

Cytotoxicity of activated rat macrophages against syngeneic islet cells is arginine-dependent, correlates with citrulline and nitrite concentrations and is identical to lysis by the nitric oxide donor nitroprusside

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Summary. Lysis of rat islet cells by syngeneic activated macrophages *in vitro* can be completely inhibited by the nitric oxide-synthase-inhibitor N^ω-methyl-L-arginine. This inhibition can be reversed by an excess of L-arginine. Time-dependent lysis of islet cells by activated macrophages is accompanied by increasing concentrations of nitrite and citrulline in the culture medium both of which are measures of nitric oxide formation derived from L-arginine. Lysis of isolated islet cells and disintegration of isolated whole islets is also obtained within 15 h by culture in the

presence of the nitric oxide generating vasodilator sodium nitroprusside. We thus conclude that nitric oxide is extremely toxic for islet cells and that nitric oxide alone and in the absence of other macrophage-generated potentially toxic products can rapidly and completely kill islet cells.

Key words: Activated macrophages, nitric oxide, nitroprusside, cytotoxic activity, islet cells, rat.

In animal models of Type 1 (insulin-dependent) diabetes mellitus macrophages were shown to be the first immunocytes that infiltrate the pancreatic islets of Langerhans in the autoimmune process [1–3]. Administration of silica particles which are selectively toxic for macrophages prevents the onset of insulinitis and diabetes [4, 5]. Activated macrophages have been shown to synthesize nitric oxide (NO) via an L-arginine-dependent pathway [6–8] as effector molecules for killing intra- and extracellular parasites [9–11] and tumour cells *in vitro* [12–14]. We have shown recently that activated macrophages, when co-cultured with syngeneic rat islet cells at a very low target:effector-cell ratio of 1:2, completely lyse islet cells but not hepatocytes or thyrocytes via soluble mediator(s) *in vitro* [15, 16]. Lysis could not be inhibited by neutralization of interleukin-1 (IL-1) and tumour necrosis factor- α (TNF- α) by specific antibodies and is dependent on L-arginine [17]. However, in these experiments we could not exclude a co-operative action of various cytotoxic effectors and NO (or a closely related reactive nitrogen-oxide intermediate). By comparison of macrophage-mediated lysis of isolated syngeneic rat islet cells with the lysis mediated by the chemical NO-donor nitroprusside, we now show for the first time, that NO without any synergistic help from other macrophage products can act as an islet toxic effector molecule.

Materials and methods

Reagents

Cells were cultured in RPMI 1640 containing 11 mmol/l glucose supplemented with 6×10^4 U/l penicillin, 60 mg/l streptomycin, 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, 10 ml/l non-essential amino acids $\times 100$, 23.8 mmol/l NaHCO₃, 10 mmol/l HEPES (Gibco, Heidelberg, FRG) and 10% heat inactivated fetal calf serum (FCS) (Boehringer Mannheim, Mannheim, FRG). N^ω-methyl-L-arginine acetate (L-NMA), sulphanilamide, N-1-naphthylethylenediamine, bovine liver rhodanese (190 U/mg), 8-bromoguanosine-3':5'-cyclic monophosphate (8-Br-cGMP), N⁶,2'-O-dibutyrylguanosine-3':5'-cyclic monophosphate (dibut-cGMP), N⁶,2'-O-dibutyrylguanosine-3':5'-cyclic monophosphate (dibut-cAMP), 3-isobutyl-1-methyl-xanthine (IBMX) and the amino acid standards were purchased from Sigma (Deisenhofen, FRG), phenylisothiocyanate, triethylamine and ninhydrin from Pierce (Oud Beijerland, Netherlands), sodium nitroprusside, potassium ferrocyanide, sodium thiosulphate and sodium thiocyanate from Merck (Darmstadt, FRG), acetonitrile and pyridine (HPLC grade) from Promochem (Wesel, FRG) and 8-(4-chlorophenylthio)-guanosine-3':5'-cyclic monophosphate (8-pCPT-cGMP) from Biolog (Bremen, FRG). Triethylamine was distilled over ninhydrin before use.

Animals

Male and female Wistar rats (200 g) from the University breeding facility received a standard diet ("ssniff-R"; Ssniff, Soest, FRG) and tap water *ad libitum*.

Macrophage-islet cell co-culture

Pancreatic islets were harvested by ductal injection of collagenase, submitted to gradient centrifugation and dissociated into a single-cell suspension exactly as described previously [15]. Macrophages were activated *in vivo* by injection of 500 μ l heat-inactivated *Corynebacterium parvum* suspension (Wellcome, Burgwedel, FRG) *i.p.* 5 days prior to isolation. Macrophages from peritoneal exudate were isolated by adherence to petri dishes pre-coated with FCS and harvested by vigorously rinsing after incubation in Ca/Mg-free HBSS at 4°C for 5 min. The resulting cell preparations consisted of more than 95% ED1⁺ ED2⁻ macrophages [16] which have the same phenotype as macrophages in inflamed (but not in normal) islets of pre-diabetic BB rats [3].

About 4×10^4 islet cells in 200 μ l were placed in 1.5 ml micro sample tubes (Sarstedt, Nümbrecht-Rommelsdorf, FRG) pre-coated with culture medium. After 8 h recovery culture, 8×10^4 peritoneal macrophages with or without L-NMA or L-arginine were carefully added to achieve a total volume of 600 μ l. The cells were allowed to settle and were incubated for the indicated intervals at 37°C in a humid atmosphere of 5% CO₂/95% air. Each experiment was performed two to three times.

Culturing of whole islets

About 20 whole islets, isolated as described above but not dissociated into single cells, were placed in 96-well flat bottom culture plates (Becton Dickinson GmbH, Heidelberg, FRG). After 1 h, nitroprusside and ferrocyanide, respectively, were added to achieve a total volume of 200 μ l. After 15 h the islets were investigated by light microscopy.

Electron microscopy

Fixation, dehydration and embedding of the cells were all carried out in the micro sample tubes in which the cells had been cultured avoiding any transfer and centrifugation steps exactly as described previously [15]. From each co-culture preparation more than 10³ islet cells from at least three ultra thin sections cut from different planes of the specimen were morphologically analysed by transmission electron microscopy. Cell viability was judged from electron density of cytoplasm together with preservation of intact structures of cellular organelles and continuity of cell membrane. Specimens were always analysed in a numbered and coded fashion; from sections analysed, all cells in the plane of section were counted. Since a cell pellet was embedded, there was no possibility for bias during sectioning.

Determination of nitrite

Supernatants of cultured cells were deproteinized by ultrafiltration (Ultrafree-MC filter units; Millipore, Eschborn, FRG) and stored at -80°C. Nitrite concentrations were determined by diazotization reaction [18]: 30 μ l supernatant were mixed with 40 μ l 1% sulphanimid in 4 mol/l HCl and 10 μ l 37% HCl. After 10 min, 30 μ l 1% N-1-naphthylethylen-diamine in methanol were added. After mixing the solution, the absorbance at 540 nm was measured in Pro-Bind assay plates (Becton Dickinson) in a microplate reader (Titer-tek Multiskan; Labsystems, Helsinki, Finland) using NaNO₂ as a standard.

Determination of citrulline

Citrulline was determined by amino acid pre-column derivatization with phenylisothiocyanate [19] and subsequent HPLC C₁₈ on a reverse-phase column (4.0 \times 125 mm Super PAC 3 μ m ODS 2; Pharmacia, Freiburg, FRG). As an internal standard, 50 nmol L-ornithine

was added. Coupling buffer (30 μ l; acetonitrile:pyridine:triethylamine:H₂O = 10:5:2:3) was added to 50 μ l culture supernatant. The solutions were dried in an exsicator by evaporation; the residues were again dissolved in 100 μ l coupling buffer plus 10 μ l phenylisothiocyanate. After 10 min at room temperature, the solutions were again evaporated to dryness. The derivatized amino acids were dissolved in 150 μ l H₂O and filtered (HV filter, 0.45 μ m, Millipore). Twenty microlitres of the resulting solutions were chromatographed at 38°C using the eluents A (25 mmol/l ammonium acetate in 0.05% phosphoric acid, pH 5.4), B (25 mmol/l ammonium acetate in 0.05% phosphoric acid, pH 5.8:acetonitrile = 1:1) and C (acetonitrile:H₂O = 7:3) at a flow rate of 1.0 ml/min. The column was equilibrated using 90% eluent A and 10% eluent B. The amino acids were separated using the following gradient: after 2 min with 90% eluent A and 10% eluent B the composition was changed linearly to 50% eluent A and 50% eluent B within 20 min. The effluent was monitored at 254 nm. After each run the column was washed for 3 min with eluent C prior to equilibration with the starting eluent for 12 min. The concentrations of citrulline were calculated comparing the peak areas of citrulline and the internal standard.

Statistical analysis

Statistical analysis was made using unpaired two-tailed Student's *t*-test.

Results

Inhibition of macrophage-mediated islet cell lysis by L-NMA

When islet cells are cultured in 1.5 ml micro sample tubes for 23 h, the cells reassociate to well-preserved pseudo-islets (Fig. 1A). Co-culturing islet cells with activated macrophages at a target:effector-cell ratio of 1:2 leads to complete lysis of beta and non-beta cells (Fig. 1B) due to soluble macrophage factor(s) [15]. This lysis is characterized by disrupted plasma membranes concomitant with loss of cytoplasmic content, swollen mitochondria, vesiculated endoplasmic reticulum and chromatin condensation within the nuclei.

N^G-methyl-L-arginine (L-NMA) specifically inhibits NO synthesis by activated macrophages [20, 21]. To investigate whether L-NMA inhibits macrophage-mediated islet-cell lysis, activated macrophages and islet cells were co-cultured for 15 h in the presence of various concentrations of L-NMA in a culture medium containing 1 mmol/l

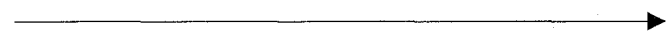
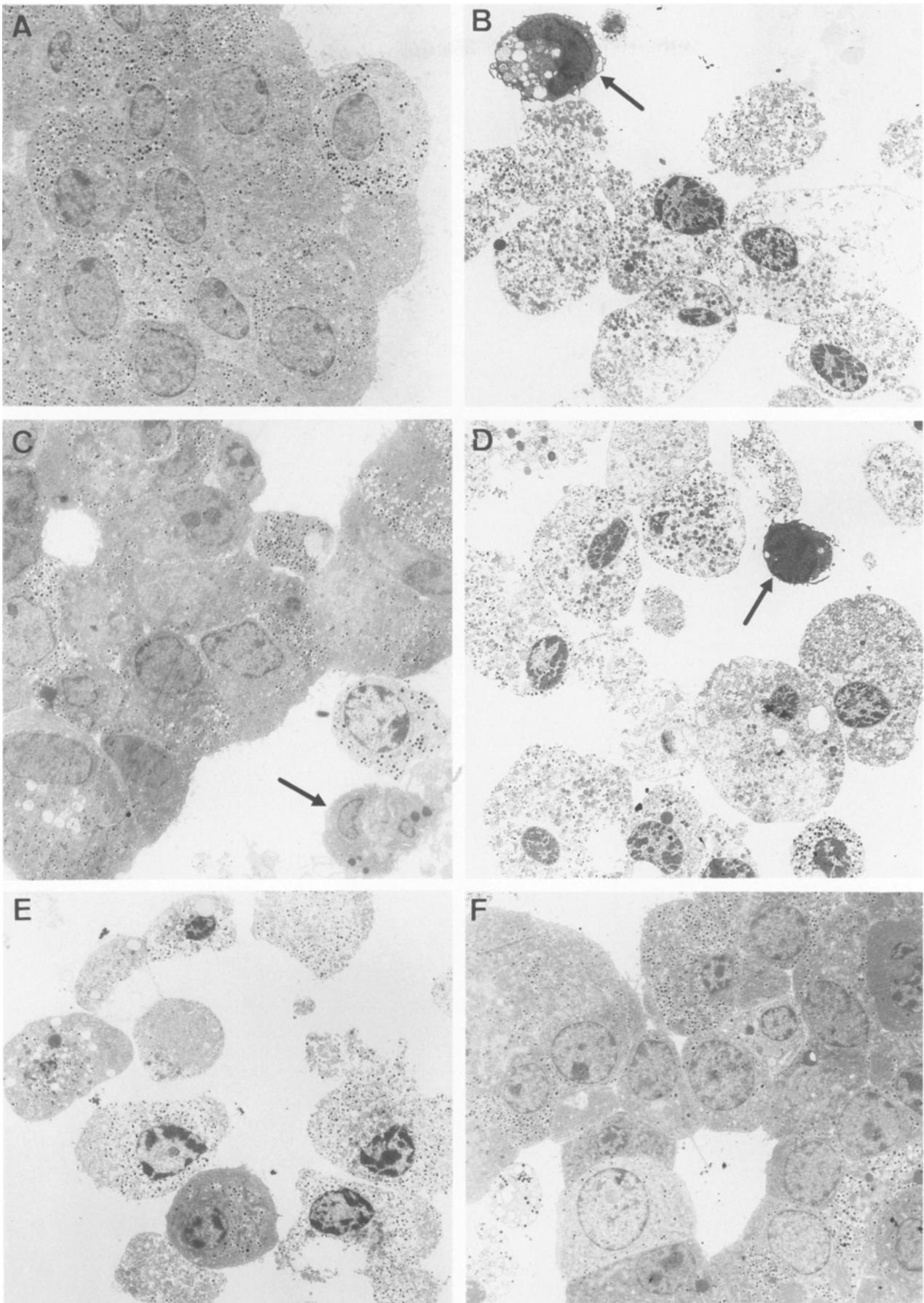


Fig. 1A-F. Low power electron micrographs of islet cells after 15 h of culture in the absence (A, E, F) and presence (B, C, D) of activated macrophages. (A) Islet cell control culture show well-preserved cells. (B) Islet cells cultured in the presence of activated macrophages result in complete lysis of the target cells. Lysis is inhibited by the addition of 0.1 mmol/l N^G-methyl-L-arginine (L-NMA) (C) and inhibition reversed by an excess (11 mmol/l) of L-arginine (D). Culturing islet cells in the presence of 60 U/ml rhodanese, 5 mmol/l thiosulphate and 0.5 mmol/l nitroprusside resulted in almost complete lysis (E) morphologically indistinguishable from lysis mediated by activated macrophages. As a control, islet cells were cultured in the presence of 60 U/ml rhodanese, 5 mmol/l thiosulphate and 2.5 mmol/l cyanide (F) which resulted in minimal lysis only demonstrating that cyanide released from nitroprusside is not the cytotoxic effector molecule under the conditions used. The arrows point to macrophages. Magnification: \times 2340



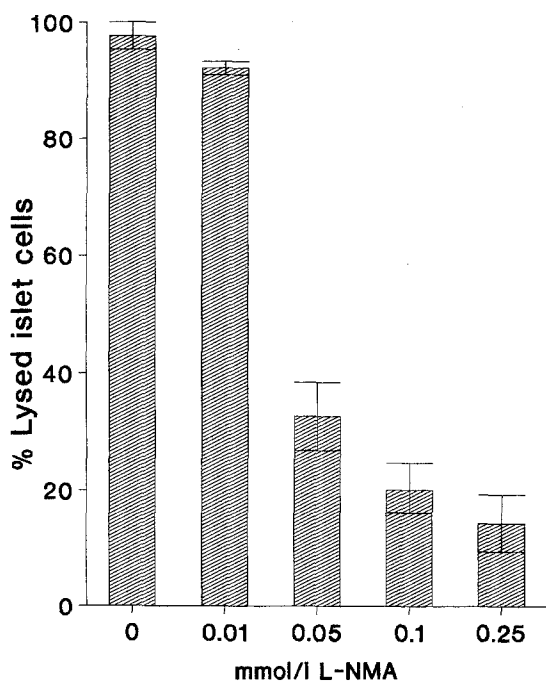


Fig. 2. Concentration-dependent inhibition of macrophage-mediated islet cell lysis by N^G -methyl-L-arginine (L-NMA). Islet cells were co-cultured for 15 h with activated macrophages in the presence of 0–0.25 mmol/l L-NMA in RPMI/10% fetal calf serum containing 1 mmol/l L-arginine. Subsequently, the cells were processed for electron microscopy. For each L-NMA concentration at least 10^3 islet cells were examined for lysis by transmission electron microscopy as described in Materials and methods. Values are means \pm SD of three-five individual specimens

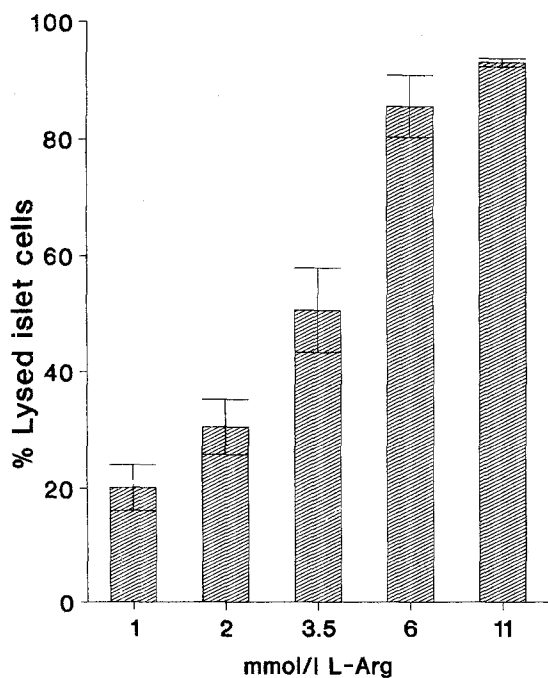


Fig. 3. L-arginine dependence of N^G -methyl-L-arginine inhibition of the macrophage mediated islet cell lysis. Islet cells were co-cultured for 15 h with activated macrophages in the presence of 0.1 mmol/l L-NMA and the indicated concentrations of L-arginine. The cells were processed and examined as described in the legend of Figure 2. Values are means \pm SD of three-five individual specimens

L-arginine. As shown in Figure 2, the inhibition of macrophage-mediated islet-cell lysis showed near-linear correlation to the L-NMA-concentration. 0.25 mmol/l L-NMA provided nearly complete protection from macrophage-mediated lysis (Fig. 1 C). The half-maximal inhibitor-concentration was calculated to be 40 μ mol/l L-NMA which is in the concentration range of half maximal L-NMA-mediated inhibition of NO-production by activated macrophages [22]. Concentrations of up to 0.25 mmol/l L-NMA were not toxic for islet cells (7.9 ± 1.1 % lysed cells).

Specificity of the inhibitory effect of L-NMA

To show that L-NMA specifically inhibits the L-arginine-dependent macrophage-mediated islet-cell lysis, the inhibition experiments with L-NMA were performed in the presence of an excess of L-arginine. Figure 3 shows that inhibition of islet-cell lysis by L-NMA is reversed by L-arginine in a near-linear concentration-dependent manner. Complete restoration of macrophage-mediated lysis of islet cells inhibited by 0.1 mmol/l L-NMA was achieved by 6–11 mmol/l L-arginine. Both, 0.1 mmol/l L-NMA and 11 mmol/l L-arginine, without macrophages added, were not considerably toxic for islet cells (24.6 ± 3.5 % lysed cells). Morphologically the observed lysis was indistinguishable from lysis mediated by macrophages without L-NMA and excess L-arginine (Fig. 1 D).

Nitrite and citrulline production correlates with macrophage-mediated islet-cell lysis

The macrophage NO-synthase converts L-arginine into L-citrulline thereby generating NO which is further oxidized to nitrite and nitrate [6]. To study whether nitrite and citrulline accumulation in the culture medium correlate with islet-cell lysis, time-dependent co-cultures with or without activated or resident macrophages were performed. Lysis of islet cells and nitrite and citrulline concentrations in the supernatants were determined, respectively.

As shown in Figure 4, islet-cell lysis increases with time accompanied by an analogous accumulation of nitrite and citrulline in the culture supernatant. Co-culture of resident macrophages and islet cells for 12 h does not result in significant target cell lysis nor in measurable concentrations of nitrite and low citrulline concentrations in the culture medium. Also, when culturing isolated islet cells without macrophages for 12 h, no nitrite and only small amounts of citrulline could be detected. Culturing 8×10^4 activated macrophages without islet cells but otherwise under the same conditions results in a similar accumulation of nitrite and citrulline not significantly different from the co-culture experiment (data not shown).

No generating compounds cause lysis of islet cells and islets in vitro

Sodium nitroprusside (NP; $\text{Na}_2[\text{Fe}^{\text{II}}(\text{CN})_5\text{NO}]$) is an inorganic complex that generates NO [23]. To investigate whether islet cells are lysed in vitro by NO derived from

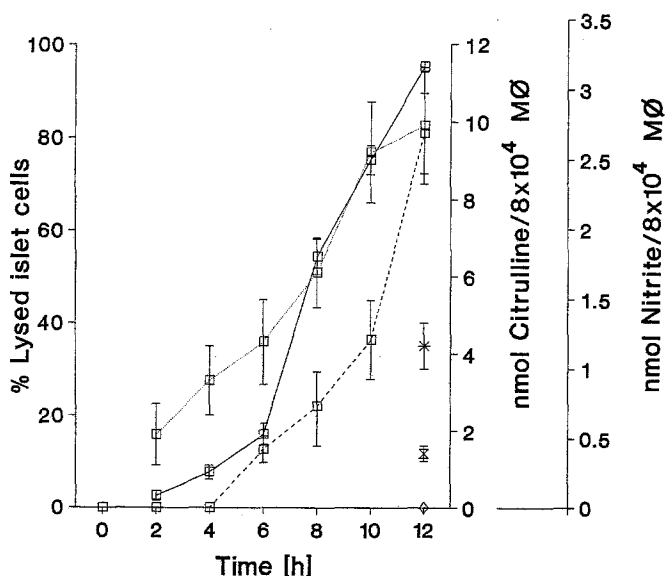


Fig. 4. Correlation of time-dependent lysis of islet cells by activated macrophages with concentrations of nitrite and citrulline in the culture medium. 4×10^4 islet cells were cultured in the presence of 8×10^4 activated (\square) or 8×10^4 resident macrophages (MØ; $\frac{1}{2}$, \star , \diamond), respectively. At the times given, supernatants were removed, deproteinized and stored at -80°C . Nitrite concentrations (dashed line, \diamond) of supernatants were determined spectrophotometrically after diazotization reaction; citrulline concentrations (dotted line, \star) were determined by phenylisothiocyanate derivatization and subsequent reverse-phase HPLC. Culture medium background levels of citrulline were subtracted. Islet-cell lysis (continuous line, $\frac{1}{2}$) was quantified as described in the legend of Figure 2. Values are means \pm SD of three individual specimens

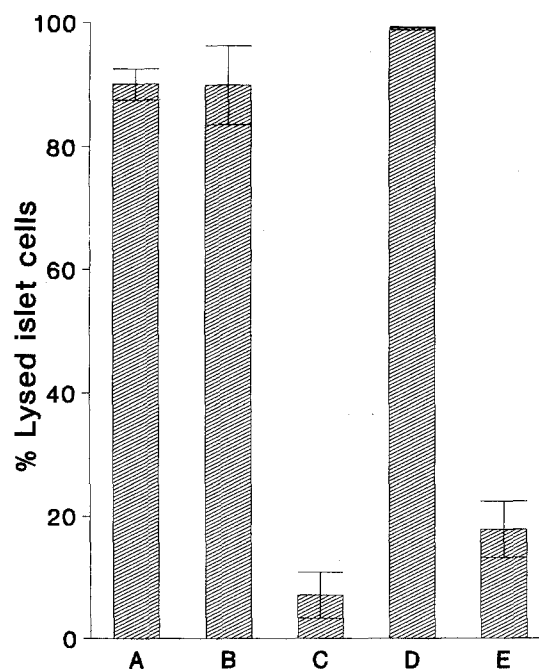


Fig. 5. Effect of nitric oxide (NO) generated by the NO donor nitroprusside (NP) on islet cells. Isolated islet cells were incubated for 15 h with the following compounds: (A) 0.5 mmol/l NP; (B) 0.5 mmol/l NP, 5 mmol/l thiosulphate, 60 U/ml rhodanese; (C) 0.5 mmol/l ferrocyanide; (D) 2.5 mmol/l cyanide; (E) 2.5 mmol/l cyanide, 5 mmol/l thiosulphate, 60 U/ml rhodanese. Cell lysis was quantified as described in the legend of Figure 2. Values are means \pm of three-four individual specimens

NP, isolated islet cells were cultured for 15 h in the presence of 0.5 mmol/l NP. Potassium ferrocyanide ($\text{K}_4[\text{Fe}^{\text{II}}(\text{CN})_6]$), a ferrous cyanide complex without nitrosyl ligand, was used as control. In contrast to activated macrophages, NP does not generate oxygen free radicals in the presence or absence of islet cells, as measured by luminol-amplified chemiluminescence (not shown).

While 0.5 mmol/l ferrocyanide was not toxic for islet cells, 0.5 mmol/l NP caused almost complete lysis of islet cells (Fig. 5). Both beta and non-beta cells were equally lysed (data not shown). Morphologically, lysis caused by NP was indistinguishable from lysis caused by activated macrophages (Fig. 1E). In addition to the rapid release of NO, NP is known to slowly release cyanide ions in aqueous solution [24]. To rule out the possibility that NP-mediated islet-cell lysis is caused by cyanide, the latter was converted to nontoxic thiocyanate by bovine liver rhodanese (thiocyanate:cyanide-sulphurtransferase, EC 2.8.1.1.) [25] which has been shown to be active at 37°C for at least 15 h in the presence of thiosulphate [26]. Rhodanese (60 U/ml) and 5 mmol/l thiosulphate protected islet cells from the toxic effect of 2.5 mmol/l cyanide but not of 0.5 mmol/l NP (Figs. 1F and 5). Neither sodium thiosulphate (5 mmol/l) nor sodium thiocyanate (2.5 mmol/l), respectively, had toxic effects on islet cells ($11.1 \pm 2.1\%$ and $2.1 \pm 1.5\%$ lysed cells, respectively).

To exclude that NP-mediated islet cell toxicity is restricted to the non-physiological state of a dispersed islet

cell suspension and inactive on whole islets, these were also cultured for 15 h in the presence and absence of 0.5 mmol/l NP or 0.5 mmol/l ferrocyanide. Islet morphology was subsequently evaluated by light microscopy. Islets cultured in the absence or presence of ferrocyanide remained intact with typical round to ovoid shape (Fig. 6A). In contrast, islets cultured in the presence of NP exhibited marked disintegration (Fig. 6B) indicative of extensive lysis.

cGMP-analogues do not induce lysis of islet cells

NO in vitro activates the cytosolic guanylate cyclase thus elevating the second messenger cGMP [27]. Indeed, NP and L-arginine have been shown to elevate cGMP-concentrations in isolated rat islets [28, 29]. We therefore tested whether high cGMP levels were sufficient triggers for toxic effects. High concentrations (1 mmol/l) of cell membrane permeable cGMP-analogues in the presence or absence of the phosphodiesterase inhibitor IBMX were added to islet-cell cultures and lysis was quantitated after 15 h. As shown in Table 1, neither IBMX alone, nor IBMX in the presence of any of the cGMP-analogues, nor the phosphodiesterase stable 8-pCPT-cGMP [30], nor, as a control, a cAMP-analogue in the presence of IBMX caused significant islet-cell lysis.

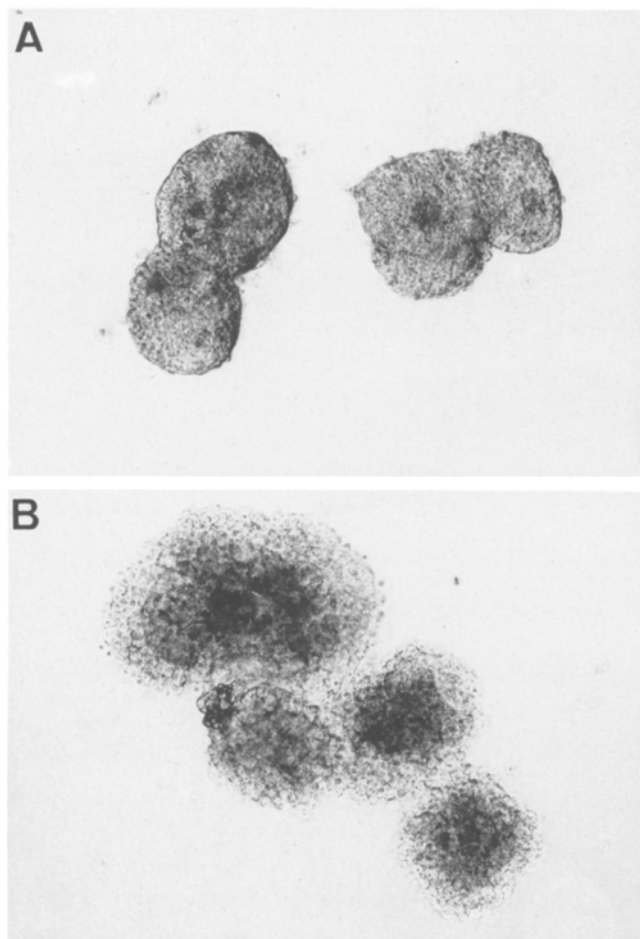


Fig. 6 A, B. Effect of nitroprusside on isolated whole islets. Light microscopy of isolated whole islets cultured for 15 h in the presence of 0.5 mmol/l ferrocyanide (A) or 0.5 mmol/l nitroprusside (B). Magnification: $\times 126$

Discussion

The results presented here show that rat islet cells are susceptible to the toxic effects of NO leading to rapid lysis within 15 h. Firstly, the L-arginine-dependent cytotoxic pathway in activated macrophages is independent from production of reactive oxygen intermediates as has been shown for macrophage cell lines exhibiting either one single or both pathways [31] and by the finding that the L-arginine antagonist L-NMA did inhibit NO but not superoxide production [20]. Although islet cells have been reported to be extremely sensitive towards reactive oxygen intermediates ($\text{OH}\cdot$, O_2^-) [32], we could show that these macrophage products apparently do not play a significant role in islet-cell lysis under the conditions used in our experiments, because L-NMA completely inhibited macrophage-mediated lysis in an L-arginine reversible manner. Secondly, formation of the strong oxidant peroxynitrite and subsequent formation of $\text{OH}\cdot$ by reaction of the two macrophage products NO and superoxide anion [33] does not seem to contribute to a great extent to islet-cell lysis, because islet cells were also lysed by the NO donor sodium nitroprusside, which does not generate oxygen radicals. These results show that indeed NO or a

Table 1. Culturing of isolated islet cells with cGMP-analogues

Compound	% islet-cell lysis
1 mmol/l IBMX	4.7 \pm 0.9
1 mmol/l 8-Br-cGMP + 1 mmol/l IBMX	17.8 \pm 3.9
1 mmol/l dibut-cGMP + 1 mmol/l IBMX	18.2 \pm 2.7
1 mmol/l 8-pCPT-cGMP	14.5 \pm 3.8
1 mmol/l dibut-cAMP + 1 mmol/l IBMX	14.9 \pm 3.7

Islet cells were cultured for 15 h in the presence of cGMP- or cAMP-analogues in the absence or presence of 3-isobutyl-1-methyl-xanthine (IBMX). Subsequently, the cells were processed for electron microscopy as described in the legend of Figure 2. Values are means \pm SD of two-three individual specimens

closely-related, as yet unidentified, reactive nitrogen-oxide intermediate is solely responsible for islet-cell lysis. It thus appears that islet cells are extremely sensitive to NO, because similar or higher concentrations of sodium nitroprusside did not show any cytotoxic effect on other tissue cells such as mesangial cells [34], osteoclasts [35] or smooth muscle cells [36].

The susceptibility of islet cells towards NO is shared by both beta and non-beta cells. However, non-beta cells are located at the periphery mostly of reassociating "pseudo-islets" and – under experimental *in vitro* conditions – are therefore the first target. In contrast, *in vivo* soluble mediators transported via blood will reach beta cells first [37, 38].

Interleukin-1 β (IL-1 β) has been reported to induce NO production in isolated whole islets [39–42]. Although in these reports impaired insulin response was taken as an indication of cytotoxicity, lysis had not been measured. Calculating an average rat islet to consist of about 2000 cells, the nitrite concentrations measured in our co-culture system are at least 10–20 times higher compared to IL-1 β -induced nitrite concentrations of whole islets.

The mechanisms by which NO mediates cytostasis of tumour cells include intracellular loss of iron [43], inactivation and degradation of Fe-S prosthetic groups of citric acid cycle enzyme aconitase [44], complex I and complex II of the mitochondrial electron transport chain [45] and inhibition of ribonucleotide reductase [46, 47], a rate-limiting enzyme in DNA synthesis and repair. In addition, NO-induced genotoxic effects have been found [48, 49]. Our morphological investigations show that co-incubation with activated macrophages as well as with NP results in chromatin condensation within the nuclei of isolated islet cells. First results indicate the occurrence of DNA strand breaks in islet cells as an early sign of toxicity (K. Fehsel, A. Jalowy, S. Qi, V. Burkart, B. Hartmann, H. Kolb, unpublished data).

The physiological role of NO may be to act as a signal transduction effector molecule specifically stimulating the cytosolic guanylate cyclase [27]. It has been shown that short-term incubation with NP or L-arginine indeed elevates the cGMP-concentration in isolated rat islets most probably via NO [28, 29]. However, in our experiments incubation of islet cells with very high concentrations of various cGMP-analogues did not result in significant cell lysis, indicating that elevation of intracellular cGMP is not sufficient to trigger islet-cell death.

In vivo administration of NO-synthase inhibitors partially prevented the onset of hyperglycaemia in low-dose streptozotocin-treated mice, suggesting that NO formation plays an important role in this animal model of Type 1 diabetes [50, 51].

In conclusion, the data presented show for the first time that NO is toxic to islet cells in the absence of other inflammatory mediators and at concentrations which were shown to be non-lytic for other cell types [34–36]. NO-mediated cytotoxicity may play a crucial role in the destruction of normal, non-transformed tissue at sites of inflammatory processes.

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References

- Kolb-Bachofen V, Epstein S, Kiesel U, Kolb H (1988) Low dose streptozotocin-induced diabetes in mice. Electron microscopy reveals single-cell insulinitis before diabetes onset. *Diabetes* 37: 21–27
- Lee KU, Kim MK, Amano K et al. (1988) Preferential infiltration of macrophages during early stages of insulinitis in diabetes-prone BB rats. *Diabetes* 37: 1053–1058
- Hanenberg H, Kolb-Bachofen V, Kantwerk-Funke G, Kolb H (1989) Macrophage infiltration precedes and is prerequisite for lymphocytic insulinitis of pre-diabetic BB rats. *Diabetologia* 32: 126–134
- Oschilewski M, Kiesel U, Kolb H (1986) Administration of silica prevents diabetes in BB rats. *Diabetes* 34: 197–199
- Lee KU, Amano K, Yoon J (1988) Evidence for initial involvement of macrophages in development of insulinitis in NOD mice. *Diabetes* 37: 989–991
- Marletta MA, Yoon PS, Iyengar R, Leaf CD, Wishnok JS (1988) Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry* 27: 8706–8711
- Hibbs JB, Taintor RR, Vavrin Z, Rachlin EM (1988) Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem Biophys Res Commun* 157: 87–94
- Stuehr DJ, Gross SS, Sakuma I, Levi R, Nathan CF (1989) Activated murine macrophages secrete a metabolite of arginine with the bioactivity of endothelium-derived relaxing factor and the chemical reactivity of nitric oxide. *J Exp Med* 169: 1011–1020
- James SL, Glaven J (1989) Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involves arginine-dependent production of reactive nitrogen intermediates. *J Immunol* 143: 4208–4212
- Green SJ, Meltzer MS, Hibbs JB, Nacy CA (1990) Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. *J Immunol* 144: 278–283
- Adams LB, Hibbs JB, Taintor RR, Krahenbuhl JL (1990) Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii*. Role for synthesis of inorganic nitrogen oxides from L-arginine. *J Immunol* 144: 2725–2729
- Hibbs JB, Vavrin Z, Taintor RR (1987) L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J Immunol* 138: 550–565
- Stuehr DJ, Nathan CF (1989) Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition of tumor target cells. *J Exp Med* 169: 1543–1555
- Keller R, Geiges M, Keist R (1990) L-arginine-dependent reactive nitrogen intermediates as mediators of tumor cell killing by activated macrophages. *Cancer Res* 50: 1421–1425
- Kröncke KD, Funda J, Berschick B, Kolb H, Kolb-Bachofen V (1991) Macrophage cytotoxicity towards isolated islet cells: neither lysis nor its protection by nicotinamide are beta-cell specific. *Diabetologia* 34: 232–238
- Appels B, Burkart V, Kantwerk-Funke G, Funda J, Kolb-Bachofen V, Kolb H (1989) Spontaneous cytotoxicity of macrophages against pancreatic islet cells. *J Immunol* 142: 3803–3808
- Kröncke KD, Kolb-Bachofen V, Berschick B, Burkart V, Kolb H (1991) Activated macrophages kill pancreatic syngeneic islet cells via arginine-dependent nitric oxide generation. *Biochem Biophys Res Commun* 175: 752–758
- Wood KS, Buga GM, Byrns RE, Ignarro LJ (1990) Vascular smooth muscle-derived relaxing factor (MDRF) and its close similarity to nitric oxide. *Biochem Biophys Res Commun* 170: 80–88
- Heinrikson RL, Meredith SC (1984) Amino acid analysis by reverse-phase high-performance liquid chromatography: pre-column derivatization with phenylisothiocyanate. *Anal Biochem* 136: 65–74
- Ding AH, Nathan CF, Stuehr DJ (1988) Release of reactive nitrogen intermediates from mouse peritoneal macrophages. *J Immunol* 141: 2407–2412
- McCall TB, Feelisch M, Palmer RJM, Moncada S (1991) Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. *Br J Pharmacol* 102: 234–238
- Lambert LE, Whitten JP, Baron BM, Cheng HC, Doherty NS, McDonald IA (1990) Nitric oxide synthesis in the CNS, endothelium and macrophages differs in its sensitivity to inhibition by arginine analogues. *Life Sci* 48: 69–75
- Feelisch M, Noack EA (1987) Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur J Pharmacol* 139: 19–30
- Leeuwenkamp OR, van Bennekom WP, van der Mark EJ, Bult A (1984) Nitroprusside, antihypertensive drug and analytical reagent; review of (photo)stability, pharmacology and analytical properties. *Pharm Weekbl* 6: 129–140
- Lang K (1933) Die Rhodanbildung im Tierkörper. *Biochem Z* 259: 243–256
- Aird BA, Horowitz PM (1988) The differential functional stability of various forms of bovine liver rhodanese. *Biochim Biophys Acta* 956: 30–38
- Ignarro LJ (1992) Haem-dependent activation of cytosolic guanylate cyclase by nitric oxide: a widespread signal transduction mechanism. *Biochem Soc Trans* 20: 465–469
- Laychock SG (1981) Evidence for guanosine 3',5'-monophosphate as a putative mediator of insulin secretion from isolated rat islets. *Endocrinology* 108: 1197–1205
- Laychock SG, Modica ME, Cavanaugh CT (1991) L-arginine stimulates cyclic guanosine 3',5'-monophosphate formation in rat islets of Langerhans and RINm5F insulinoma cells: evidence for L-arginine: nitric oxide synthase. *Endocrinology* 129: 3043–3052
- Francis SH, Noblett BD, Todd BW, Weels JN, Corbin JD (1988) Relaxation of vascular and tracheal smooth muscle by cyclic nucleotide analogs that preferentially activate purified cGMP-dependent protein kinase. *Mol Pharmacol* 34: 506–517
- Iyengar R, Stuehr DJ, Marletta MA (1987) Macrophage synthesis of nitrite, nitrate and N-nitrosamines: precursors and role of the respiratory burst. *Proc Natl Acad Sci USA* 84: 6369–6373
- Malaisse WJ, Malaisse-Lagae F, Sener A, Pipeleers DG (1982) Determinations of the selective toxicity of alloxan to the pancreatic B cell. *Proc Natl Acad Sci USA* 79: 927–930
- Radi R, Beckman JS, Bush KM, Freeman BA (1991) Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J Biol Chem* 266: 4244–4250

34. Garg UC, Hassid A (1989) Inhibition of rat mesangial cell mitogenesis by nitric oxide-generating vasodilators. *Am J Physiol* 257: F60–F66
35. MacIntyre I, Zaidi M, Towhidul ASM et al. (1991) Osteoclastic inhibition: an action of nitric oxide not mediated by cyclic GMP. *Proc Natl Acad Sci USA* 88: 2936–2940
36. O'Connor KJ, Knowles RG, Patel KD (1991) Nitrovasodilators have proliferative as well as antiproliferative effects. *J Cardiovasc Pharmacol* 17 [Suppl 3]: S100–S103
37. Bonner-Weir S, Orci L (1982) New perspectives on the microvasculature of the islets of Langerhans in the rat. *Diabetes* 31: 883–889
38. Samols E, Stagner JI, Ewart RB, Marks V (1988) The order of islet microvascular cellular perfusion is B→A→D in the perfused rat pancreas. *J Clin Invest* 82: 350–353
39. Southern C, Schulster D, Green IC (1990) Inhibition of insulin secretion by interleukin-1 β and tumor necrosis factor- α via an L-arginine-dependent nitric oxide generating mechanism. *FEBS Lett* 276: 42–44
40. Welsh N, Eizirik DL, Bendtzen K, Sandler S (1991) Interleukin-1 β -induced nitric oxide production in isolated rat pancreatic islets requires gene transcription and may lead to inhibition of the Krebs cycle enzyme aconitase. *Endocrinology* 129: 3167–3173
41. Corbett JA, Lancaster JR Jr, Sweetland MA, McDaniel ML (1991) Interleukin-1 β -induced formation of EPR-detectable iron-nitrosyl complexes in islets of Langerhans. *J Biol Chem* 266: 21351–21354
42. Welsh N, Sandler S (1992) Interleukin-1 β induces nitric oxide production and inhibits the activity of aconitase without decreasing glucose oxidation rates in isolated mouse pancreatic islets. *Biochem Biophys Res Commun* 182: 333–340
43. Hibbs JB, Taintor RR, Vavrin Z (1984) Iron depletion: possible cause of tumor cell cytotoxicity induced by activated macrophages. *Biochem Biophys Res Commun* 123: 716–723
44. Drapier JC, Hibbs JB (1986) Murine cytotoxic activated macrophages inhibit aconitase in tumor cells. *J Clin Invest* 78: 790–797
45. Granger DL, Lehninger AL (1982) Sites of inhibition of mitochondrial electron transport in macrophage-injured neoplastic cells. *J Cell Biol* 95: 527–535
46. Lepoivre M, Fieschi F, Coves J, Thelander L, Fontcave M (1991) Inactivation of ribonucleotide reductase by nitric oxide. *Biochem Biophys Res Commun* 179: 442–448
47. Kwon NS, Stuehr DJ, Nathan CF (1991) Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide. *J Exp Med* 174: 761–767
48. Wink DA, Kasprzak KS, Maragos CM et al. (1991) DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* 254: 1001–1003
49. Nguyen T, Brunson D, Crespi CL, Penman BW, Wishnok JS, Tannenbaum SR (1992) DNA damage and mutation in human cells exposed to nitric oxide in vitro. *Proc Natl Acad Sci USA* 89: 3030–3034
50. Lukic ML, Stosic-Grujicic S, Ostojic N, Chan WL, Liew FY (1991) Inhibition of nitric oxide generation affects the induction of diabetes by streptozotocin mice. *Biochem Biophys Res Commun* 178: 913–920
51. Kolb H, Kiesel U, Kröncke KD, Kolb-Bachofen V (1991) Suppression of low dose streptozotocin induced diabetes in mice by administration of nitric oxide synthase inhibitor. *Life Sci* 49: PL213–PL217

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