Suppression of nitric oxide toxicity in islet cells by α-tocopherol

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Abstract We show here that preincubation of pancreatic islet cells with α-tocopherol significantly improves their resistance to toxic doses of nitric oxide (NO). No protection was afforded by other antioxidants such as vitamin C or glutathione-monoethyl ester. The pathway of NO induced islet cell death involves DNA damage and excessive activation of poly(ADP-ribose)polymerase leading to irreversible depletion of intracellular NAD^+. α-Tocopherol was found to interfere at early steps of this pathway, by preventing the occurrence of DNA strand breaks. This indicates that α-tocopherol directly interacts with NO or its reactive intermediates. We conclude that α-tocopherol is not only part of the cellular defence system against oxygen radicals but also protects eukaryotic cells from NO toxicity.

Key words: Nitric oxide; Antioxidant; Vitamin E; Vitamin C; Pancreatic islet cell

1. Introduction

Nitric oxide (NO) has been identified as a potent and pleiotropic mediator of numerous physiological and immunological reactions. Although the picture of NO functions is far from complete, it is now clear that this radical accounts for the activity of endothelium-derived relaxing factor, acts as neurotransmitter, prevents platelet aggregation and is a major defence molecule of immune cells against parasites, tumour cells and mycobacteria [1,2].

Due to its radical nature NO interacts with several constituents of cells, including heme groups, iron sulfur clusters of enzymes, sulfhydryl residues and DNA [1,2]. While some of these actions serve physiological or immune defence purposes, an undesired effect is the damage of bystander cells and of the NO producing cell itself [3,4].

This situation is reminiscent of the role of oxygen radical intermediates (ROI) in eukaryotic cells. Oxygen radicals, on the one hand, serve as physiological mediators, such as in NF_kB activation, and as immune defence molecules [5,6]. On the other hand, ROI display undesired toxicity towards the cells of the body. Several mechanisms have been identified which provide protection from ROI toxicity in eukaryotic cells, these include ROI inactivating enzymes, as well as hydrophilic and lipophilic antioxidants [7,8].

Recent data suggest that NO may equal or surpass oxygen radicals in contributing to tissue damage during inflammation [3,4,9]. Moreover, vascular damage in long-term diabetic animals could be partly prevented by the administration of oxygen radical scavengers but was fully suppressed in rats receiving an inhibitor of NO formation [10].

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In type 1 (insulin-dependent) diabetes, ROI as well as NO are thought to contribute to islet β cell destruction [9,11,12]. In vitro, NO was identified as the most potent islet-toxic product of inflammatory macrophages [13]. These findings led us to search for possible endogenous scavengers of undesired NO toxicity. In an earlier study we analysed several potent hydrophilic antioxidants and the enzymes superoxide dismutase and catalase for their ability to protect islet cells from NO induced cell death. None of the compounds tested improved islet cell survival which indicated that the oxygen radical defence system of eukaryotic cells may not be involved in prevention of undesired NO toxicity. During the continuation of these studies we now have identified one member of the cellular oxidants defence system which provides protection from NO toxicity in islet cells, α-tocopherol.

2. Materials and methods

2.1. Islet cells

Pancreatic islet cells were isolated from Wistar rats (provided from our own breeding colony) by injection of a collagenase solution in the pancreatic duct (Serva GmbH, Heidelberg, Germany; 0.37 U/mg, 1.5 mg/ml in Hank's balanced salt solution (HBSS; Gibco-Europe, Heidelberg, Germany)) as described [14]. After 35 min of incubation at 37°C the islets were enriched by centrifugation of the dispersed pancreatic tissue on a Ficoll gradient (Ficoll 400, Pharmacia GmbH, Freiburg, Germany). Subsequently, the islets were dissociated in single cells by treatment with 2 mg/ml trypsin (Boehringer-Mannheim, Mannheim, Germany) in Ca^{2+}- and Mg^{2+}-free HBSS (Gibco). For the experiments the islet cells were resuspended in modified RPMI 1640 (Gibco) with a reduced glucose concentration of 4 mM and 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% fetal calf serum (FCS; Gibco).

2.2. Antioxidants

RRR-α-tocopherol (α-tocopherol) was a kind gift of Henkel KGaA (Düsseldorf, Germany), vitamin C (ascorbic acid) was purchased from Sigma and glutathione-monoethyl ester (GME) was purchased from Bachem Biochemica GmbH (Heidelberg, Germany). Stock solutions of the compounds were freshly prepared in modified RPMI 1640 (vitamin C, GME) or ethanol (α-tocopherol) before each experiment. The final concentration of ethanol in the samples with α-tocopherol never exceeded 1% and control experiments showed no detectable effects of 1% ethanol on cell viability or DNA integrity. Also, ethanol did not interfere with NO toxicity in islet cells.

2.3. Nitric oxide cytotoxicity assay

For the analysis of nitric oxide induced islet cell toxicity 2×10^4 islet cells were seeded in triplicates in 120 μl modified RPMI 1640 per well of 1/2-area flat-bottom microtiter plates (Costar, Cambridge, MA, USA). The cells were incubated for different time intervals with various concentrations of α-tocopherol or other potentially protective compounds and exposed to NO for 18 h. In some samples the α-tocopherol was removed by washing the samples three times with fresh culture medium before the exposure to NO. NO was generated chemically by the addition of 0.45 mM sodium nitroprusside (sodium pentacyanonitrosylferrate(II); Merck, Darmstadt, Germany) which releases NO

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during decomposition. Rhodanese (EC 2.8.1.1; 8 U/well; Sigma, Deisenhofen, Germany) and $Na_2S_2O_3$ (5 mM; Merck) were added to scavenge cyanide ions potentially released during the decomposition of sodium nitroprusside [15]. The viability of the islet cells was evaluated by the Trypan blue exclusion assay at the end of the incubation periods. To determine the percentage of dead cells at least 200 cells per well were counted [15].

To analyze DNA damage islet cells were cultivated in Lab-Tec chamber slides (Nunc, Naperville, IL, USA) until adherence 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 [16]. 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 non-specific antibody binding, the cells were incubated for 10 min in phosphate buffered saline with 0.1% thimerosal and 10% FCS. The biotinylated dUTP, incorporated by polymerase-activity at sites of DNA damage, was detected by the peroxidase reaction (Vectastain-Kit, Camon, Wiesbaden, Germany) using diaminobenzidine as substrate. Nuclei with DNA strand breaks appeared brown. At least 100 cells per chamber were evaluated microscopically and the percentage of cells with stained nuclei was determined.

2.4. Statistics

Differences between mean values were analyzed by Student's *t*-test, two-sided.

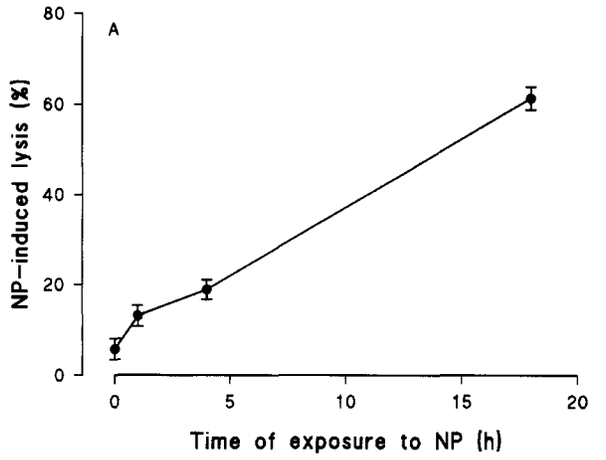
3. Results

When freshly isolated pancreatic islet cells were exposed to NO generated by sodium nitroprusside, plasma membrane lysis was seen only after several hours of incubation (Fig. 1A). For analysis of possible protective activity, α-tocopherol was added to islet cell cultures 6 h prior to addition of the NO donor sodium nitroprusside. In the absence of α-tocopherol, NO-induced islet cell lysis was 65 ± 9% (mean ± S.D.). Significant protection from NO toxicity was observed in the presence of 10–1000 μM α-tocopherol (Fig. 1B). Near-maximal effect was already seen at 10 μM (47% protection from cell lysis) with only minor increased protection with 100-fold higher α-tocopherol levels (60% protection at 1 mM).

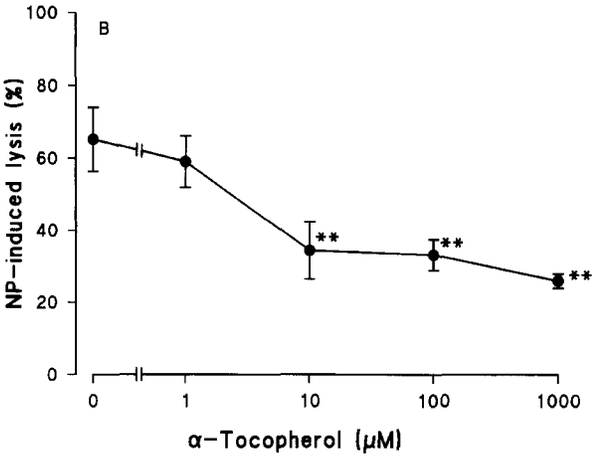
The protective effect was not further improved by extending the time of contact between α-tocopherol and islet cells. As shown in Fig. 2A near-maximal effects of α-tocopherol were already reached with 3 h of preincubation. α-Tocopherol was still partially protective when added immediately prior to the NO donor (at 0 h; Fig. 2A). This rapid action of exogenous α-tocopherol allowed us to analyse whether α-tocopherol exerted its effect on early or late steps of NO toxicity. To reach significant protection the addition of α-tocopherol could be delayed for 2 h, but when given 4 h after the addition of NP cell lysis was not reduced (Fig. 2B).

To exclude a possible direct interaction between NO and α-tocopherol islet cells were preincubated with 100 μM α-tocopherol. After 6 h, the vitamin-containing medium was removed by washing the samples with fresh culture medium. Then NP was added and the lysis of the islet cells was determined after 18 h. As shown in Table 1, preincubation with α-tocopherol reduced islet cell lysis from 64.1% to 44.5%. The protection almost reached the level which was achieved when α-tocopherol was present 6 h before and during the 18 h incubation period with NP (41.7%).

Since DNA strand breaks were observed previously by us as



A



B

Fig. 1. (A) Kinetics of NO induced islet cell lysis. Sodium nitroprusside (NP; 0.45 mM) was added to the islet cells and cell lysis was determined by the Trypan blue exclusion method after various incubation periods. (B) Dose-dependent protection of islet cells from NO induced lysis by α-tocopherol. Increasing doses of α-tocopherol were added to the islet cells 6 h prior to the addition of sodium nitroprusside (NP; 0.45 mM). Islet cell lysis was determined after 18 h of incubation. Data show mean ± S.D. from 3 experiments. $^{**}P < 0.001$ compared to the samples without α-tocopherol.

crucial early events in the destruction of islet cells by NO [17], we determined whether α-tocopherol would prevent DNA damage. As shown in Fig. 3, the percentage of cells with recognizeable DNA damage after 4 and 18 h of incubation with the NO donor was strongly reduced in the presence of 100 μM α-tocopherol.

Finally we compared the potential of α-tocopherol, vitamin C and glutathione to prevent NO toxicity. Significant protection was only seen for α-tocopherol (Fig. 4). Various combinations of the compounds did not provide better protection than α-tocopherol alone (data not shown).

4. Discussion

The data show for the first time that α-tocopherol protects

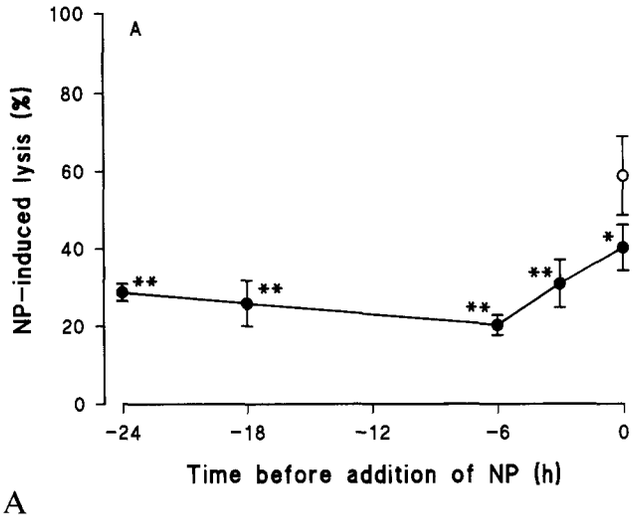


Fig. 2. Effect of α-tocopherol addition before or after initiation of NO exposure. α-Tocopherol (100 μM) was added to the islet cells at various time points before (A) or after (B) the addition of sodium nitroprusside (NP; 0.45 mM). Control samples were incubated with sodium nitroprusside alone (○). The lysis of the islet cells was determined after 18 h of incubation in the presence of sodium nitroprusside (●). Data show mean ± SD from 3 experiments. $^{*}P < 0.01$, $^{**}P < 0.001$ compared to the sample without the addition of α-tocopherol at 0 h.

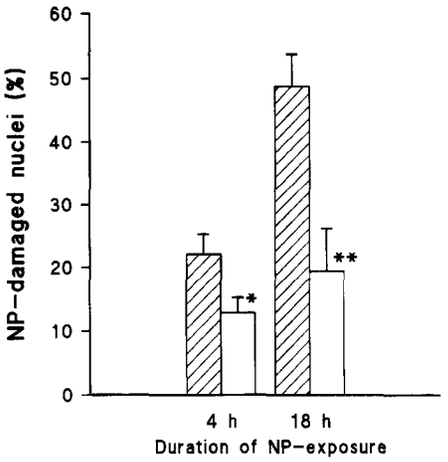


Fig. 3. Reduction of NO induced DNA damage in islet cells by α-tocopherol. Islet cells were preincubated in the absence (hatched bars) or in the presence (open bars) of 100 μM α-tocopherol for 6 h before the addition of sodium nitroprusside (NP; 0.45 mM). The percentage of cells with DNA damage was determined by the in situ nick translation technique after 4 h and 18 h of incubation with the NO donor. Data show mean ± S.D. from 3 experiments. $^{*}P < 0.01$, $^{**}P < 0.001$ compared to the samples treated with NP alone.

eukaryotic cells from NO cytotoxicity. Previously, pancreatic islet cells have been found to be highly susceptible to NO released by inflammatory macrophages or chemical NO donors [14,15,17,18]. Islet cell destruction by NO was significantly suppressed in the presence of ≥ 10 μM α-tocopherol while slight or no protection was afforded by vitamin C or glutathione-monoethyl ester, respectively. The failure of vitamin C or glutathione to significantly improve islet cell survival supports the concept that oxygen radical scavengers are not effective in preventing NO toxicity [15,19,20]. We conclude that antioxidants in general do not mediate defence against NO toxicity, with α-tocopherol as an exception.

What is the mechanism of the protective action of α-tocopherol? The possibility of a direct scavenging of exogenous NO by α-tocopherol present in the culture medium was ruled out in experiments in which α-tocopherol was washed out before the addition of the NO donor sodium nitroprusside. Even under these conditions α-tocopherol exerted a protective effect on islet cells. This observation implies that islet cells have the ability to take up sufficient amounts of α-tocopherol to improve their defence potential against radicals. Recent studies had shown that NO mediated lysis of islet cells is a process which takes at least 4–6 h for completion [15]. The kinetic studies performed indicate that α-tocopherol exerts its protective action during the first hours of exposure to NO. Since sodium nitroprusside was found to release NO at a low rate in aqueous solutions [21] it can be expected that islet cell toxic concentrations of the NO will be reached only after several hours of incubation with the NO donor. This may explain the partial protective effect of α-tocopherol even when the vitamin was added immediately prior to sodium nitroprusside.

The pathway of NO induced cell lysis has been determined by us recently [17,22]. In the early stage it involves DNA strand breaks followed by activation of the DNA repair enzyme

Table 1
Effect of preincubation with α-tocopherol on NO-induced islet cell lysis

Treatment	Islet cell lysis (%)		
	Control	α-Tocopherol incubation period	
		−6 h to 0 h	−6 h to 18 h
None	11.0 ± 2.2	13.0 ± 1.0	12.0 ± 2.2
Nitroprusside (0.45 mM)	64.1 ± 2.2	44.5 ± 2.6*	41.7 ± 2.7*

Islet cells were preincubated for 6 h in the absence or presence of 100 μM α-tocopherol. Then in a set of samples the α-tocopherol was removed by washing the samples. NP (0.45 mM) was added at $t = 0$ and the incubation was continued for 18 h in the absence or presence of α-tocopherol. Islet cell lysis was determined by the Trypan blue exclusion assay and expressed as percent of positive cells. Data show mean ± S.D. from 6 experiments.

$^{*}P < 0.001$ compared to the controls treated with NP alone.

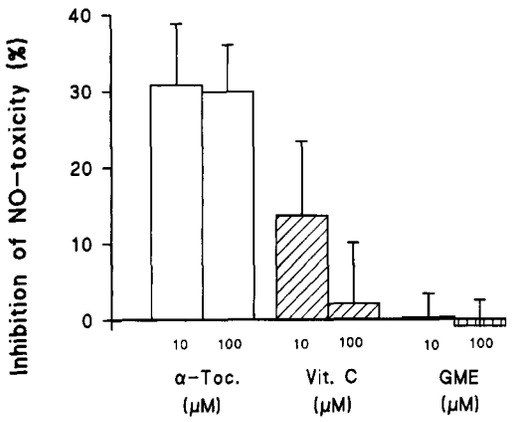


Fig. 4. Potential of α-tocopherol, vitamin C and glutathione-monoethyl ester to prevent NO induced islet cell lysis. Islet cells were incubated with various doses of α-tocopherol (α-Toc.), vitamin C (Vit. C) and glutathione-monoethyl ester (GME) 6 h prior to the addition of the NO donor sodium nitroprusside (0.45 mM). Islet cell lysis was determined after 18 h of exposure to sodium nitroprusside. Controls treated with the NO donor alone were lysed to 65.2 ± 8.8%. Data show mean ± S.D. from 3 experiments.

poly(ADP-ribose)polymerase. The excessive synthesis of poly-ADP-ribose from NAD^+ causes rapid depletion of intracellular NAD^+ stores and subsequent cell lysis. This NAD^+ depletion pathway has been suggested previously to account for streptozotocin toxicity in islet cells and for oxygen radical toxicity in various cell types including islet cells [23]. Other known toxic actions of NO, such as the inhibition of the electron transfer chain in mitochondria, of aconitase and glyceraldehyde-3-phosphate dehydrogenase or the release of intracellular iron do not appear to be involved. Direct evidence for dominance of this mechanism of cell death came from the analysis of cells with a disrupted poly(ADP-ribose)polymerase gene [24]. Protection from cell death by α-tocopherol therefore must be due to interference with the NAD^+ depletion pathway. DNA damage is an early step of this pathway and indeed we found a strong reduction of NO induced DNA strand breaks in the presence of α-tocopherol. We therefore conclude that α-tocopherol suppresses toxicity of NO towards nuclear DNA and thereby prevents induction of the NAD^+ depletion pathway.

This conclusion is supported by earlier studies in bacteria where α-tocopherol was found to suppress the mutagenic, i.e. DNA damaging, action of NO [25]. Interestingly, this study of NO mutagenicity in bacteria also showed little protection by other antioxidants beside α-tocopherol. The only other effective compound was β-carotene [25]. NO-induced alterations of mammalian DNA resemble those seen in cancers of the colon, liver and lungs [26]. Thus, like oxygen radicals, NO is a potential mutagen and may be a factor contributing to carcinogenesis.

It will be difficult to determine in which compartment of eukaryotic cells and by which mechanism α-tocopherol blocks the damaging effect of NO on DNA. In this context a report by de Groot et al. is of interest showing that in a lipophilic solvent α-tocopherol is oxidized to its quinone derivative when in contact with NO gas [27]. Furthermore, it was found that α-tocopherol in low-density lipoprotein is oxidized by peroxynitrite, a product of the reaction between nitric oxide and superoxide [28]. These findings demonstrate that α-tocopherol may directly interact with NO or NO-derived reactive intermediates.

The protective effect of α-tocopherol was found to be dose-dependent, but even at a level of 1 mM exogenous α-tocopherol did not completely prevent NO-induced islet cell death. Possibly, islet cells do not accumulate sufficient quantities of the vitamin. On the other hand, NO toxicity may also affect compartments of the cell with little α-tocopherol content. Finally, NO displays a wide range of chemical reactivities and under certain conditions resembles the positively charged nitrosonium ion or the negatively charged nitroxyl anion rather than the neutral radical [29,30]. α-Tocopherol may not interfere with all of these NO species.

Our findings are very likely relevant to the situation in vivo. NO has been shown to be generated at high levels by the inducible NO synthase at sites of inflammation [3,4,31,32]. The administration of inhibitors of NO synthases has been found to decrease or prevent tissue damage in a large number of chronic inflammatory diseases including immune complex glomerulonephritis, graft versus host disease and arthritis [3,4]. A role of NO in the pathogenesis of type I diabetes has been suggested [33,34], and the administration of large doses of α-tocopherol prevented spontaneous autoimmune diabetes in the NOD mouse [35] while only minor effects were seen in the BB rat diabetes model [36,37]. Taken together these data imply that α-tocopherol participates not only in the cellular oxygen radical defence system but also mediates protection from undesired NO toxicity. α-Tocopherol thus qualifies as a member of the cellular NO defence system, when the majority of compounds remain to be identified.

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