Determinants of the selective toxicity of alloxan to the pancreatic B cell

(glutathione peroxidase/*tert*-butyl hydroperoxide)

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ABSTRACT   The diabetogenic agent alloxan exerts a prefer-ential cytotoxic effect on the pancreatic B cell. The determinants of such a tissue specificity were investigated. Alloxan accumulated rapidly in liver and pancreatic islets but much more slowly in muscle. The activity of glutathione peroxidase and the resistance to exogenous peroxide were ≈20 times higher in liver and kidney than in islets, intermediate values being found in exocrine pan-creas and muscle. These findings suggest that the selective cyto-toxicity of alloxan to the pancreatic B cell is attributable to the conjunction of two features: a rapid cellular uptake of the drug and an exquisite sensitivity of the B cell to peroxide.

Alloxan has been known for about four decades to induce dia-betes mellitus in animals by causing a rather selective necrosis of insulin-producing cells (1, 2). There is evidence that the gen-eration of hydrogen peroxide, superoxide anion radicals, and hydroxyl radicals plays a critical role in the cytotoxicity of alloxan (3–6). However, there was so far no biochemical explanation for the selectivity of this cytotoxic action toward the pancreatic B cell. We have investigated the determinants of such selec-tivity from a double standpoint. First, we have explored whether different tissues vary from one another by their ca-pacity to accumulate alloxan. Second, we have assessed their sensitivity to peroxide radicals by treating them with various concentrations of *tert*-butyl hydroperoxide (*tert*-BuOOH).

MATERIALS AND METHODS

Yeast glutathione reductase (EC 1.6.4.2) was purchased from Boehringer (Mannheim, Federal Republic of Germany), alloxan was from Sigma, *tert*-BuOOH was from Aldrich-Europe (Beerse, Belgium), [^{14}C]urea and [6,6'(n)-^{3}H]sucrose were from the Ra-diochemical Centre (Amersham, England), D-[U-^{14}C]glucose was from New England Nuclear (Boston, MA), and [2-^{14}C]alloxan was from California Bionuclear (Sun Valley, CA). The latter preparation contained 9.2% alloxanic acid, as judged by TLC (7).

All experiments were carried out with tissues removed from fed albino rats. The incubation medium was a bicarbonate buf-fered solution (139 mM Na$^+$/5 mM K$^+$/1 mM Ca^{2+}/1 mM Mg^{2+}/24 mM HCO$_3^-$/124 mM Cl$^-$) containing bovine albumin (5 mg/ml) and equilibrated against 95% O$_2$/5% CO$_2$ (vol/vol). The methods used to measure reduced and oxidized glutathione (GSH and GSSG, respectively) (8), glucose oxidation (9), and insulin release (10) in isolated islets have been described. The oxidation of D-[U-^{14}C]glucose by single hemidiaphragms (149.0 ± 3.3 mg of wet weight; n = 80), by groups of four pieces each of pancreatic tissue (26.8 ± 0.8 mg; n = 108), and by single slices of kidney (25.3 ± 1.3 mg; n = 60), or liver (38.2 ± 1.8 mg; n

= 80) was measured by a method similar to that used for the islets, except for the higher volume of incubation medium (0.5–2.0 ml).

The tissue uptake of [2-^{14}C]alloxan (0.6 mM), D-[U-^{14}C]glu-cose (0.6 or 11.1 mM), and [^{14}C]urea (1.0 mM) was measured over 5 min of incubation at 23°C in media containing [6,6'(n)-^{3}H]sucrose (0.6 mM) after previous incubation of the tissues for 30 min at 23°C in a glucose-free nonradioactive medium con-taining sucrose (0.6 mM). After incubation, the islets (incubated in groups of 30 islets each in 0.1 ml of medium) were separated from the incubation medium by centrifugating the latter through a layer of di-*n*-butyl phthalate (11), whereas liver slices (10.1 ± 0.5 mg; n = 48) or hemidiaphragms (155.8 ± 8.9 mg; n = 19) were separated from the medium on a Millipore filter. The radioactivity of the tissue samples was determined by liquid scintillation assay in Instafluor II (Packard, Downers Grove, IL.) or Lumagel (Lumac, Basel, Switzerland), the liver and muscle samples being first digested overnight in hyamine hy-droxide (Packard). The radioactivity of the incubation medium was examined under identical conditions (i.e., in the presence of suitable amounts of nonradioactive tissue). The distribution space of [^{3}H]sucrose averaged 3.39 ± 0.29 nl per islet (n = 24), 601 ± 33 nl/mg of wet weight in liver slices (n = 48), and 280 ± 15 nl/mg of wet weight in hemidiaphragms (n = 19). The apparent space of distribution of the ^{14}C-labeled compound was always corrected for the [^{3}H]sucrose space measured in the same sample.

For measuring GSH peroxidase activity (EC 1.11.1.9), tissue samples (1000 islets, 5 mg of diaphragm, 1.5 mg of pancreas, or 0.5 mg of kidney or liver) were sonicated (twice, 5 sec each; islets, liver) or homogenized by hand (diaphragm, pancreas, kidney) in 1.0 ml of the usual incubation medium (except for the absence of albumin). After centrifugation (15 min, 4°C, 800 × g), samples of the homogenate were brought to a final volume of 0.1 ml in (final concentrations) 250 mM Na$_2$HPO$_4$/HCl buf-fer, pH 7.0/4 mM EDTA/4 mM nicotinamide/2 mM Na$_3$N/3 mM GSH/0.75 mM NADPH/containing GSSG reductase at 5 μg/ml and 0.5 mM *tert*-BuOOH, which was added to initiate the reaction. After 15 min of incubation at room temperature, the reaction was halted by addition of 0.3 ml of 0.3 M HCl. The NADP$^+$ formed was converted to a fluorescent product by in-cubation for 10 min at 60°C with 1.0 ml of 6 M NaOH. NADP$^+$ (2.5–25 nmol) and GSSG (2.5–10 nmol) standards were treated in exactly the same manner throughout the procedure and yielded linear dose-related responses. Neither *tert*-BuOOH nor the islet homogenate (in the absence of *tert*-BuOOH) affected the readings obtained with these standards. The readings ob-tained with the tissue samples were corrected for both the blank value found in the absence of tissue and *tert*-BuOOH and the

Abbreviations: *tert*-BuOOH, *tert*-butyl hydroperoxide; GSSG and GSH, oxidized glutathione and reduced glutathione, respectively.

Table 1. Effect of alloxan and *tert*-BuOOH on pancreatic islets

	GSH, pmol per islet	GSH/GSSG	D-[U-^{14}C]glucose oxidation, (pmol/60 min) per islet	Insulin release, (microunits/ 60 min) per islet
		Experiment 1		
Control	52.1 ± 4.9 (5)	32.1 ± 1.2 (5)	19.6 ± 1.3 (12)	166.5 ± 12.6 (10)
Alloxan	34.1 ± 5.3 (5)*	18.6 ± 2.9 (5)†	8.0 ± 0.7 (11)‡	26.6 ± 7.2 (10)‡
		Experiment 2		
Control	58.2 ± 4.3 (5)	29.5 ± 4.9 (4)	28.7 ± 1.2 (34)	179.8 ± 8.9 (10)
tert-BuOOH	36.6 ± 3.6 (5)†	13.8 ± 1.6 (5)§	2.1 ± 0.5 (25)‡	59.9 ± 7.3 (10)‡

In experiment 1, islets were incubated at 4°C for 30 min in the absence of glucose with or without 10 mM alloxan and then at 37°C for 30 min (for GSH assay) or for 60 min in the presence of glucose (11.1 mM). In experiment 2, islets were incubated at 37°C for 30 min in the presence of 11.1 mM glucose with or without 2 mM *tert*-BuOOH and then further incubated under the same conditions for 30 min (for GSH assay) or for 60 min. Results represent mean ± SEM; values in parentheses represent n.
* $P < 0.05$.
† $P < 0.005$.
‡ $P < 0.001$.
§ $P < 0.02$.

increases in fluorescence due to the same tissue homogenate (in the absence of *tert*-BuOOH) and to *tert*-BuOOH (in the absence of homogenate). The reaction velocity was expressed as (μmol/60 min)/mg of protein, measured using bovine albumin as the standard (12). With all tissues, the reaction velocity was proportional to the volume of homogenate used in the assay. Each individual result represents the mean of determinations made with three different volumes of homogenate (10, 20, and 40 μl), and each determination was carried out in triplicate.

A series of experiments was carried out with dispersed islet cells (15,000–22,500 cells per sample) separated into B-cell and non-B-cell populations (13). The method used to measure GSH peroxidase activity in these populations was similar to that used for whole islets.

All results are presented as mean ± SEM.

RESULTS

Alloxan decreased the GSH content and inhibited both glucose oxidation and glucose-stimulated insulin release in pancreatic islets (Table 1). These effects were mimicked by *tert*-BuOOH (Table 1). During 90 min of incubation at 37°C, H_2O_2 (0.2 mM) decreased insulin release evoked by glucose (16.7 mM) from the control value of 287 ± 29 to 123 ± 24 (microunits/90 min) per islet ($n = 8$ for both groups; $P<0.001$).

As shown in Table 2, when islets were incubated for 5 min at 23°C, the distribution space of [2-^{14}C]alloxan (0.6 mM), in excess of extracellular space, was not significantly different ($P>0.4$) from that of D-[U-^{14}C]glucose (11.1 mM), which is so rapidly transported in B cells as to ensure almost instantaneous equilibration of the intracellular and extracellular concentra-

tions of free glucose (14, 15). In liver slices, the distribution space of [2-^{14}C]alloxan largely exceeded ($P<0.001$) the intracellular space (Table 2). During 5 min of incubation at 23°C, the uptake of [2-^{14}C]alloxan (0.6 mM) by liver (259 ± 18 pmol/mg of wet weight; $n = 10$) was ≈10 times higher than that of D-[U-^{14}C]glucose (29 ± 4 pmol/mg of wet weight; $n = 10$) at the same concentration. This situation was not attributable to contamination of [2-^{14}C]alloxan by radioactive alloxanic acid. Indeed, when [2-^{14}C]alloxan (0.6 mM) was converted to alloxanic acid by heating at 37°C for 120 min (7), it yielded a distribution space (in excess of extracellular fluid) no larger than 31 ± 5 nl/mg of wet weight ($n = 9$) [i.e., ≈7% of the value found over the same length of incubation (5 min) and at the same temperature (23°C) with unmodified [2-^{14}C]alloxan]. Thus, alloxanic acid penetrates as poorly in liver as in islet cells (7). In muscle, the distribution space of [2-^{14}C]alloxan was ≈1/10 that in liver and 1/5 the intracellular space (Table 2). The rate of [2-^{14}C]alloxan uptake by hemidiaphragms [25.0 ± 2.2 (pmol/5 min)/mg of wet weight; $n = 7$] was somewhat lower than the rate of D-[U-^{14}C]glucose uptake [47.3 ± 5.6 (pmol/5 min)/mg of wet weight; $n = 6$], both measurements being made at 23°C and at the same concentration (0.6 mM) of either alloxan or glucose.

When *tert*-BuOOH was used as the substrate, the activity of GSH peroxidase was much higher in liver and kidney than in exocrine pancreas and muscle and lowest in pancreatic islets (Table 2). The activity of GSH peroxidase [(pmol/60 min) per cell] was comparable in the B-cell (0.21 ± 0.02; $n = 3$) and non-B-cell (0.17 ± 0.05; $n = 3$) populations. To assess the functional significance of the differences in GSH peroxidase activity, we examined the influence of *tert*-BuOOH on glucose oxidation. We selected *tert*-BuOOH because its intracellular access, in

Table 2. Uptake of alloxan, activity of GSH peroxidase, and sensitivity to *tert*-BuOOH in different tissues

Tissue	[2-^{14}C]alloxan distribution space*	Intracellular space*†	GSH peroxidase activity, (μmol/60 min)/ mg of protein	*tert*-BuOOH (minimal effective dose), mM
Islets	801 ± 124 pl per islet (12)	924 ± 91 pl per islet (12)	1.0 ± 0.3 (3)	0.2
Muscle	42 ± 4 nl/mg‡ (7)	215 ± 44 nl/mg‡ (6)	4.0 ± 0.8 (4)	0.5
Pancreas	ND	ND	5.1 ± 0.6 (4)	2.0
Kidney	ND	ND	19.1 ± 2.2 (4)	5.0
Liver	432 ± 30 nl/mg† (10)	231 ± 33 nl/mg† (10)	28.2 ± 3.1 (4)	5.0

Results represent mean ± SEM; values in parentheses represent n. ND, Not determined.
* Corrected for [^{3}H]sucrose space.
† Distribution space of either D-[U-^{14}C]glucose (islets) or [^{14}C]urea (muscle and liver).
‡ Results expressed per mg of wet weight.

contrast to that of alloxan, was unlikely to display any marked tissue specificity. Agents, such as *tert*-BuOOH, that affect the redox state may either increase or decrease D-[U-^{14}C]glucose oxidation. This dual effect does not depend solely on the balance between oxidation in the pentose shunt and the Krebs cycle (16); it has also been observed with nutrients such as L-[U-^{14}C]glutamine (data not shown). Therefore, the sensitivity to *tert*-BuOOH was judged from the changes in glucose oxidation, independently of whether they consisted of an increase or a decrease in $^{14}CO_2$ output. As little as 0.2 mM *tert*-BuOOH was sufficient to cause a 50% decrease in glucose oxidation by islets (Fig. 1). The concentration of *tert*-BuOOH had to reach 0.5 and 2.0 mM, respectively, to significantly affect glucose oxidation in muscle and exocrine pancreas. Kidney and liver were even more resistant to *tert*-BuOOH, requiring a concentration of 5.0 mM to alter glucose oxidation. There was a close correlation ($r = 0.942$; $P < 0.02$) between the activity of GSH peroxidase in each tissue and the lowest concentration of *tert*-BuOOH affecting significantly glucose oxidation in the same tissue (Table 2). There was also a significant correlation ($r = 0.947$; $P < 0.02$) between the relative magnitude (percent decrease) of the inhibitory action of *tert*-BuOOH on glucose oxidation by pancreatic islets and the concentration of glucose (2.8–27.8 mM) present in the preincubation and incubation media (data not shown). In other words, glucose protected the islet cells against the inhibitory action of *tert*-BuOOH in a dose-related fashion.

DISCUSSION

Our results show that alloxan decreases the islet GSH content and confirm that alloxan inhibits glucose oxidation (17) and glucose-stimulated insulin release (18) by pancreatic islets. These functional effects of alloxan are presumably relevant to the cytotoxic action of the drug. They were qualitatively reproduced by *tert*-BuOOH, a finding consistent with the view that the cytotoxicity of alloxan is related to the generation of peroxides (3–6).

Glucose, which protects islet cells against the inhibitory action of alloxan on both glucose oxidation and insulin release (17, 18) and increases the generation rate of reducing equivalents (16), also protected the islet cells against the inhibitory action of *tert*-BuOOH on glucose oxidation. The effect of alloxan on islet function was examined after prior incubation at 4°C in the absence of glucose to avoid both the inactivation of alloxan and the protective effect of glucose. In examining the sensitivity to *tert*-BuOOH, all tissues were first incubated at 37°C in the presence of glucose so that both incubations were carried out under identical conditions and, hence, the oxidation of glucose was measured in a close-to-steady state.

It is unlikely that the specific toxicity of alloxan to the B cell is accounted for solely by the cellular uptake of the drug (7, 19) because alloxan accumulates at least as rapidly and extensively in liver as in islets (20, 21). Nevertheless, the uptake of alloxan may be required for the cytotoxic effect. Thus, a low rate of alloxan uptake may explain why certain tissues other than islets are little affected by the diabetogenic agent. In this respect, there appear to exist definite analogies between glucose and alloxan transport. For instance, alloxan is slowly taken up by muscle, in which glucose transport is a rate-limiting step of its metabolism. Such is not the case in hepatocytes or B cells (14, 15, 22). These analogies should be considered in light of the structural similarity between alloxan and D-glucose, as judged by stereospecific criteria (23). They may also account for the resistance to alloxan of non-B-islet cells such as the glucagon-producing cell, in which glucose transport apparently represents a rate-limiting and insulin-sensitive process (24–26).

In search of a second factor conditioning the sensitivity to alloxan, we have measured in several tissues the activity of GSH peroxidase, an enzyme catalyzing the reduction of peroxides.
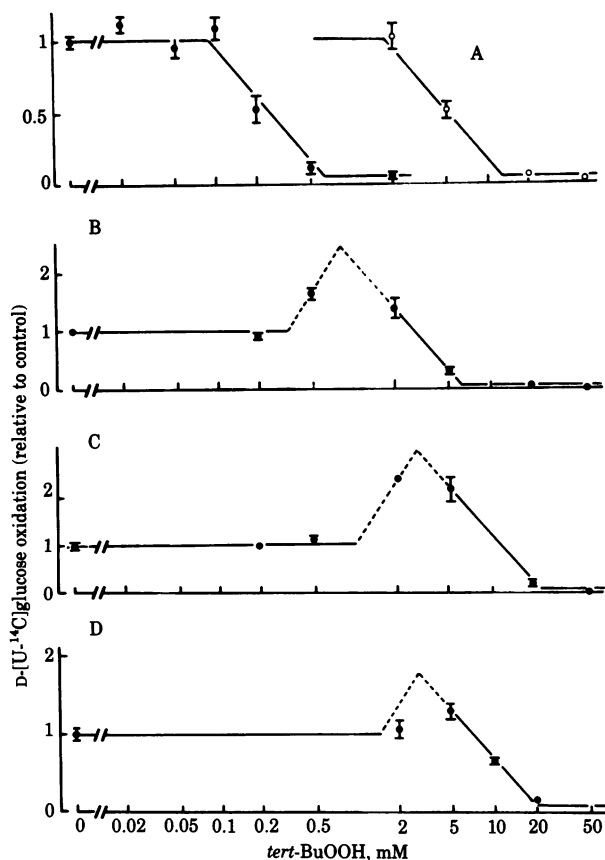

FIG. 1. Effect of various concentrations of *tert*-BuOOH (logarithmic scale) on rate of D-[U-^{14}C]glucose oxidation by pancreatic islets [A(●)], hemidiaphragms (B), pieces of pancreatic tissue (C), and slices of kidney (D) and liver [A (○)]. All measurements were carried after a 30-min incubation followed by a 60-min incubation with 11.1 mM glucose and *tert*-BuOOH (stated concentration) present in both incubation media. Rate of glucose oxidation is expressed relative to the mean control value for the same experiment(s) in the absence of *tert*-BuOOH. As measured over a 60-min incubation, these control values were 28.7 ± 1.2 pmol per islet, 0.85 ± 0.05 nmol/mg of muscle, 1.16 ± 0.10 nmol/mg of pancreas, 1.05 ± 0.06 nmol/mg of liver, and 5.88 ± 0.78 nmol/mg of kidney, using wet weight of tissue as reference ($n = 20$–48). Results are mean ± SEM for 7–25 measurements. The SEM is not shown whenever it did not exceed the size of the mean point. The scale of the ordinates was chosen in each case so that the descending segments of all curves would have identical slopes. The dotted segments of each line were then drawn by extrapolation. Maximal oxidation rates shown in B, C, and D correspond to the values that would be reached if, as in pancreatic islets and liver, a 7-fold increase in *tert*-BuOOH concentration were required to decrease glucose oxidation from its highest to its lowest value.

The activity was much less in islets than in other tissues, in good agreement with recent data obtained in our laboratory by a different technique (27). To assess the functional significance of the difference in peroxidase activity, we have examined the sensitivity of intact cells to *tert*-BuOOH, the substrate used in the measurement of peroxidase activity in tissue homogenates. There was a close correlation between these two series of determinations.

These findings suggest that the selective cytotoxicity of alloxan to the pancreatic B cell results from the coincidence of two features: a rapid cellular uptake of the drug, also seen in liver, and an exquisite sensitivity to peroxide, itself attributable to low peroxidase activity (GSH peroxidase, catalase). This interpretation is further supported by the observation that islets removed from guinea pigs, which are more resistant than rats to the diabetogenic action of alloxan (28), are ≈1/5 as sensitive as rat islets to *tert*-BuOOH (29).

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