoassays. The control sample had an insulin concentration of 360 pmol/l (60 μIU/ml). The assays of the mitochondrial electron transport activities were performed as described in METHODS (cf. Fig. 3 legend). The data in column c (average of triplicates) were obtained in frozen-thawed rat heart mitochondria respiring NADH. The data in columns d and e (means of five separate experiments with standard deviation) were obtained in coupled submitochondrial particles. The data in column f (average of triplicate) were obtained as polarographic measurements of state-3 respiration in coupled rat liver mitochondria with 12 mmol/l glutamate and 4 mmol/l malate as NAD-linked substrates. The state-3 activity of oxygen uptake was induced by 2 mmol/l ADP and usually was sixfold faster than in the absence of ADP. a, insulin release from rat islets (26); b, insulin release from hamster insulinoma HIT-T15 cells; c, NADH oxidase (broken rat heart mitochondria); d, NADH:DB reductase (average, submitochondrial particles); e, NADH oxidase (average, submitochondrial particles); f, glutamate respiration (coupled rat liver mitochondria); g, succinate:Q-*1*:DCIP reductase (submitochondrial particles); h, superoxide dismutase activity (erythrocytes) (27).

inhibitor enables effective incorporation into the mitochondrial membrane regardless of its sidedness. The rapid incorporation of Vacor is also indicated by the fact that its inhibition of complex I activity does not depend on the time of incubation with the mitochondrial preparations (at least up to 30 min). The inhibition properties suggest that Vacor behaves similarly to the complex I inhibitors capsaicin, meperidine (Demerol) (21,22), and uncharged pyridine analogs (23–25). However, the mode of action of Vacor is clearly different from the pyridinium derivatives of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which are more potent in intact mitochondria than in submitochondrial particles and show a marked time dependence in their inhibition (23–25).

To understand whether complex I inhibition could be responsible for the diabetogenic action of Vacor, we have established a quantitative correlation between the action of Vacor in mitochondria and its cytotoxic effects in the pancreatic islet cells. For instance, Vacor at 0.1 mmol/l inhibits by over 80% both the insulin release in insulinoma cells and the activities of complex I in mitochondria (Fig. 3). Moreover, Vacor potently inhibits insulin release in insulinoma cell lines with half-maximal effect at an external concentration of 0.023 mmol/l (Fig. 3, results not shown). This value is lower than the average I_{50} for the electron transport activity of complex I in mitochondrial preparations, but similar to the half-maximal effect on the membrane potential generation of complex I in submitochondrial particles (results not shown). Vacor inhibition of insulinoma cells also matches that previously reported in rat islets (cf. 26).

DISCUSSION

This work demonstrates that the rodenticide Vacor is a specific inhibitor of complex I of the mitochondrial respiratory chain (Fig. 2) at the concentrations that produce an effective inhibition of insulin release (Fig. 3). Thus, inhibition of complex I is identified as the likely biochemical mechanism for the diabetogenic action of Vacor in human subjects. The site of Vacor inhibition is at the level of ubiquinone reduction by complex I, near the site of action of rotenone and of the neurotoxic metabolite of MPTP, which induces parkinsonism (23–25). Our unpublished data suggest that the mode of inhibition apparently resembles that of the psychotic drug meperidine (Demerol) more than that of rotenone. Further studies are required to substantiate this possibility.

Previous reports considered that Vacor interferes with nicotinamide metabolism (6,8,12), or damages DNA structure (8), or alternatively inhibits the activity of superoxide dismutase (27). Figure 3 compares the effects of Vacor at 0.1 mmol/l on various activities of animal tissues. Clearly, only the inhibition of respiratory complex I correlates well with the toxic effect on the release of insulin in islet β-cells (26) (Fig. 3). It is worth noting that the average I_{50} of ~0.05 mmol/l for complex I inhibition relates well also with the LD_{50} of Vacor in humans, which has been deduced to be about 5 mg/kg of body weight, corresponding to a plasma level of around 0.2 mmol/l (8).

Hence, we suggest that the specific destruction of the islet β-cells caused by Vacor intoxication (6,9) primarily derives from inhibition of complex I function in pancreatic islets. The β-cells of pancreatic islets resemble neuronal cells in many respects (28–30), and in particular, they are extremely dependent on mitochondrial oxidative phosphorylation (31). The diabetogenic and neurological conditions caused by Vacor intoxication (6–8) could reflect the fact that complex I function is especially critical in the insulin-secreting β-cells and some neurons of the central nervous system (31–33). Other alternatives are that pancreatic β-cells possess crucial metabolic pathways that are particularly susceptible to the effects of Vacor on mitochondrial function (M.D.E., I.R. Mackay, unpublished observations) or that Vacor, by inhibiting complex I, might enhance the formation of radicals contributing to its cytotoxic effects.

Previous connections between mitochondrial function and diabetes derived from animal models in which the diabetic conditions are induced by treatment with streptozotocin, which damages the mitochondria of islet β-cells by decreasing their content of mtDNA (34,35), and from the association of familial cases of diabetes to mutations in mtDNA (33,35). The present finding that Vacor, which is highly diabetogenic to humans, specifically acts as an

inhibitor of mitochondrial respiration adds further weight to the possibility that mitochondrial malfunction may be a common cause in initiating or precipitating the pathological conditions of diabetes. In this light, Vacor inhibition of complex I may represent a biochemical paradigm for studying the metabolic induction of IDDM, and a potential parallel to the neurotoxic action of the pyridinium metabolite of MPTP (23,25), which constitutes a model for the pathogenesis of Parkinson's disease (36).

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