INFLUENCE OF TRACE METALS ON ALLOXAN CYTOTOXICITY IN PANCREATIC ISLETS

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1. Introduction

Alloxan injection into animals causes pancreatic β-cell necrosis, insulin deficiency and diabetes mellitus [1]. From protection experiments in vivo alloxan diabetogenicity was inferred [2] to be mediated by the sequenced generation of dialuric acid, superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$). In support of this hypothesis in vitro experiments with isolated pancreatic islets and islet cells showed that the cytotoxicity of alloxan is counteracted by superoxide dismutase, catalase, and non-enzymic scavangers of the hydroxyl radical [3]. The strong and indiscriminate reactivity of $OH^{\cdot}$ with cell constituents can explain why alloxan rapidly affects as diverse phenomena as insulin synthesis [4], insulin release [5], glucose oxidation and oxygen consumption [6], univalent cation pumping [7] and trypan blue exclusion [8,9] in mammalian islet cells, and the mannitol permeability [10] in fish islets.

The generation of $OH^{\cdot}$ from $O_2^{\cdot-}$ and H_2O_2 (Haber-Weiss reaction) in biological systems appears to require catalysis by traces of transitional metals [11–14] and may be seen as the summation of the reduction of ferric ion by superoxide ($Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2$) and the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow OH^{\cdot} + OH^- + Fe^{3+}$); the analogous reactions with Cu^+/Cu^{2+} as catalyst have also been proposed [15]. Therefore, to test further the free radical hypothesis of alloxan diabetogenicity we have investigated how metal ions and chelators influence the toxic action of alloxan on Rb^+ accumulation by isolated pancreatic islets. The effect of alloxan was found to be inhibited by diethylenetriaminepentaacetic acid (Detapac), a chelator with marked ability to suppress the metal-catalyzed Haber-Weiss reaction in biochemical systems [12–14]. In addition, exogenous Cu^{2+} was found to inhibit Rb^+ accumulation in a manner seemingly additive to that of alloxan.

2. Materials and methods

Male 9 month ob/ob-mice from the non-inbred Umeå colony were starved overnight. Pancreatic islets were isolated by free-hand microdissection without the use of collagenase [16]. Dissection was performed at 14°C with the pancreas immersed in a buffer of the same composition as Krebs-Ringer bicarbonate [17] except that the bicarbonate was replaced by 20 mM 4-(2-hydroxyethyl)-1-piperazinesulphonic acid (Hepes) (pH 7.4) and that the buffer was equilibrated with ambient air.

The validity of $^{86}Rb^+$ accumulation in ob/ob-mouse islets as a parameter for studying alloxan cytotoxicity in vitro has been described [3,7]. Batches of 3 islets were incubated in 1 ml non-radioactive medium for 40 min. Two batches were incubated with the substance to be tested, while two others were incubated without test substance. After 30 min we added 2 μl of a stock solution of 250 mM alloxan in 10 mM HCl to one batch within each pair. HCl (2 μl, 10 mM) alone was added to the other member of the pair. Incubation was continued for 10 min. Alloxan did not noticeably alter the pH of the incubation medium.

All batches of islets were then incubated in 200 μl of medium labelled with 20 μM ^{86}RbCl (267–570 Ci/mol) and 8 μM [6,6′-^{3}H]sucrose (2000 Ci/mol). The radioactive medium contained neither alloxan nor test substance. After 120 min the islets were transferred to small pieces of aluminium foil, freed of contaminating fluid with a micro-pipette and plunged into melting

isopentane. After freeze-drying overnight (−40°C, 0.1 Pa) they were weighed on a quartz fiber balance, dissolved in Hyamine and Instafluor and analyzed for ^{86}Rb and ^{3}H in a liquid-scintillation counter. The islet content of Rb^+ in excess of the extracellular sucrose space was calculated by using samples of incubation medium as external standards in the liquid-scintillation counting procedure.

To study whether alloxan reacted directly with test substances in the incubation medium, the decrease of alloxan-dependent light absorbance (A_{290}) was followed in Hepes-buffered medium to which had been added alloxan alone or alloxan in combination with test substance [3].

Alloxan monohydrate was from United States Biochem. Corp., Cleveland, OH. Collagenase was from Worthington Biochem. Corp., Freehold, NJ. $^{86}RbCl$ and [6,6′-$^{3}H_2$]sucrose were from The Radiochemical Centre, Amersham, Bucks. Detapac was from Sigma Chemical Co., St Louis, MO. Transferrin from Kabi AB, Stockholm, was exposed to an excess of Fe^{3+} and separated from free iron by filtration on Sephadex G-25.

3. Results

Figure 1 shows the dose-dependent inhibitory action of alloxan on Rb^+ accumulation by microdissected islets. A comparison of the dose–response curves for alloxan in the presence and absence of 5 or 10 μM $CuSO_4$ shows that Cu^{2+}, too, inhibited the Rb^+ accumulation. The shape of the curves suggests that this effect was additive to that of alloxan. In contrast, 10 μM $NaSO_4$, 10 μM $FeSO_4$, or 5 μM $ZnSO_4$ had no inhibitory effect, alone or in combination with alloxan.

The influence of chelators is summarized in table 1. Whereas Fe(II)EDTA or Fe(III)transferrin lacked significant effects, the alloxan action was clearly inhibited by Detapac. The effect of Detapac was specifically to protect against alloxan, since Detapac alone did not affect Rb^+ accumulation.

By measuring the decrease of A_{290} in cell-free medium, we observed no direct reaction between the ions or ion chelators on the one hand and alloxan on the other.

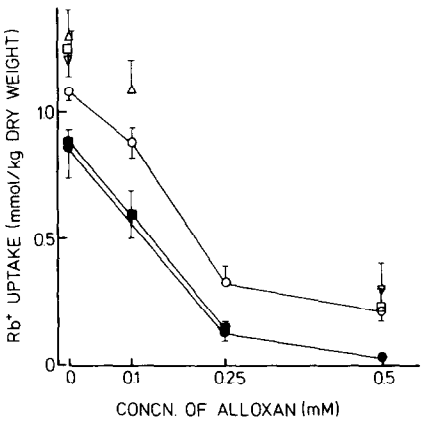

Fig.1. Islet uptake of Rb^+ after treatment with various concentrations of alloxan alone (○) or alloxan in combination with 5 μM $CuSO_4$ (■), 10 μM $CuSO_4$ (●), 10 μM $FeSO_4$ (▽), 10 μM Na_2SO_4 (□), or 5 μM $ZnSO_4$ (△). Islets were exposed to the inorganic salts during 30 min of preliminary incubation. During the last 10 min of preliminary incubation, alloxan was also present as indicated. All islets were then incubated for 120 min in $^{86}Rb^+$-labelled medium lacking alloxan and the inorganic salts under study. The islet cell uptake of Rb^+ in excess of the [6,6′-^{3}H]sucrose (extracellular) space is given as mean values; SEM is indicated when bigger than the symbol for the mean value. In incubations without test salts, the numbers of experiments were: 71 (0 mM alloxan); 16 (0.1 mM alloxan); 20 (0.25 mM alloxan); and 33 (0.5 mM alloxan). In the absence of alloxan the numbers of experiments were 6 ($FeSO_4$, $ZnSO_4$) or 14–16 (5 or 10 μM $CuSO_4$, Na_2SO_4); the other points are based on 4–12 experiments, typically 6 or 8. Effects of salts in parallel test and control incubations were assessed by Wilcoxon's rank sum test for paired data (two-tailed); $P < 0.01$ for effect of 10 μM $CuSO_4$ in absence of alloxan, and $P = 0.02$ for effect of 10 μM $CuSO_4$ in presence of 0.25 mM alloxan.

4. Discussion

The fact that Detapac counteracted the effect of alloxan supports the hypothesis that traces of metal are involved as catalysts in alloxan diabetogenic action. Others have shown that Detapac effectively inhibits the metal-dependent generation of OH· in cell-free iron/xanthine/xanthine oxidase systems [12–14]. Detapac was therefore proposed as a valuable probe of the involvement of an 'iron-catalyzed Haber-Weiss reaction' in biochemical systems [13]. In contrast, EDTA or Fe-EDTA may rather increase the production of radical [13,14], although inhibitory effects of EDTA have also been observed in a bacterial

Table 1
Effects of alloxan, Detapac, Fe(II)EDTA and Fe(III)transferrin on the Rb^+ accumulation by microdissected islets

Test substances	No. of expts.	Islet cell uptake of Rb^+ (mmol/kg dry wt)			
		No alloxan		Alloxan	
		Control	Test	Control	Test
Detapac, 2 mM	13	1.07 ± 0.05	1.03 ± 0.08	0.26 ± 0.07	0.49 ± 0.08[a]
Fe(III)transferrin, 5 μM	8	1.07 ± 0.16	1.19 ± 0.24	0.25 ± 0.11	0.22 ± 0.08
$FeSO_4$, 10 μM, + EDTA, 15 μM	8	0.99 ± 0.14	0.87 ± 0.12	0.36 ± 0.05	0.36 ± 0.08

[a] $P < 0.01$

During preliminary incubation for 30 min, islets were not exposed (control) or exposed (test) to the test substances listed to the left. During the last 10 min of preliminary incubation, alloxan was or was not included in the medium as indicated; alloxan was 0.5 mM in experiments with Detapac and transferrin, and 0.25 mM in experiments with EDTA. All islets were then incubated for 120 min in $^{86}Rb^+$-labelled medium lacking alloxan and test substances. The islet cell uptake of Rb^+ in excess of the [6,6'-^{3}H]sucrose (extracellular) space is given as mean values ± SEM for the stated numbers of experiments. The significance of differences between parallel test and control incubations were assessed by Wilcoxon's rank sum test for paired data (two-tailed)

system [15]. It cannot be completely ruled out that Detapac protected against alloxan by reacting directly with $OH^\cdot$ rather than by chelating a metal catalyst, but such a mechanism seems unlikely. Thus, ≤ 3 mM Detapac was reported not to influence hydroxylation by the NADH/methosulphate system [14] or of salicylate and 4-hydroxycinnamate by horseradish peroxidase in the presence of dihydroxyfumarate [13].

The present failure of exogenous Fe^{2+}, Fe(II)EDTA, or Fe(III)transferrin to enhance the cytotoxic action of alloxan indicates that the islet tissue contains endogenous catalysts in amounts sufficient to yield a maximum toxic response. Although Rb^+ accumulation was more inhibited by alloxan in combination with Cu^{2+} than by alloxan alone, the effects of Cu^{2+} and alloxan appeared to be additive rather than synergistic. Therefore, exogenous Cu^{2+}, too, probably did not function as a catalyst in the Haber-Weiss reaction. The mechanism of Cu^{2+} toxicity in islet cells is unclear, but several explanations are obviously conceivable in view of the known toxicity of Cu^{2+} against erythrocyte membranes [18] and of organic mercury compounds against islet cells [19,20].

Since the ability of Detapac to penetrate islet cells has not been measured, it should be emphasized that the alloxan-induced generation of $OH^\cdot$ probably occurs near the outer surface of the cells. It has long been suggested that alloxan reacts with the islet cell plasma membranes [10,21]. Strong support for this notion is provided by the fact that mannitol is an extracellular space marker in isolated islets [10,22,23] and yet can protect against alloxan [3], presumably by scavanging hydroxyl radicals. Because the hydroxyl radical is highly reactive and therefore shortlived in biological systems, its ability to traverse an intact plasma membrane must be extremely limited.

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