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FUSIDIC ACID SUPPRESSES NITRIC OXIDE TOXICITY IN PANCREATIC ISLET CELLS

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Abstract—Earlier preclinical and clinical trials indicate that fusidic acid, a triterpenoid compound originally described as an antimicrobial drug may protect islet beta cells from destruction in type I (insulin-dependent) diabetes mellitus. Since nitric oxide appears to be an important mediator of inflammatory islet cell death we analysed whether fusidic acid interferes with nitric oxide production or action. We report here that fusidic acid dose-dependently inhibits lysis of isolated islet cells by activated macrophages, a process mediated by nitric oxide. In the presence of 100 μM fusidic acid macrophage-mediated islet cell lysis was reduced from 52.5 to 1.7% ($P < 0.001$). Fusidic acid only slightly affected macrophage function and did not inhibit the release of nitric oxide. We therefore tested whether fusidic acid suppresses nitric oxide toxicity in target cells. Isolated islet cells were exposed to the nitric oxide donor nitroprusside which led to DNA strand breaks and plasma membrane lysis. DNA strand breaks were reduced from 54.6 to 34.9% ($P < 0.001$) in the presence of 100 μM fusidic acid and cell lysis was reduced from 60.1 to 27.5% with 100 μM ($P < 0.001$). In the presence of 500 μM fusidic acid DNA strand breaks and cell lysis were reduced further to 27.1 and 10.7%, respectively ($P < 0.001$). No protection by fusidic acid was observed when cells were exposed to oxygen radicals or the alkylating beta cell toxin streptozotocin. The suppression of nitric oxide toxicity by fusidic acid was not due to its known inhibitory action on protein biosynthesis and thus represents a hitherto unknown activity of this drug.

Key words: fusidic acid; pancreatic islet cells; nitric oxide; reactive oxygen intermediates; streptozotocin

In the search for new immunomodulatory drugs recent preclinical and clinical trials revealed the potential of the steroid fusidic acid for the therapy of organ specific autoimmune disorders. Fusidic acid was found to preserve pancreatic beta cell function in an animal model [1] and possibly in human type I (insulin-dependent) diabetes mellitus [2] and showed beneficial effects in the therapy of Crohn's disease [3] and chronic uveitis [4].

Fusidic acid, a tetracyclic triterpenoid compound originally isolated from a strain of fusidium, exerts strong antimicrobial activity against various strains of staphylococcus and mycobacterium [5]. The drug inhibits protein biosynthesis by interfering with ribosomal translocation during the elongation process in procaryotic [6] and eukaryotic mitochondrial translation systems [7].

At present the most detailed information on the protective effects of fusidic acid in autoimmune diseases is available from studies in models of human type I diabetes. Recent analysis has revealed T-cell immunosuppressive properties of the drug [8] possibly resembling those of cyclosporin A [9], which was used in therapeutic trials in newly diagnosed type I diabetic patients [10, 11]. The preservation of pancreatic beta cell function may be explained by the ability of fusidic acid to suppress the release of islet cell toxic interleukin 1 (IL-1) [9] from mononuclear cells and to prevent the inhibitory effect of IL-1 on beta cell function [12].

Previous studies have shown that NO† is the major mediator of IL-1 induced modulation of islet cell function [13] and of IL-1 induced islet cell lysis [14]. Furthermore NO is the main mediator of macrophage mediated islet cell toxicity [15]. The present study therefore was undertaken to investigate the potential of fusidic acid to interfere with NO production or action.

MATERIALS AND METHODS

Pancreatic islet cells. Pancreatic islet cells were isolated from Wistar rats (provided from our own breeding colony) by ductal injection of a collagenase solution [Serva GmbH, Heidelberg, F.R.G.; 0.37 U/mg, 1.5 mg/mL in HBSS (Gibco Europe, Heidelberg, F.R.G.)] as described previously [16]. After 40 min of incubation (37°) the islets were enriched from the dispersed tissue by centrifugation on a Ficoll density gradient (Ficoll 400, Pharmacia GmbH, Freiburg, F.R.G.). Single cell suspensions were prepared by dissociating the whole islets in Ca^{2+}- and Mg^{2+}-free HBSS (Gibco) in the presence of 2 mg/mL trypsin (Boehringer Mannheim, Mannheim, F.R.G.). For the subsequent experiments the islet

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† Abbreviations: NO, nitric oxide; ROI, reactive oxygen intermediates; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; L-NMA, N^G-methyl-L-arginine; FCS, foetal calf serum; HBSS, Hank's balanced salt solution; LPS, lipopolysaccharide.

cells were resuspended in modified RPMI 1640 with a reduced glucose concentration of 4 mM. The medium contained a standard concentration of 1.14 mM L-arginine and was supplemented with 25 mg/L ampicillin, 120 mg/L penicillin, 270 mg/L streptomycin (Serva), 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mL/L non-essential amino acids (100×, Gibco), 2 g/L $NaHCO_3$, 2.38 g/L HEPES (Serva) and 10% (v/v) FCS (Gibco).

Macrophages. Peritoneal cells were harvested from Wistar rats pretreated with an intraperitoneal injection of 500 μL inactivated Corynebacterium parvum suspension (Wellcome, Burgwedel, F.R.G.). Macrophages were enriched by adherence on FCS-coated petri dishes (1 hr, 37°, 5% CO_2). The resulting population of activated macrophages (>95% ED1 positive cells [16]) was resuspended in modified RPMI 1640 and used as effector cells in the assay for macrophage-mediated islet cell toxicity.

Macrophage-mediated islet cell toxicity. For the determination of macrophage-mediated cytotoxicity islet cells were labelled by incubation (20 hr, 37°, 5% CO_2) with 5 μCi/mL L-[4,5-^{3}H]leucine (sp. act. 177 Ci/mmol, Amersham Buchler, Braunschweig, F.R.G.). This method proved to be especially useful for the gentle labelling of highly sensitive pancreatic islet cells [17]. As shown in previous studies controlled by electron microscopy, this labelling method yields reliable results on the lysis of islet cells independent of their metabolic and insulin secretory activity [16]. The labelled islet cells (5×10^3 cells in 100 μL modified RPMI 1640) were seeded in triplicate in round-bottom microtiter plates and preincubated (37°, 5% CO_2) for 3 hr in the presence of various concentrations of fusidic acid (sodium salt, Sigma, Deisenhofen, F.R.G.) or 100 μM L-NMA (Sigma). The macrophages (2×10^5 cells in 100 μL modified RPMI 1640) were added and the incubation continued in the presence of the drugs. After 18 hr the amount of radiolabel was determined in the supernatant of the islet cell-macrophage cocultures, of islet cells alone and of islet cells completely lysed by Triton X-100 [16]. The spontaneous release of [^{3}H]leucine from the islet cells in the absence of effector cells was <30% of the total [^{3}H]leucine release. The specific lysis of the islet cells was calculated according to the formula:

$$\text{Specific lysis (\%)} = 100 \times \frac{(\text{cpm}_{\text{test}} - \text{cpm}_{\text{spontaneous}})}{(\text{cpm}_{\text{total}} - \text{cpm}_{\text{spontaneous}})}$$

Chemically induced islet cell toxicity. The analysis of chemically induced islet cell cytotoxicity was performed in 1/2 area flat-bottom microtiter plates (Costar, Cambridge, MA, U.S.A.). To achieve a cell density on the bottom of the culture wells comparable to the conditions of the macrophage coculture 2×10^4 islet cells were seeded in 120 μL modified RPMI 1640 per well in triplicate. The cells were preincubated for 3 hr with various concentrations of fusidic acid or chloramphenicol (Sigma) before exposure to the toxic agents for a further 18 hr. NO-mediated cytotoxicity was investigated by incubating the islet cells in the presence of the NO-donor nitroprusside (sodium pentacyanonitrosylferrate (II), 0.45 mM, Merck, Darmstadt, F.R.G.). Under *in vitro* conditions in the presence of cellular constituents 1 mM nitroprusside was found to release NO in a concentration range of about 1 nmol/mL/min [18, 19] without releasing detectable amounts of reactive oxygen intermediates. In a recent study it was demonstrated that macrophage mediated islet cell lysis is identical to lysis by nitroprusside [20]. Rhodanese (EC 2.8.1.1, 8 U/well, Sigma) and $Na_2S_2O_3$ (5 mM, Merck, Darmstadt, F.R.G.) were added to scavenge cyanide ions potentially released during the decomposition of nitroprusside [21]. Islet cell toxic ROI were generated by the addition of xanthine oxidase (EC 1.1.3.22, 12.5 mU/mL, Sigma) and hypoxanthine (50 μM, Sigma) to the islet cell culture [22]. The beta cell toxin streptozotocin (Boehringer Mannheim) was added to the islet cell culture at a final concentration of 0.8 mM.

Determination of cell viability. Macrophage and islet cell viability was evaluated by the Trypan blue exclusion assay. To determine the percentage of dead cells at least 200 cells per well were counted [21].

Assay for mitochondrial activity. Mitochondrial activity was determined in macrophages and islet cells (1×10^5 cells in 200 μL modified RPMI 1640) cultivated in triplicate in 96 well round-bottom microtiter plates (37°, 5% CO_2) for 20 hr in the presence of various concentrations of fusidic acid. To assess residual mitochondrial activity at the end of the incubation period the culture supernatants were replaced by 100 μL of an MTT solution [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, 1 mg/mL modified RPMI 1640, Sigma] according to the method originally described by Mosmann [23] and adapted for islet cells [22, 24]. The incubation was continued for 4 hr and the resulting formazan crystals were dissolved in 50 μL isopropanol. The optical density of the solution was determined in an eight-channel photometer at 540 nm (reference wavelength: 690 nm) [22].

Chemiluminescence assay. The generation of ROI from macrophages was determined in a six-channel bioluminescence analysing system (Biolumat LB 9505 C, Berthold, Wildbad, F.R.G.). Peritoneal macrophages (1×10^6 cells) were incubated in modified RPMI 1640 in round bottom vials. The release of ROI was induced by the addition of zymosan (50 μg/mL, Sigma) and the resulting chemiluminescence activity was monitored for 60 min using luminol (0.5 mM, Sigma) as detecting agent [25].

Assay for nitric oxide release. The production of NO from macrophages was assessed by detection of its oxidized form, nitrite (NO_2^-), using the Griess reaction [26]. Briefly, 1×10^5 macrophages were cultivated in 200 μL modified RPMI 1640 in the wells of 96-well round-bottom microtiter plates. Serial dilutions of fusidic acid were added and in a set of samples the macrophages were stimulated with 1 ng/mL LPS (from *Escherichia coli* 026:B6, Sigma). After 20 hr of incubation (37°, 5% CO_2) 120 μL of the supernatant were removed from each well and the concentration of the accumulated NO_2^- was determined by the Griess reaction which is based on the diazotation of NO_2^- with sulfanilamide followed

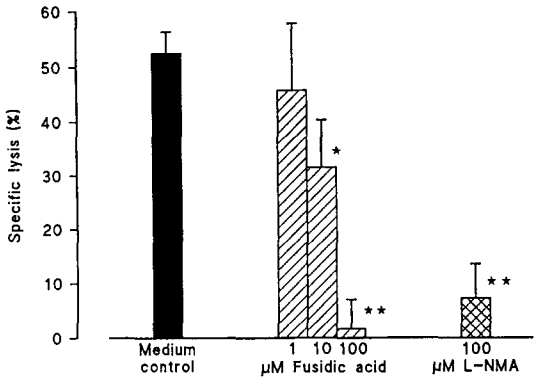

Fig. 1. Dose-dependent inhibition of macrophage-mediated islet cell lysis by fusidic acid. [^{3}H]Leucine labelled islet cells were cocultured with macrophages in the presence of 100 μM L-NMA or increasing concentrations of fusidic acid as described in Materials and Methods. After 18 hr the specific lysis of the islet cells was calculated from the amount of radiolabel released into the supernatant of the coculture according to the formula given in Materials and Methods. The data shown are means ± SD from three experiments. *$P < 0.01$, **$P < 0.001$.

by azo-coupling with *N*-(1-naphtyl)ethylendiamine. The optical density of the resulting azo-pigment solution was measured at 540 nm and the NO_2^- concentrations were calculated from a standard curve obtained with $NaNO_2$. The background content of NO_2^- in the medium was below the detection limit of the Griess assay (1 nmol NO_2^-/200 μL).

Detection of DNA-strand breaks. To analyse DNA damage islet cells were cultivated in Lab-Tec chamber slides (Nunc, Naperville, IL, U.S.A.) and exposed to the various experimental treatments. Then the cells were air-dried, fixed in acetone (10 min) and subjected to the *in situ* nick translation procedure as described previously [27]. Briefly, 50 μL of the nick translation mixture (4 U/100 μL Kornberg polymerase, EC 2.7.7.7, Boehringer Mannheim; 3 μM biotinylated dUTP; 3 μM each dGTP, dCTP, dATP; 50 mM Tris–HCl pH 7.5; 5 mM $MgCl_2$; 0.1 mM dithiothreitol) were added on the fixed cells for 9 min at room temperature. To block unspecific antibody binding, the cells were incubated for 10 min in PBS with 0.1% (w/v) thimerosal and 10% (v/v) FCS. The biotinylated dUTP, incorporated by polymerase-activity at sites of DNA damage, was detected by the peroxidase reaction (Vectastain-Kit, Camon, Wiesbaden, F.R.G.) with diaminobenzidine as substrate. Nuclei with DNA strand breaks appeared brown. The resulting staining pattern of the cells was illustrated in detail in a previous study [27]. At least 100 cells per chamber were evaluated microscopically and the percentage of cells with stained nuclei determined.

Statistical analysis. For statistical analysis of the data the Student's *t*-test was used.

RESULTS

Incubation of isolated islet cells with activated macrophages led to 52.5 ± 4.6% lysis (mean ± SD) within 18 hr of coculture. In the presence of L-NMA, an inhibitor of NO synthase [28], islet cell lysis was almost completely suppressed, confirming that target cell death is NO dependent (Fig. 1). When fusidic acid was present during the cocultivation period a dose-dependent inhibition of lysis was observed. A concentration of 100 μM fusidic acid resulted in an almost complete survival of the islet cells (specific lysis 1.7 ± 5.8%).

To exclude possible toxic effects of fusidic acid on macrophages or islet cells, the cells were incubated with increasing concentrations of the drug. After 18 hr the cells were analysed for their viability by the trypan blue exclusion assay and their residual respiratory activity assessed by the capacity of the cells to convert the tetrazolium salt MTT into its formazan product. As shown in Table 1 the macrophages and the islet cells retained their viability and respiratory activity even in the presence of 100 μM fusidic acid. Additional analysis of the islet cells revealed no significant effects on the spontaneous release of radiolabel and on islet cell DNA integrity as determined by nick translation. A concentration of 1000 μM of fusidic acid caused strong damage in macrophages as well as in islet cells.

Further analysis of macrophage functions was performed to investigate the effect of fusidic acid on the release of the islet cell toxic mediators NO and ROI (Fig. 2). As detected by the Griess method the basic and LPS induced formation of NO was not altered in the presence of fusidic acid. Measurement of the chemiluminescence activity revealed only a slight reduction in zymosan-induced ROI release from a peak activity of $3.8 \pm 0.6 \times 10^6$ cpm to $2.4 \pm 0.4 \times 10^6$ cpm only at the maximum concentration of fusidic acid (100 μM). These observations clearly show that fusidic acid has no direct effect on NO and ROI formation from macrophages since even under optimum stimulatory conditions the drug did not affect the release of these mediators. These results further indicate that fusidic acid does not directly scavenge macrophage-derived NO or ROI nor does it interfere with the detection systems for these mediators.

We then studied whether fusidic acid interferes with NO toxicity in islet cells. For this purpose islet cells were exposed to the NO donor nitroprusside (0.45 mM) in the presence of absence of fusidic acid. As shown in Fig. 3 the presence of fusidic acid (100 μM) reduced the extent of NO-induced islet cell lysis from 60.1 ± 3.6% to 27.5 ± 4.8% ($P < 0.001$) and the percentage of cells with NO induced DNA strand breaks from 54.6 ± 5.1% to 34.9 ± 4.6% ($P < 0.001$). At 500 μM of fusidic acid lysis and DNA strand breaks were further reduced to 10.7 ± 2.3% and 27.1 ± 3.1%, respectively ($P < 0.001$).

When islet cells were exposed to the beta cell toxin streptozotocin or to ROI generated by the xanthine oxidase/hypoxanthine system, the addition of fusidic acid did not cause any protection (Fig. 4). Finally, we tested the protective potential of chloramphenicol, a drug whose inhibitory activity on protein biosynthesis is similar to that of the antibiotic fusidic acid. However, chloramphenicol

Table 1. Effect of fusidic acid on macrophages and islet cells

Fusidic acid (μM)	Macrophages		Islet cells			
	Mitochondrial activity (%)	Cell viability (%)	Mitochondrial activity (%)	Cell viability (%)	DNA damage (%)	^{3}H-release (cpm)
0	100	99 ± 1	100	83 ± 3	15 ± 8	2789 ± 710
1	116 ± 34	98 ± 1	77 ± 11	80 ± 2	ND	3037 ± 652
10	102 ± 14	98 ± 1	84 ± 18	81 ± 2	ND	3032 ± 720
100	91 ± 25	97 ± 2	75 ± 21	82 ± 2	21 ± 1	2512 ± 539
1000	7 ± 8	<5	14 ± 3	66 ± 4	67 ± 4	4127 ± 623

After 18 hr of incubation with fusidic acid the cells were analysed for residual mitochondrial activity by the MTT assay [the control without fusidic acid was set at 100% (ΔOD = 0.25 for macrophages and ΔOD = 0.19 for islet cells)], for viability by the trypan blue exclusion method and for DNA-damage by the *in situ* nick translation technique. In parallel the spontaneous release of radiolabel was determined from [^{3}H]leucine labelled islet cells. The data shown are means ± SD from three experiments performed in triplicate or quadruplicate. ND, analysis not performed.

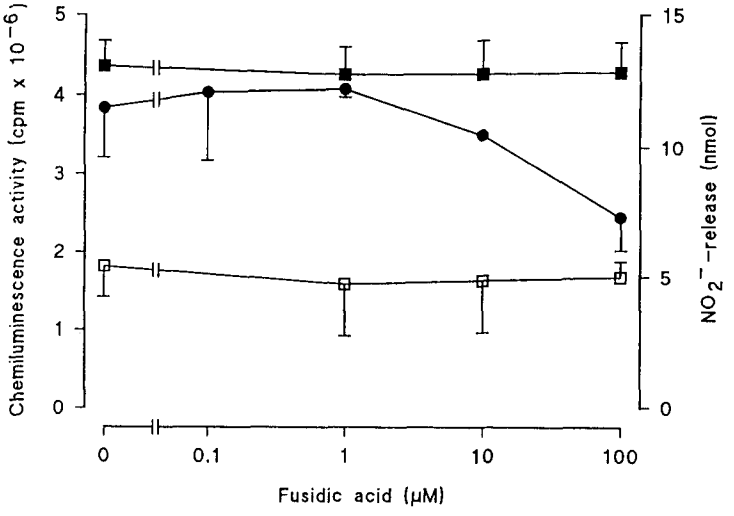

Fig. 2. Effect of fusidic acid on the release of NO and ROI from macrophages. As described in Materials and Methods the release of NO was assessed indirectly by the Griess method which detects the concentration of NO_2^- (nmol NO_2^-/200 μL modified RPMI 1640) accumulated in the supernatant of untreated (□) or LPS treated macrophages (■). The release of ROI from zymosan-stimulated macrophages (●) was determined in a bioluminescence analysing system. Data represent means ± SD from three experiments.

did not reduce NO-induced islet cell lysis even at a concentration of 1000 μM (Fig. 4).

DISCUSSION

The lysis of islet cells by activated macrophages can be completely prevented by inhibiting NO release but not by neutralizing IL-1 and/or TNFα (tumour necrosis factor α) [15]. Fusidic acid prevented islet cell lysis to a degree similar to the NO synthase inhibitor L-NMA. We therefore analysed the possible effects of fusidic acid on NO release and action in this model.

Since it is known that fusidic acid suppresses the formation of macrophage mediators [9] we investigated the effect of the drug on the release of NO from macrophages. Surprisingly, fusidic acid had no effect on NO-release from macrophages and the formation of ROI, which may interact with NO to form highly cytotoxic compounds [29], also remained nearly unchanged.

In this respect the mode of action of fusidic acid clearly differs from that of the immunosuppressive drug cyclosporin A. There is evidence that cyclosporin A reduces NO release from activated macrophages [30] and inhibits the formation of ROI from mononuclear cells [31], whereas fusidic acid showed none of these effects on the macrophages in our test system.

Fusidic acid obviously did not impair macrophage

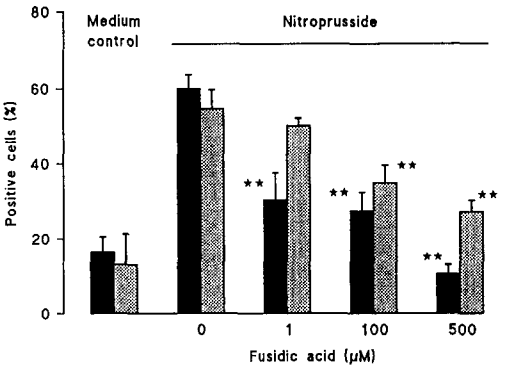

Fig. 3. Fusidic acid inhibits NO-mediated islet cell lysis and DNA damage. Islet cells were exposed to nitroprusside-generated NO in the presence of increasing concentrations of fusidic acid for 18 hr. The percentage of lysed cells (solid bars) was determined by trypan blue uptake and the percentage of cells with DNA damage (dotted bars) was assessed by the *in situ* nick translation procedure. Thiosulfate and rhodanese caused an islet cell lysis of 8.1 ± 2.3% and 9.0 ± 2.2%, respectively. Data shown are means ± SD from three experiments. *P < 0.01, **P < 0.001.

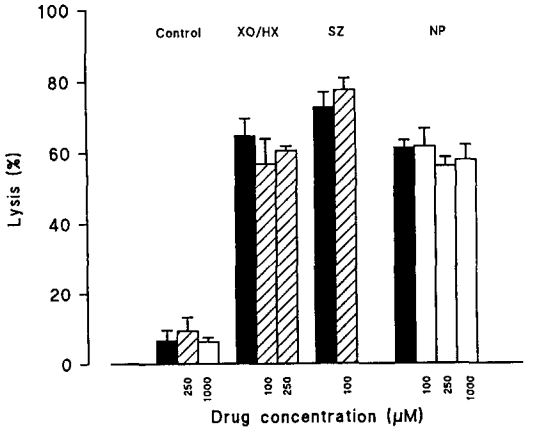

Fig. 4. Fusidic acid has no effect on islet cell lysis mediated by streptozotocin or by enzymatically generated ROI. As described in Materials and Methods islet cells were exposed to xanthine oxidase/hypoxanthine (XO/HX), streptozotocin (SZ) or nitroprusside (NP) alone (solid bars) and in the presence of various doses of fusidic acid (hatched bars) or chloramphenicol (open bars). The percentage of lysed cells was determined by the trypan blue exclusion assay. Data shown are means ± SD from three experiments. *P < 0.01, **P < 0.001.

viability since up to a concentration of 100 μM the drug caused neither toxicity nor inhibition of mitochondrial respiration. This good tolerance corresponds to previous findings with human mononuclear cells exposed to fusidic acid [9]. The effective drug concentrations in our studies were in a range comparable to the serum concentrations of about 100 μg/mL which can be expected *in vivo* after long term treatment with fusidic acid [8, 9].

These data led us to assume that fusidic acid interferes with NO toxicity at the level of target cells. Previous studies had shown that the major consequences of NO action in islet cells were DNA strand breaks and plasma membrane lysis [15, 27, 32]. We used both parameters to analyse for the protective effects of fusidic acid. To avoid the presence of macrophages in the test system we used nitroprusside as chemical donor of NO. The toxic action of nitroprusside on islet cells is similar to that of macrophage-induced islet cell lysis [20]. Both NO-induced DNA strand breaks and plasma membrane lysis were inhibited to a different extent in the presence of fusidic acid. The fusidic acid concentration (IC_{50}) required to reduce NO-induced DNA strand breaks to about 50% was more than 100 μM, whereas the IC_{50} for NO- (and also macrophage-) induced islet cell lysis was only about 1–10 μM. This strongly suggests that the mechanisms involved in NO-induced DNA damage are less sensitive to inhibition by fusidic acid. Therefore it may be concluded that fusidic acid does not exert its protective effect primarily by preventing NO-induced DNA damage.

Interestingly, fusidic acid did not provide protection against damage induced by ROI or by the alkylating effects of streptozotocin. These observations distinguish fusidic acid from the only other drug identified by us as protecting islet cells from NO, i.e. nicotinamide. The latter compound also confers protection against ROI and streptozotocin [21, 33, 34]. However, the lack of fusidic acid mediated protection against streptozotocin toxicity *in vitro* does not necessarily mean that the drug fails to protect against streptozotocin-induced islet cell death *in vivo*. When administered in mice at multiple low doses [35] streptozotocin is thought to induce initial damage by alkylating the DNA in islet cells [36]. Subsequently, islet infiltrating macrophages [37] will cause the majority of cell death by releasing the islet cell toxic mediator NO. During this phase of active autoimmune reactivity fusidic acid may be able to protect islet cells from the damage induced by macrophage-derived NO. The use of fusidic acid in the suppression of streptozotocin-induced diabetes may in fact be promising since the drug has already been successfully used to preserve islet cells and to reduce diabetes incidence in diabetes-prone BioBreeding (BB) rats [38].

The antibiotic activity of fusidic acid results from its inhibitory action on mitochondrial protein biosynthesis [7]. We therefore tested another antibiotic inhibitor of mitochondrial protein synthesis, chloramphenicol [39], but did not find any protective action in islet cells. Earlier studies had shown that cycloheximide, an inhibitor of cytoplasmic protein biosynthesis, also does not suppress NO toxicity in islet cells [40]. These findings render it improbable that inhibition of protein biosynthesis accounts for the protective action of fusidic acid. The suppression of NO toxicity in islet cells thus represents a hitherto unknown activity of the steroid

fusidic acid. The data warrant further analysis of fusidic acid as a cell protective compound in NO-mediated inflammatory conditions.

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