

Oxygen free radical scavengers protect rat islet cells from damage by cytokines

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Summary. A possible role for oxygen free radicals in mediating the cytotoxic effects of cytokines in islets was sought by the use of agents that scavenge free radicals. Rat islet cell monolayer cultures were incubated for 6 days with t-butylhydroperoxide, alloxan, streptozotocin, or the cytokines, interleukin 1, tumour necrosis factor, and interferon gamma, with or without together with the oxygen free radical scavenger combination of dimethylthiourea and citiolone, and islet cell lysis was measured in a 51 chromium cytotoxicity assay. The free radical scavengers significantly inhibited the islet cell cytotoxic effects of t-butylhydroperoxide and alloxan, but not streptozotocin. Similarly, the cytotoxic effects of the cytokine combinations of interleukin 1 plus tumour necrosis factor, in-

terferon gamma plus tumour necrosis factor, and interferon gamma plus interleukin 1 were significantly inhibited by the free radical scavenger combination of dimethylthiourea and citiolone. These results suggest that the cytokine products of macrophages and lymphocytes infiltrating islets in Type 1 (insulin-dependent) diabetes may contribute to B-cell damage by inducing the production of oxygen free radicals in the islet cells.

Key words: Islet cells, cytotoxicity, oxygen free radicals, interleukin 1, tumour necrosis factor, interferon gamma, alloxan, streptozotocin.

There is increasing evidence that pancreatic islet B-cell destruction in Type 1 (insulin-dependent) diabetes mellitus is the consequence of immune/inflammatory cell-mediated processes, both in human subjects [1, 2], and in rodent models with spontaneous Type 1 diabetes [3–7]. A variety of mononuclear cells (T lymphocytes, macrophages, and natural killer cells) have been implicated in islet B-cell destruction in these studies. Whereas it is not known what may elicit the appearance of immune/inflammatory mononuclear cells in islets, their destructive effects may result from direct contact with islet B-cells and/or from secretion of inflammatory products (proteases and free radicals) and immunomodulatory peptides (cytokines).

Recently, it has been demonstrated by several groups [8–16] that the cytokines, interleukin 1 (IL-1), tumour necrosis factor (TNF), and interferon gamma (IFN- γ), acting either alone or more potently in combination, can impair insulin secretion and may be cytotoxic to islet cells in vitro. The cellular and molecular mechanisms responsible for mediating the effects of cytokines on islet B-cells, however, are unknown.

Among the various mechanisms proposed for cell killing by cytokines and other cytotoxic molecules,

generation of oxygen free radicals in the target cell has been implicated [17]. Such a mechanism for cytokine-mediated islet cell injury was first suggested by Mandrup-Poulsen et al. [18], and is based on the observations that islet cells possess very low oxygen free radical scavenging enzyme activities [19, 20], and are exquisitely vulnerable to free radicals [20]. In the present report, we demonstrate that the oxygen free radical scavengers, dimethylthiourea and citiolone, can significantly protect islet cell monolayer cultures from lysis by the oxygen free radical generators, t-butylhydroperoxide and alloxan, as well as by toxic combinations of the cytokines, IL-1, TNF, and IFN- γ .

Materials and methods

Islet cell monolayer cultures

Islets were isolated from the pancreases of adult Sprague Dawley rats (Biosciences Animal Services, University of Alberta, Edmonton, AB Canada) and dissociated into single cells by methods previously described [15]. The islet cells were suspended at 0.4×10^6 /ml in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing

Table 1. Effects of oxygen free radical scavengers on rat islet lysis by tert-butylhydroperoxide (t-BHP)

Scavenger		Islet cell lysis		
Citilone (mmol/l)	DMTU (mmol/l)	% ⁵¹ Cr release	% Inhibition by scavenger	<i>p</i>
0	0	46.5 ± 3.0	-	NS
0	1	42.3 ± 2.4	8.9 ± 1.5	NS
0	3	38.5 ± 3.4	17.2 ± 4.7	NS
0	10	33.4 ± 4.1	28.6 ± 5.0	<0.05
1	0	39.2 ± 4.5	15.6 ± 4.2	NS
1	1	26.6 ± 0.8	42.3 ± 5.7	<0.01
1	3	22.1 ± 2.1	52.7 ± 3.6	<0.001
1	10	25.5 ± 2.6	45.2 ± 5.6	<0.01
3	0	33.2 ± 2.6	33.3 ± 6.4	<0.05
3	1	19.3 ± 2.0	58.5 ± 4.4	<0.001
3	3	21.3 ± 4.4	54.1 ± 5.7	<0.01
3	10	22.5 ± 4.7	50.8 ± 5.5	<0.01

Islet cell cultures were incubated in medium containing 0.15 mmol/l t-BHP, without and with the oxygen free radical scavengers, citilone and/or dimethylthiourea (DMTU) in the concentrations shown. Islet cell lysis was measured as % ⁵¹Cr release from prelabelled islet cultures after 6 days of incubation. Values are means ± SEM for 4–5 experiments. The % inhibition of t-BHP-induced islet cell lysis was determined in each experiment and is shown together with the statistical significances (*p*) of the differences between islet cell lysis by t-BHP in the absence and the presence of scavengers, using the paired Student's *t*-test

Table 2. Effects of oxygen free radical scavengers on rat islet cell lysis by different cytotoxins

Cytotoxin (mmol/l)	Islet cell lysis			
	Without scavengers (% ⁵¹ Cr release)	With scavengers (% ⁵¹ Cr release)	<i>p</i>	
t-BHP	0.05	1.4 ± 0.5	2.2 ± 0.8	NS
	0.10	19.6 ± 5.8	6.0 ± 1.2	<0.05
	0.15	47.3 ± 8.8	20.3 ± 5.6	<0.01
	0.20	67.3 ± 6.3	52.1 ± 4.8	<0.05
Alloxan	2.0	5.9 ± 1.9	9.0 ± 2.2	NS
	2.5	17.1 ± 6.0	19.1 ± 2.9	NS
	3.0	44.7 ± 2.3	30.6 ± 2.3	<0.01
	3.5	63.4 ± 9.0	37.7 ± 7.7	<0.05
Streptozotocin	0.05	4.2 ± 2.5	6.8 ± 4.6	NS
	0.10	34.7 ± 9.4	26.8 ± 9.0	NS
	0.15	72.6 ± 8.1	70.5 ± 3.5	NS
	0.20	79.7 ± 5.3	79.9 ± 5.6	NS

Islet cell cultures were incubated in medium containing the cytotoxins shown, without and with the free radical scavenger combination of 1 mmol/l dimethylthiourea and 3 mmol/l citilone. Islet cell lysis was measured as % ⁵¹Cr release from prelabelled islet cultures after 6 days of incubation. Values are means ± SEM for 4–5 experiments. Statistical significances (*p*) are shown for the differences between islet cell lysis by each cytotoxin in the absence and the presence of scavengers, using the paired Student's *t*-test. t-BHP = tert-butylhydroperoxide

11 mmol/l glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml amphotericin B, 12 mmol/l HEPES, and 10% heat-inactivated fetal calf serum (complete medium). To promote islet cell re-aggregation and monolayer formation, 0.1 mmol/l 3-isobutyl-1-methylxanthine (IBMX) was added to the medium [15]. The cells were seeded in this medium (4 × 10⁴/well) in 96-well half-area microculture plates (Costar, Cambridge, Mass., USA), and incubated in a humidified incubator at 37°C in 95% air/5% CO₂. After 5–7 days, the islet cells had reaggregated in small clusters of

10–100 cells attached to the bottom of the wells and spread out in monolayer. The cultures were then washed and reincubated in complete medium (without IBMX) for 3 days.

Islet cell cytotoxicity assay

Islet cell monolayer cultures were labelled by incubation for 24 h at 37°C with 1 µCi ⁵¹Cr sodium chromate (New England Nuclear, Boston, Mass., USA) in 170 µl complete medium per well, then washed 4 times, reincubated for 96 h in ⁵¹Cr-free complete medium, and washed again 4 times. Test agents (t-butylhydroperoxide, alloxan, streptozotocin, cytokines) were added to the monolayers in the absence and presence of the free radical scavengers, dimethylthiourea and citilone (170 µl/well in quadruplicate) and the plates were incubated at 37°C for 6 days. An aliquot (100 µl) of supernatant medium was collected from each well and counted in a gamma counter. Percent specific cell lysis was calculated as 100% × (test medium cpm – spontaneous cpm)/(total cpm – spontaneous cpm). Spontaneous release of ⁵¹Cr was measured in wells incubated in medium alone and this was <12% of total ⁵¹Cr release, measured by dissolving the cells in 4% Triton X-100 for 16 h.

Cytokines and chemicals

Recombinant (r) human TNF-α (4 × 10⁷ U/mg) was kindly provided by J. Kaumeyer (Genentech, San Francisco, Calif., USA), r human IL-1-β (10⁸ U/mg) was purchased from Genzyme (Boston, Mass., USA), and r rat IFN-γ (4 × 10⁶ U/mg) was purchased from Amgen Biologicals (Thousand Oaks, Calif., USA). Alloxan (Sigma, St. Louis, Mo, USA), and streptozotocin (STZ) (Upjohn, Kalamazoo, Mich., USA) were dissolved in citrate buffer (pH 4.6) as 0.5 mol/l stock solutions and diluted in complete medium immediately before use. Tert-butylhydroperoxide (t-BHP), and N-acetyl-homocysteine-thiolactone (citilone) were purchased from Sigma, and dimethylthiourea (DMTU) from Aldrich (Milwaukee, Wis., USA). Stock solutions of DMTU (1 mol/l) citilone (0.5 mol/l), and t-BHP (0.1 mol/l) were freshly prepared in distilled water and diluted in complete medium immediately before use.

Statistical analysis

Data are presented as mean values ± SEM, and statistical analysis was done with Student's two-tailed *t*-test for paired data.

Results

Free radical scavengers protect against t-BHP and alloxan

The islet cell cytotoxic action of the oxygen free radical generator, t-BHP was inhibited in a dose-dependent fashion by either of the two oxygen free radical scavengers, DMTU or citilone, and combination of the two scavengers produced additive effects (Table 1). Thus, 0.15 mmol/l t-BHP produced 46.5 ± 3.0% lysis of islet cells, and this was decreased by 28.6 ± 5.0% (*p* < 0.05) by 10 mmol/l DMTU, 33.3 ± 6.4% (*p* < 0.05) by 3 mmol/l citilone, and by 50.8 ± 5.5% (*p* < 0.01) by the

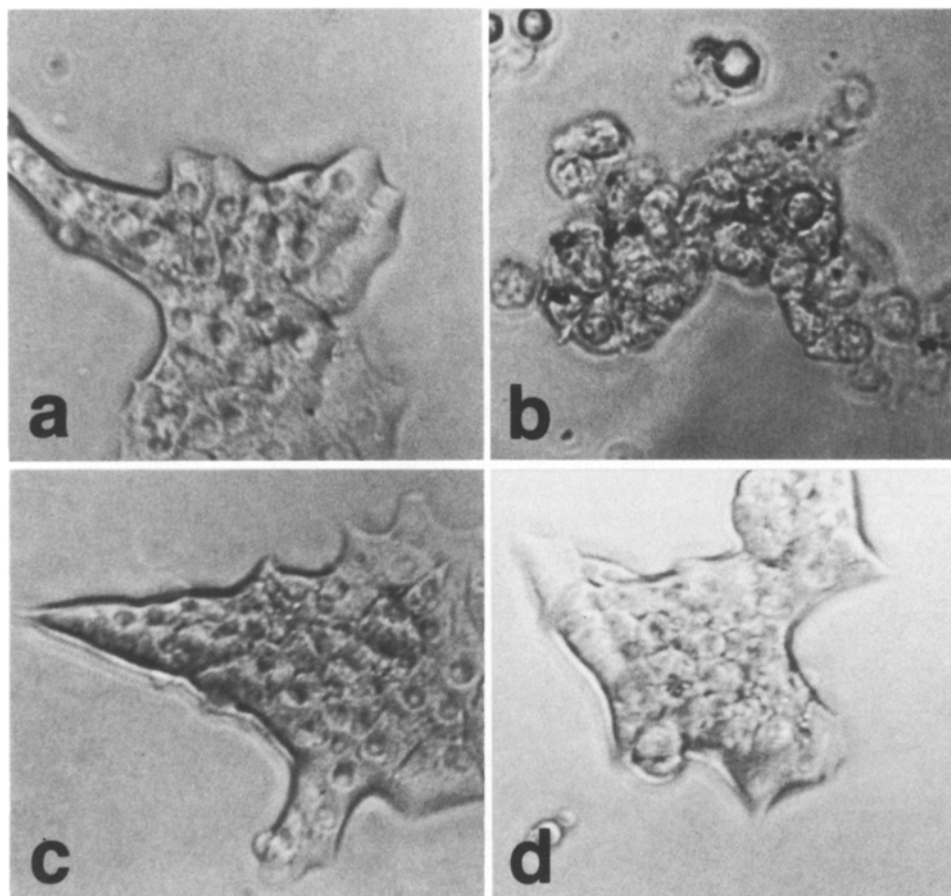


Fig. 1 a-d. Phase contrast photomicrographs (x250) of rat islet cells in monolayer culture after incubation for 6 days in RPMI-1640 complete medium (control, **a**) and the same medium with further supplements (**b-d**). The combination of interferon gamma (IFN- γ) (10 U/ml) and tumour necrosis factor (TNF) (10^3 U/ml) caused islet cell necrosis (**b**). Dimethylthiourea (DMTU) (1 mmol/l) together with citiolone (3 mmol/l) did not affect the islet cells (**c**). The addition of DMTU and citiolone to TNF plus IFN- γ protected the islet cells from the toxic effects of the cytokines (compare **d** and **b**)

combination of 10 mmol/l DMTU and 3 mmol/l citiolone. The strongest inhibition of the cytotoxic effect of t-BHP was obtained with the combination of 1 mmol/l DMTU and 3 mmol/l citiolone ($58.5 \pm 4.4\%$ inhibition, $p < 0.001$). Therefore, this combination of concentrations of DMTU (1 mmol/l) and citiolone (3 mmol/l) was used in subsequent experiments.

In addition to inhibiting the islet cytotoxic effect of t-BHP, the free radical scavenger combination of DMTU and citiolone also protected against the cytotoxicity of the islet B-cell toxin, alloxan, but not streptozotocin (Table 2). The greatest protection by the free radical scavengers occurred at intermediate levels of islet cell lysis (30–50% ^{51}Cr release) produced by either t-BHP or alloxan ($p < 0.01$). In contrast, the free radical scavengers did not provide significant protection against streptozotocin-induced islet cell lysis.

Free radical scavengers protect against cytokines

The free radical scavenger combination of 1 mmol/l DMTU and 3 mmol/l citiolone was then tested in incubations with cytokines (Table 3). IL-1 (1.0 U/ml), IFN- γ (10 U/ml), and TNF (10^3 U/ml) were not cytotoxic as single agents, however, combinations of IL-1 and TNF, IFN- γ and TNF, and IFN- γ and IL-1 exerted dose-dependent cytotoxic effects, as we have reported pre-

viously [15]. These cytokine-induced lytic effects were inhibited by the free radical scavenger combination of DMTU and citiolone, and the greatest protection against islet cell lysis occurred at intermediate levels of cytotoxicity (30–45% ^{51}Cr release) delivered by any of the cytokine combinations. The free radical scavengers were more protective against combinations of IFN- γ and TNF (50–60% inhibition, $p < 0.01$) than against combinations containing IL-1 (IL-1 plus TNF, and IFN- γ plus IL-1) (20–35% inhibition, $p < 0.05$ –0.01). The protective effect of DMTU and citiolone against IFN- γ plus TNF-mediated lysis of the islet cell monolayers was also evident by phase contrast microscopy (Fig. 1).

Discussion

We have previously reported that the cytotoxic effects of cytokines on islet cells can be monitored and quantitated in a ^{51}Cr release cytotoxicity assay using rat islet cells in monolayer culture [15]. In the present study, we have used this assay to investigate a possible role for oxygen free radicals in mediating the cytolytic effects of cytokines on islet cells, since cytokines are known to induce free radical generation in other cell types [17].

Table 3. Effects of oxygen free radical scavengers on rat islet cell lysis by different cytokine combinations

Cytokine (U/ml)	Islet cell lysis		
	Without scavengers (% ⁵¹ Cr release)	With scavengers (% ⁵¹ Cr release)	<i>p</i>
IL-1 1.0	4.5 ± 1.0	5.3 ± 1.5	NS
IL-1 1.0 + TNF 10	9.0 ± 2.6	7.8 ± 2.5	NS
IL-1 1.0 + TNF 10 ²	17.5 ± 2.5	10.4 ± 3.0	< 0.05
IL-1 1.0 + TNF 10 ³	30.7 ± 2.7	20.7 ± 2.4	< 0.01
IFN-γ 10	5.9 ± 1.2	2.6 ± 1.2	NS
IFN-γ 10 + TNF 10	9.9 ± 2.7	4.8 ± 2.0	< 0.05
IFN-γ 10 + TNF 10 ²	33.2 ± 5.0	14.4 ± 3.1	< 0.01
IFN-γ 10 + TNF 10 ³	43.2 ± 6.7	21.7 ± 4.0	< 0.01
TNF 10 ³	1.9 ± 0.6	3.1 ± 1.0	NS
IFN-γ 10 + IL-1 0.1	31.4 ± 3.1	20.5 ± 4.0	< 0.05
IFN-γ 10 + IL-1 1	42.8 ± 3.9	33.7 ± 4.3	< 0.05
IFN-γ 10 + IL-1 10	45.7 ± 3.8	43.8 ± 3.5	NS

Islet cell cultures were incubated in medium containing the cytokines shown, without and with the free radical scavenger combination of 1 mmol/l dimethylthiourea and 3 mmol/l citiolone. Islet cell lysis was measured as % ⁵¹Cr release from prelabelled islet cultures after 6 days of incubation. Values are means ± SEM for 4–6 experiments. Statistical significances (*p*) are shown for the differences between islet cell lysis by each cytokine or cytokine combination in the absence and the presence of scavengers, using the paired Student's *t*-test. IL-1 = interleukin 1; TNF = tumour necrosis factor; IFN-γ = interferon gamma

First, we identified oxygen free radical scavengers which significantly inhibited the cytotoxic effect of known free radical generators, the non-specific cytotoxin, t-BHP, and the islet B-cell specific toxin, alloxan. We showed that both t-BHP and alloxan are highly toxic to islet cells, and that this toxicity can be significantly inhibited by the combination of two free radical scavengers, DMTU and citiolone. DMTU and citiolone are scavengers of hydroxyl free radicals ([•]OH), considered as the most reactive and damaging of the oxygen-derived free radicals. In addition to scavenging of [•]OH [21], citiolone has the ability to increase superoxide dismutase (SOD) activity in rat islets [22]. SOD catalyses the conversion of superoxide radicals (O₂^{•-}) to hydrogen peroxide (H₂O₂), thereby decreasing the amounts of O₂^{•-} available to form [•]OH. Thus, DMTU and citiolone would be expected to have additive effects in protecting against free radical-induced cell injury, as we observed using this combination of scavengers to counter the cytotoxic effects of the oxygen free radical generator, t-BHP.

Interestingly, the free radical scavenging combination of DMTU and citiolone significantly inhibited the islet cell cytotoxic effect of alloxan and not streptozotocin. It is well established that both alloxan and streptozotocin are selective B-cell toxins, however the mechanisms that impart their selective toxicity remain controversial. There is evidence that alloxan and streptozotocin may act to kill B-cells by different mechanisms [23]. Our findings that the free radical scavengers inhibited alloxan and not streptozotocin-induced islet cell killing are in agreement with other studies which

suggest that alloxan acts through generation of hydroxyl radicals whereas streptozotocin may act via other mechanisms [24, 25].

Since the free radical scavenging combination of DMTU and citiolone significantly inhibited the cytotoxic effects of the free radical generators, t-BHP and alloxan and also significantly inhibited the cytotoxic effects of cytokine combinations (TNF+IL-1, IFN-γ + TNF, and IFN-γ + IL-1), it follows that these cytokine combinations may be toxic by leading to the formation of oxygen free radicals in islet cells. An oxygen free radical-mediated mechanism for cytokine-induced islet cell injury is compatible with the report by Eizirik that IL-1/TNF-induced islet cell toxicity is associated with inhibition of oxidative metabolism of glucose [13], since interference with mitochondrial energy production is recognized as an early manifestation of oxygen free radical-mediated injury [26].

The cytokines, IL-1, TNF, and IFN-γ have been reported to stimulate oxygen free radical production during the respiratory burst in a variety of cell types, such as macrophages [27] and polymorphonuclear leukocytes [28] in association with host defense against micro-organisms and tumours. Stimulation of oxygen free radical production in pancreatic islet cells, however, might be expected to have lethal consequences to islet cells since islet cells possess very low free radical scavenging enzyme activities [19, 20], and therefore are exquisitely vulnerable to free radical-induced injury [20]. Cytokine-induced oxygen free radicals are primarily products of arachidonic acid metabolism in many cell types, and we have recently observed that inhibitors of arachidonic acid metabolism can protect islet cells from the cytotoxic effects of TNF and IFN-γ. It remains to be directly demonstrated, however, that cytokines stimulate increased arachidonate metabolism and free radical production in islet cells.

Evidence exists to suggest involvement of oxygen free radicals in immune-mediated damage to islet B-cells, *in vivo*. Thus, desferrioxamine, an inhibitor of hydroxyl radical formation, and nicotinamide, a poly (ADP-ribose) synthetase inhibitor and a weak free radical scavenger, protected islet cells from immune destruction in allograft rejection and in low-dose streptozotocin-induced insulinitis [29]. Also, treatment of spontaneously diabetic NOD mice with nicotinamide and desferrioxamine [30], or with superoxide dismutase [31], protected islet grafts from immune damage and consequent recurrence of diabetes. Activated macrophages in the insulinitis infiltrate [32] might be the source of islet-damaging free radicals, and activated peritoneal macrophages have been reported to be cytotoxic to islet cells [33]. In addition, our findings suggest that the cytokine products of lymphocytes and macrophages in the islet infiltrate might contribute to immune-mediated damage by stimulating the generation of oxygen free radicals in the islet cells.

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