Alloxan-induced Free Radical Production in Isolated Cells

Selective Effect on Islet Cells

KOHTARO ASAYAMA, FRITZ NYFELER, DENIS ENGLISH, SIMON J. PILKIS, AND IAN M. BURR

SUMMARY

Chemiluminescence, as a direct measure of oxygen free radical production, induced in isolated cells, hepatocytes, and red cells by the action of alloxan has been measured. The assay system used luminol, 3 μM, for signal amplification. The buffer used was Krebs-Ringer bicarbonate with 16 mM Hepes, pH 7.4. This buffer did not react with alloxan in the absence of cells. Some chemiluminescence was noted from all cells in the absence of alloxan. In the presence of alloxan, reactions occurred within seconds and islet cells were significantly more reactive to alloxan than either red cells or hepatocytes as defined by alloxan dose-response curves with fixed cell numbers or fixed surface areas. These data indicate a cell specificity for an early action of alloxan perhaps mediated at the cell membrane. DIABETES 1984; 33:1008–11.

A variety of agents with direct or indirect reducing activity have been demonstrated to protect against alloxan-induced β-cell damage.[1-3] More specifically, scavengers of oxygen free radicals also protect against alloxan toxicity.[4-8] These studies, together with the demonstrated ability of dialuric acid (a reduced form of alloxan) to produce oxygen free radicals on auto-oxidation[9,10] in cell-free systems, have provided indirect evidence that alloxan cytotoxicity could be mediated by free radical production.

Recent studies in our laboratory have provided direct confirmation of the ability of alloxan to produce free radicals in the presence of pancreatic islets[11] in a buffer system that by itself does not support such free radical production.

However, the reason for the high susceptibility of pancreatic β-cells to alloxan cytoxicity remains to be clarified. It seemed probable that alloxan would generate free radicals in all tissue cells and that direct demonstration of this effect in islet cells per se does not provide evidence for the selectivity or otherwise of the effect. In the present study, alloxan-induced luminol chemiluminescence was measured in different tissues of rat: dispersed islet cells, hepatocytes, and erythrocytes. If indeed dose-response curves of alloxan-induced chemiluminescence were similar in all tissues, the relative selectivity of alloxan for islet β-cells could not be considered to be due primarily to this particular effect of alloxan.

From the Departments of Pediatrics and Physiology, Vanderbilt University Medical Center, Nashville, Tennessee.
Address reprint requests to Ian M. Burr, M.D., Department of Pediatrics, Vanderbilt University Medical Center, Nashville, Tennessee 37232.
Received for publication 14 October 1983 and in revised form 22 February 1984.

MATERIALS AND METHODS

Alloxan monohydrate, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), ethyleneglycol-bis-(β-aminoethyl ether)-N,N′-tetraacetic acid (EGTA), and deoxyribonuclease I (925 U/mg) were obtained from Sigma Chemical Co. (St. Louis, Missouri). Collagenase CLS IV (170 U/mg, for the digestion of rat prancreas) and CLS I (for the digestion of rat liver) were products of Worthington Biochemicals (Freehold, New Jersey). Trypsin (10 ×, 2.5%) in modified Hanks' balanced salt solution was purchased from Flow Laboratories (McLean, Virginia). Other chemicals were reagent grade.

All dispersed cell preparations were isolated from male Sprague-Dawley rats (250–300 g) in the morning after an overnight fast, and cells were counted in a hemocytometer. All cell preparations were washed 3 times with Krebs-Ringer solution (pH 7.4) containing 20 mM Hepes [4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid], 5 mM D-glucose, and no calcium; cells were resuspended in the same buffer and kept at 37°C under 95% O_2/5% CO_2 until measurement.

Islets were obtained by a previously described modification of the method of Lacy and Kostianovsky.[12] The isolated islets (700–1500) were suspended in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 16 mM Hepes, 1 mM EGTA, 0.1% bovine serum albumin, 2.4 mM D-glucose, and no calcium. Islet disruption was performed at room temperature for 10 min by gentle aspiration using a Pasteur pipette

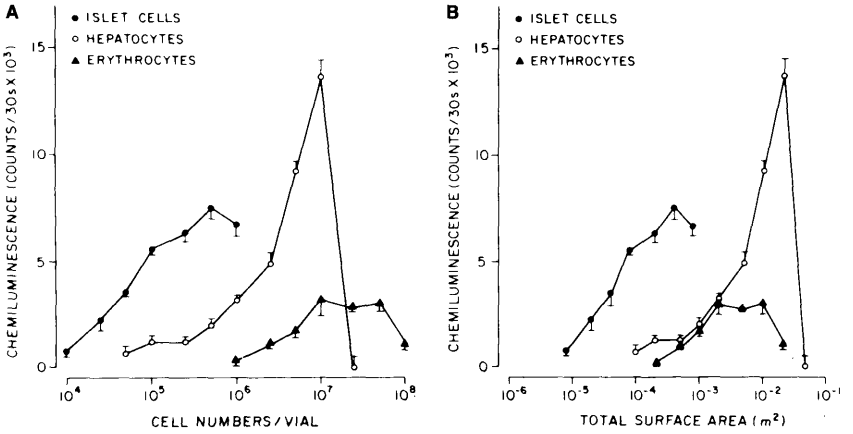

FIGURE 1. Alloxan-induced chemiluminescence for 3 cell types at various (A) concentrations of cells and (B) total cell surface area calculated from mean cell diameters. The data are the results of quadruplicate measurements for each cell type. The brackets indicate SEM.

in the presence of 1 mg/ml of trypsin and 10 μg/ml of deoxyribonuclease I. By this procedure, 2–6 × 10^6 cells were recovered as dispersed islet cells. As determined by trypan blue dye exclusion, more than 95% of the cells were viable when the cells were incubated in the same buffer at 37°C under 95% O_2/5% CO_2. Electron micrographic studies revealed intact organelles in the β-cells of this cell preparation. Hepatocytes were prepared by the method of Berry and Friend,[13] with modifications as described previously.[14] Hyaluronidase was omitted. The viability as determined by trypan blue exclusion was 90–95%. The leakage of lactate dehydrogenase during 60-min incubation was 2–5%. The hepatocytes prepared by this procedure were metabolically intact as indicated by their responsiveness to insulin[15] and glucagon.[14]

Rat erythrocytes were obtained from heparinized blood after removal of plasma and buffy coat by centrifugation.

The assay method for the alloxan-induced luminol chemiluminescence has been previously reported.[11] The buffer used was Krebs-Ringer bicarbonate buffer containing 16 mM Hepes (pH 7.4). Five-milliliter aliquots were sealed into airtight, glass counting vials immediately after preparation and then incubated in a waterbath (37°C) for 6 min before measurement of chemiluminescence. For each sample, the baseline luminescence from the cells in the presence of luminol was measured over the 30-s period from 20 to 50 s after placing the vial in the counting position of the liquid scintillation counter. The luminescence induced by alloxan was measured again over the 30-s period from 20 to 50 s after addition of alloxan. The baseline luminescence was subtracted from the stimulated value for each sample, and the data were expressed as counts/30 s.

The stock solution of alloxan was freshly prepared just before use, dissolved in cold saline, adjusted to pH 2 with hydrochloric acid, and kept on ice. Three series of experiments were performed on each cell type. In the first series, the alloxan concentration was kept constant at 100 μM and the cell numbers were varied. This series of experiments was performed to define the optimum conditions needed for defining the dose responses for each cell type. Increased density of cells could be anticipated to lead ultimately to quenching. This was observed with 10^6 islet cells, with 10^8 red cells, and 2.5 × 10^7 hepatocytes.

In the second series, the cell number was kept constant and the alloxan concentration was varied over the range of 50 μM to 1 mM.

In the third series of experiments, the number of cells used for each cell type was such that the cell surface area was kept constant from cell type to cell type (of a value approximating the optimal response observed in islet cells, as defined in the first two experimental series) and the alloxan concentration was varied from 50 μM to 2 mM. Cell surface areas were calculated from cell diameters defined by electron microscopy (courtesy of Dr. Mary Grey, Department of Pathology, Vanderbilt University Medical School). Diameters were 8 μm (rbc), 16 μm (islet cells), and 25 μm (hepatocytes).

RESULTS

Chemiluminescence at various concentrations of cells and a fixed concentration of alloxan. Figure 1A shows the alloxan-induced chemiluminescence in the presence of 3 μM of luminol and 100 μM of alloxan at various concentrations of cells for the 3 cell types. At these concentrations of alloxan and luminol, the minimum number of cells generating a significant chemiluminescence was 10^4 cells for islet cells, 5 × 10^4 cells for hepatocytes, and 10^6 cells for erythrocytes. The peak response values (mean ± SEM) were 7443 ± 524 counts/30 s (at 5 × 10^5 cells/vial for islet cells, 13770 ± 766 counts/30 s (at 10^7 cells/vial) for hepatocytes, and 2973 ± 465 counts/30 s (at 5 × 10^7 cells/vial) for erythrocytes. The mean responses of islet cells, for concentrations ranging from 5 × 10^4 to 10^6 cells/vial, were higher than those of hepatocytes at these cell concentrations, and of erythrocytes at all cell concentrations measured. (Direct comparison of responsiveness for each cell type at cell concentrations higher than 10^6 cells/vial was impossible because of the quenching observed with islet cells at these concentrations.)

When the results of this first series of experiments were recalculated according to surface area, the marked increase in responsiveness of islet cells remained evident (Figure 1B).

Chemiluminescence at various concentrations of alloxan and a fixed concentration of cells. The alloxan-induced luminescence in the presence of 2.5 × 10^5 cells/vial for each cell type, at alloxan concentrations from 50 μM to 1 mM, are shown in Figure 2.

As can be seen in Figure 2, the dose response to alloxan for islet cells was well to the left of the curves for hepatocytes

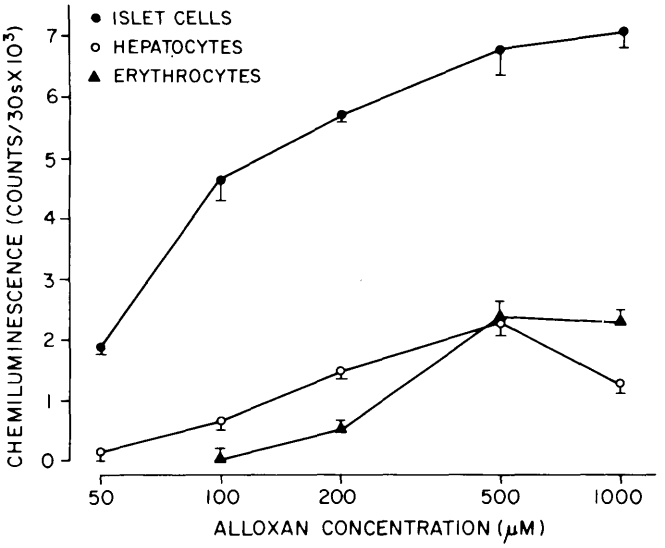

FIGURE 2. The dose-response curve of chemiluminescence in response to alloxan in the presence of 2.5 × 10^5 cells/vial for each cell type. The data are the results of triplicate (for islet cells) or quadruplicate (for hepatocytes and erythrocytes) measurements. The brackets indicate SEM.

and erythrocytes. At all concentrations, islet cell responsiveness was over double that observed with the other cell types. The maximum response was obtained at 500 μM of alloxan for erythrocytes and hepatocytes, and at 1 mM for islet cells.

Chemiluminescence at various concentrations of alloxan and a fixed surface area of cells. The alloxan-induced luminescence in the presence of 2 × 10^{-4} m^2 cell surface area/vial for each cell type, at alloxan concentrations from 50 μM to 2 mM, are shown in Figure 3. At all concentrations of alloxan, the dose-response curve for islet cells was again well to the left of the curves for the other cell types. Again, at any given alloxan concentration, chemiluminescence from islet cells exceeded that of the other cells by at least twofold. Erythrocyte and hepatocyte responses were similar. The individual peak responses were observed at 1 mM for islet cells and erythrocytes, and at 500 μM for hepatocytes.

DISCUSSION

The observation that alloxan could induce free radical production in a controlled system in the presence, but not in the absence, of islets[11] provided direct support for the hypothesis that alloxan cytotoxicity is induced, at least in part, by free radical production. However, these data raised questions regarding the tissue specificity of the effect. The present study was undertaken to provide some answer to this question. Indeed, this study has demonstrated that, in the presence of constant alloxan concentrations, the chemiluminescence response obtained is dependent on the number of cells and that curves plotting cell number against response indicate that islet cells are more sensitive to this effect of alloxan than either hepatocytes or red cells. Furthermore, the dose-response curve of chemiluminescence in response to alloxan induced in islet cells was well to the left of the dose-response curves for hepatocytes and red cells when the cell number was kept constant, indicative of a marked increase in sensitivity.

However, it is possible that chemiluminescence could be related to surface area rather than to cell number. Therefore, the alloxan dose-response curves were recalculated against surface area from the data shown in Figure 1A. When this was performed, islet cells were much more responsive than were erythrocytes and hepatocytes at cell concentrations at which the chemiluminescence of islet cells was measurable. This prompted a third series of experiments in which cell surface area was kept constant and the dose response to alloxan was defined. In this study, it was again evident that, per unit surface area, islet cells were more responsive than either of the other cell types. However, the differences between hepatocytes and erythrocytes in response per unit surface area were minimal.

These data suggest a tissue selectivity for the acute, perhaps earliest, action of alloxan. That is, it indicates that islet cells accumulate more oxygen free radicals more rapidly than the other cell types on exposure to alloxan and that this occurs within seconds of addition of alloxan. It can, therefore, be concluded that either islet cells have higher affinity for alloxan, a more efficient free radical generating system, and/or a less effective scavenging system.

The possibility of an increase in "affinity" for alloxan could represent a more reactive membrane and/or a greater uptake of alloxan into the cell. Watkins et al.[16,17] have proposed that the action of alloxan is primarily dependent on interaction with membrane-linked thiol groups. The rapidity of action of alloxan in generating free radicals, within seconds as demonstrated in the present study, is certainly compatible with this concept. Less direct indices of an alloxan effect are also demonstrable within minutes of exposure, e.g., leakage of insulin,[18] depolarization,[19] decreased Rb uptake,[2] and, hence, tend to support a membrane effect. An alternate explanation, that selective alloxan uptake accounts for its selective effects on free oxygen radical production, appears unlikely as alloxan uptake by liver[20-22] appears to be equally

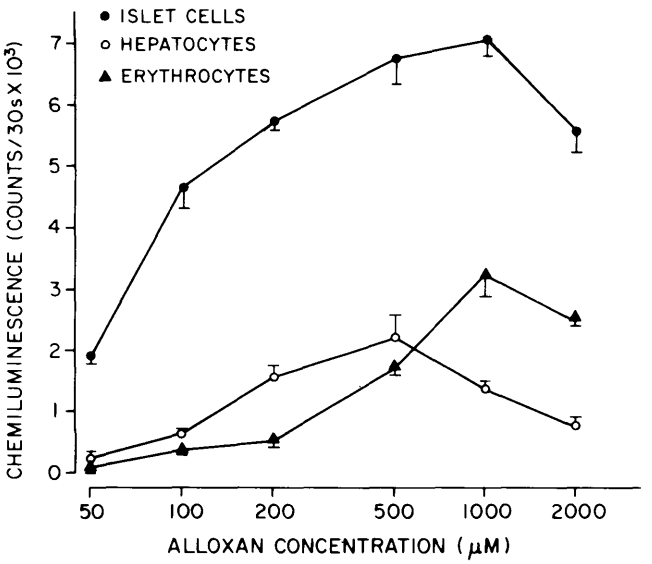

FIGURE 3. The dose-response curve of chemiluminescence in response to alloxan. The total surface area of each cell type was kept at 2 × 10^{-4} m^2. The data are the results of triplicate (for islet cells) or quadruplicate (for hepatocytes and erythrocytes) measurements. The brackets indicate SEM.

as rapid as its uptake by islets, whereas islets were more sensitive to alloxan-induced free radical production than were hepatocytes.

The possibility that the relatively selective effect of alloxan on islet cells is consequent upon relatively enhanced production of free radicals by islet cells has been suggested. Others have suggested that the relative selectivity could reside in differing degrees of ionization of membrane-bound thiol groups from cell type to cell type.[16,17] The alloxan data in this paper are compatible with these concepts.

The final possibility to account for the enhanced sensitivity of islet cells is that they may have a decreased ability to scavenge oxygen free radicals. This possibility is supported by the data indicating that superoxide dismutase (SOD) and catalase can inhibit alloxan-induced free radical production in islets[11] and that both agents can protect against alloxan-induced diabetes[6,8] and DNA strand breaks.[23] In support of this possibility, Malaisse has demonstrated decreased glutathione peroxidase activity in islets.[20] However, these data are indirect; furthermore, no significant difference in SOD activity has been observed between islets and other less susceptible tissues.[24]

In conclusion, rapid production of oxygen free radicals by alloxan can be demonstrated in a number of tissues under conditions in which the buffer used cannot itself support such free radical production. Islet cells are significantly more responsive to this effect of alloxan than are red cells or hepatocytes. The effect is observed in seconds and is compatible with a membrane-related phenomenon. Thus, there is significant tissue selectivity in an extremely early event induced by alloxan, perhaps related to differences between membrane constituents of the differing cell types. While prior indirect evidence strongly supported the hypothesis that the effect of alloxan was mediated, at least in part, through free radical generation, these data provide direct evidence for this assumption. Furthermore, these studies indicate that the relative selectivity of alloxan for islet cells could be related to this selectivity of net free radical production.

REFERENCES

[1] Rerup, C. C.: Drugs producing diabetes through damage of the insulin secreting cells. Pharmacol. Rev. 1970; 22:485–518.

[2] Idahl, L. A., Lernmark, A., Sehlin, J., and Täljedal, I. B.: Alloxan toxicity *in vitro*: inhibition of rubitium ion pumping in pancreatic B-cells. Biochem. J. 1977; 162:9–18.

[3] Slonim, A. E., Surber, M. L., Page, D. L., Sharp, R. A., and Burr, I. M.: Modification of chemically induced diabetes in rats by vitamin E. J. Clin. Invest. 1983; 71:1282–88.

[4] Heikkila, R. E., Winston, B., and Cohen, G.: Alloxan induced diabetes—evidence for hydroxyl radical as a cytotoxic intermediate. Biochem. Pharmacol. 1976; 25:1085–92.

[5] Heikkila, R. E., and Cabbat, F. S.: Protection against alloxan-induced diabetes in mice by hydroxyl radical scavenger dimethylurea. Eur. J. Pharmacol. 1978; 52:57–60.

[6] Grankvist, K., Marklund, S., Sehlin, J., and Täljedal, I. B.: Superoxide dismutase, catalase and scavengers of hydroxyl radical protect against the toxic action of alloxan on pancreatic islet cells *in vivo*. Biochem. J. 1979; 182:17–25.

[7] Fisher, L. J., and Hamburger, S. A.: Dimethylurea: a radical scavenger that protects isolated pancreatic islets from the effects of alloxan and dihydrooxyfumarate exposure. Life Sci. 1980; 26:1405–1409.

[8] Fisher, L. J., and Hamburger, S. A.: Inhibition of alloxan action in isolated pancreatic islets by superoxide dismutase, catalase and metal chelator. Diabetes 1980; 29:213–16.

[9] Cohen, G., and Heikkila, R. E.: The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid, and related cytotoxic agents. J. Biol. Chem. 1974; 249:2447–52.

[10] Grankvist, K.: Alloxan-induced luminol luminescence as a tool for investigating mechanism of radical-mediated diabetogenicity. Biochem. J. 1981; 200:685–90.

[11] Asayama, K., English, D., Slonim, A. E., and Burr, I. M.: Chemiluminescence as an index of drug-induced free radical production in pancreatic islets. Diabetes 1984; 33:160–63.

[12] Lacy, P. E., and Kostianovsky, M.: Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes 1967; 16:35–39.

[13] Berry, M. N., and Friend, D. S.: High-yield preparation of isolated rat liver parenchymal cells. J. Cell. Biol. 1969; 43:506–20.

[14] Claus, T. H., Pilkis, S. J., and Park, C. R.: Stimulation by glucagon of the incorporation of U-^{14}C labeled substrates into glucose by isolated hepatocytes from fed rats. Biochim. Biophys. Acta 1975; 404:110–23.

[15] Pilkis, S. J., Chrisman, T. D., El-Maghrabi, M. R., Colosia, A., Fox, E., Pilkis, J., and Claus, T. H.: The action of insulin on hepatic fructose 2,6-bis phosphate metabolism. J. Biol. Chem. 1983; 258:1495–1503.

[16] Watkins, D., and Cooperstein, S. J.: Effect of alloxan on islet tissue permeability: protection and reversal by dithiols. J. Pharmacol. Exp. Ther. 1976; 199:575–82.

[17] Watkins, D., Cooperstein, S. J., and Fiel, S.: Studies on the selectivity of alloxan for the B-cells of the islets of Langerhans: effect of pH on the *in vitro* action of alloxan. J. Pharmacol. Exp. Ther. 1979; 208:184–89.

[18] Gunnarsson, R., and Hellerström, C.: Acute effects of alloxan on the metabolism and insulin secretion of the pancreatic B-cells. Horm. Metab. Res. 1973; 5:404–409.

[19] Mathews, E. K., Dean, P. M., and Sakamoto, Y.: The biological activity of the islet cell membrane. *In* Handbook of Experimental Pharmacology 32, Part 2, Insulin II. Hasselblatt, A., and Bruchhansen, F., Eds. New York, Springer-Verlag, 1975:157–73.

[20] Malaisse, W. J., Malaisse-Lagae, F., Sener, A., and Pipeleers, D. G.: Determinants of the selective toxicity of alloxan to the pancreatic B-cell. Proc. Natl. Acad. Sci. USA 1982; 79:927–30.

[21] Landau, B. R., and Renold, A. E.: The distribution of alloxan in the rat. Diabetes 1954; 3:47–50.

[22] Janes, R. G., and Winnick, T.: Distribution of C^{14}-labeled alloxan in the tissues of rat and its mode of elimination. Proc. Soc. Exp. Biol. Med. 1953; 81:226–29.

[23] Uchigata, Y., Yamamoto, H., Kawamura, A., and Okamoto, H.: Protection by superoxide dismutase, catalase, and poly (ADP-ribose) synthetase inhibitors against alloxan- and streptozotocin-induced islet DNA strand breaks and against the inhibition of proinsulin synthesis. J. Biol. Chem. 1982; 257: 6084–88.

[24] Grankvist, K., Marklund, S. L., and Täljedal, I. B.: CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. Biochem. J. 1981; 199:393–98.