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Involvement of advanced lipooxidation end products (ALEs) and protein oxidation in the apoptotic actions of nitric oxide in insulin secreting RINm5F cells

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Abstract

We have explored the impact of nitric oxide (NO) exposure on oxidation damage of lipids, and proteins, and the contribution of this type of damage to the activation of the apoptotic program in insulin secreting RINm5F cells. Exposure of cells to NO donors and to interleukin-1 beta (IL-1β) led to generation of lipooxidation products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Addition of superoxide dismutase (SOD) and catalase (Cat) to cells decreased by 50% MDA and 4-HNE production induced by IL-1β. Over-expression of Mn-SOD in cells conferred a remarkable decrease (75%) in IL-1β-induced lipid peroxidation. These data suggest that peroxynitrite ($ONOO^-$) mediates peroxidative damage to lipids in this cell system. Inhibitors of advanced lipooxidation end products (ALEs) formation such as aminoguanidine (AG) and pyridoxamine (PM) prevented partially apoptotic events triggered by NO such as DNA fragmentation, caspase-3 activation and cytochrome *c* release from mitochondria. These findings indicate that ALEs are involved in NO-induced apoptosis. In fact, NO-induced carbonylation of PARP protein preceded its apoptotic degradation and inhibitors of ALEs formation prevented both events. We thus propose that carbonylation of proteins is instrumental in linking NO-dependent lipid oxidation and apoptosis in this cell system.

Keywords: RINm5F cells; Apoptosis; Nitric oxide; Interleukin-1β; ALEs; Protein carbonylation

1. Introduction

Evidence gathered over the last decade supports the notion that NO generation is involved in the control of cell death [1,2]. High levels of NO induce release of apoptogenic molecules from mitochondria and degradation of the anti-apoptotic molecule Bcl-2, resulting in apoptosis [3,4]. On the other hand, PKG-dependent protection from apoptotic death by low concentrations of NO has been reported [5,6]. Thus, the picture is emerging that NO contributes to the control of apoptosis in biological systems, although the underlying mechanisms remain to be fully deciphered [7–10]. In insulin secreting cells, apoptotic events such as Bcl-2 breakdown, cytochrome *c* release and caspase activation dissociate from the classical action of NO on the GC/PKG system [11,12]. On the other hand, nitrogen-derived free radicals mediate apoptotic actions of NO in some biological systems [9,13–15]. Thus, interaction of NO with oxygen free radicals at the hydrophobic phase of membranes yields reactive intermediates such as N_2O_3, N_2O_4, NO_2 which promote peroxidative damage to lipids yielding reactive end products such as MDA and 4-HNE [13,16–21]. NO-derived radicals also react with proteins through generation of oxidized derivatives of amino acids such as nitrotyrosine, nitrosothiols and carbonyl derivatives [22–27]. Alternatively, protein carbonylation could result from reaction of lipid end products with side chains of amino acids such as Lys, Arg, Pro and Thr.

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Abbreviations: AG, aminoguanidine; ALEs, advanced lipooxidation end products; DETA/NO, 2,2′-(hydroxynitrosohydrazono)bis-ethanimine; DNPH, 2,4-dinitrophenylhydrazine; 4-HNE, 4-hydroxynonenal; IL-1β, interleukin-1 beta; LDH, lactate dehydrogenase; L-NMMA, *N*-methyl-L-arginine; MDA, malondialdehyde; NO, nitric oxide; NOS2, inducible nitric oxide synthase; SOD, superoxide dismutase; $ONOO^-$, peroxynitrite; PARP, poly (ADP-ribose) polymerase; PM, pyridoxamine; SIN-1, 3-morpholinosydnonimine; SNAP, *S*-nitroso-*N*-acetyl-penicillamine.

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Little evidence is available in the literature on the contribution of protein carbonylative stress to NO-induced apoptosis. We have thus explored this topic and we report that NO-induced protein carbonylation is associated with peroxidative damage to lipids. Nucleophiles that block the formation of MDA–amino acid and 4-HNE–amino acid adducts such AG and PM are used as tools to assess the relationship between lipid and protein oxidative damage and NO-induced apoptotic cell death [28–31]. The finding that inhibition of carbonylation protects cells from NO-induced cell death suggests that oxidative damage of proteins is involved in the regulation of apoptosis.

2. Experimental procedures

2.1. Materials

Aminoguanidine and methanesulfonic acid were from Fluka; IL-1β was from R&D systems; DETA/NO was from RBI; DEVD-AFC, polyclonal anti-Mn-SOD, monoclonal anti-PARP were from Calbiochem; RPMI 1640 was from BioWhittaker; cell death detection ELISA^{plus}, streptomycin, penicillin, geneticin, glutamine, amphotericin B, SOD, catalase, 4(2-aminoethyl)-benzenesulfonyl fluoride (peflablock), pepstatin A and leupeptin were from Roche; aprotinin was from Bayer; protein G-Sepharose and PVDF membranes were from Amersham Pharmacia Biotech; polyclonal anti-macNOS was from Transduction Laboratories; colorimetric assay of lipid peroxidation (LPO-586 method) was from Bioxytech S.A.; monoclonal anti-cytochrome *c* (7H8.2C12) was from Pharmingen; PM, SIN-1, SNAP, L-NMMA, *N*-methyl-2-phenylindole, acetonotrile, 1,1,3,3-tetramethoxypropane, DNPH, polyclonal anti-DNP, anti-mouse and anti-rabbit peroxidase conjugate, trypsin and other chemicals were from Sigma.

$ONOO^-$ was synthesized from $NaNO_2$ and H_2O_2 as in [18]. Aliquots were stored at -80° preserved from light.

2.2. Cell culture and transfection

RINm5F cells were cultured in RPMI 1640 supplemented with 10% FBS (BioWhittaker), 100 μg/mL streptomycin, 100 U/mL penicillin G, 2.5 μg/mL amphotericin B in a humidified atmosphere of 5% CO_2 at 37°. Cells (6×10^6) in 800 μL of serum-free RPMI 1640 were transfected by electroporation with a single pulse of 300 V and 1200 μF capacitance with 5 μg of the expression plasmid prcCMV/Mn-SOD, this construction was provided by Dr. J.A. Meléndez (Department of Biochemistry and Molecular Biology, Albany Medical College, New York). Transfected cells were selected with geneticin (400 mg/L).

2.3. Detection of DNA fragmentation

Cells were scraped off the plates and centrifuged at 700 *g* for 10 min, washed with PBS and suspended in lysis buffer. DNA fragmentation into nucleosomes was determined with the cell death detection ELISA^{plus} according to the manufacturer's instructions.

2.4. Measurement of caspase activity

Cells were spun at 700 *g* for 3 min, washed twice in PBS and suspended in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, and 1 mM EGTA, supplemented with a cocktail of protease inhibitors containing 1 mM pefablock, 100 U/mL aprotinin and 10 μg/mL leupeptin. After 30 min incubation on ice, cells were lysed with 40 strokes in a homogenizer. Following removal of particulate matter by centrifugation at 13,000 *g* for 15 min, supernatants were supplemented with 0.5 mM EDTA and 2 mM DTT. Before measurements, the clarified supernatants were incubated with 20 mM DTT on ice for 30 min in order to revert to a denitrosylated state. DTT was removed by passing samples through a Sephadex G-25 column equilibrated with caspase buffer containing 25 mM HEPES pH 7.5, 0.1% (w/v) CHAPS, 10 mM DTT, 100 U/mL aprotinin, 1 mM pefablock. The supernatants were supplemented with 0.5 mM EDTA and 2 mM DTT. Aliquots containing 50 μg of protein were added to 225 μL of freshly prepared caspase buffer supplemented with 100 μM of caspase-3 substrate DEVD-AFC, and incubated for 2 hr at 37°. The reaction was stopped by addition of 725 μL of ice-cold buffer. Fluorescence was measured using an excitation wavelength of 400 nm and emission wavelength of 505 nm.

2.5. Western blot analyses

For PARP detection, cells were collected by centrifugation at 700 *g* for 3 min at 4°, washed twice with ice-cold PBS and centrifuged at 700 *g* for 3 min. Cells were lysed by suspending the pellet in 62.5 mM Tris–HCl, pH 6.8; 6 M urea; 10% glycerol; 2% SDS; 0.00125% bromophenol blue; 5% β-mercaptoethanol and subsequent sonication for 15 s and incubation at 65° for 15 min. 20 μg of protein were then resolved on 7.5% SDS–polyacrylamide gel and blotted onto PVDF membranes. Membranes were incubated for 4 hr at room temperature with anti-PARP monoclonal antibody Ab-2 (diluted 1:2000).

For cytochrome *c* detection, cells were collected by centrifugation at 700 *g* for 3 min at 4°, washed twice with ice-cold PBS and centrifuged at 700 *g* for 3 min, cell pellets were suspended in 60 μL of extraction buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM HEPES pH 7.5, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 1 mM pefablock, 10 μg/mL pepstatin A, and 10 μg/mL leupeptin. After 30 min of incubation on ice, cells were homogenized by 40 strokes with a Teflon pestle; homogenates were then spun at 13,000 *g* for 15 min and supernatants equivalent to 10 μg of protein were resolved on 12.5% SDS–polyacrylamide gel and then blotted to PVDF membranes. Membranes were incubated for 6 hr at room

temperature with anti-cytochrome *c* monoclonal antibody 7H8.2C12 (diluted 1:2000).

For NOS2 and SOD detection, cells (5×10^6) were homogenized in 50 μL of 20 mM HEPES pH 7.5 supplemented with 10 mM EGTA, 40 mM glycerophosphate, 1% NP-40, 25 mM $MgCl_2$, 140 mM NaCl, 1 mM DTT, 2 mM sodium orthovanadate, 50 μM phenarsine oxide, 1 mM pefablock, 10 μg/mL pepstatin A, 10 μg/mL leupeptin and 100 U/mL aprotinin. Lysis was carried out at 4° for 1 hr. Extracts were centrifuged for 15 min at 13,000 *g*. 30 μg of supernatant protein was resolved on 7.5% for NOS2 and 12.5% for Mn-SOD SDS–PAGE electrophoresis and blotted to PVDF membranes that were incubated for 2 hr at room temperature with anti-macNOS and anti-Mn-SOD antibodies (dilution 1:2000). In all cases, the excess of primary antibody was removed by three washes with 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20 (TBST); membranes were then incubated with anti-mouse IgG conjugated with peroxidase (1:20,000) for 30 min, and immunoreactive bands were detected by enhanced chemiluminescence [32].

2.6. PARP derivatization by dinitrophenylhydrazine (DNPH) and immunochemical detection

Cells were collected by centrifugation at 700 *g* for 3 min at 4°, washed twice with ice-cold PBS and centrifuged at 700 *g* for 3 min, cell pellets were suspended in 50 μL of lysis buffer (20 mM Tris–HCl pH 7.5, 137 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, supplemented with cocktail of proteases inhibitors containing 1 mM pefablock, 10 μg/mL pepstatin A and 10 μg/mL leupeptin). Lysis was carried out at 4° for 30 min. Cell lysates were sonicated three times for 10 s on ice. Lysates were centrifuged at 13,000 *g* for 15 min. 500 μg of protein of clarified supernatants were precipitated with anti-PARP antibody (2 μg/mg protein) for 4 hr at 4°. 20 μL 50% (v/v). Protein G-Sepharose was then added and the mixture was incubated for 4 hr at 4° followed by centrifugation to recover the protein G-Sepharose pellet. The immunoprecipitates were washed three times with PBS and derivatized with 50 μL of 10 mM DNPH (in 2 N HCl) for 1 hr at room temperature. DNPH-derivatized samples were neutralized in 50 μL of 10 mM sodium phosphate buffer pH 7.45 containing 0.1% SDS, 30% glycerol, 5% 2-β-mercaptoethanol and resolved by 7.5% SDS–PAGE. Proteins were blotted onto PVDF membranes. Detection of protein carbonylation was detected with anti-DNP antibody. Immunoreactive bands were detected by enhanced chemiluminescence.

2.7. Assay of cell toxicity

Cells were carefully collected from culture and centrifuged at 700 *g* for 3 min. Supernatants were collected for analysis of lactate dehydrogenase (LDH) activity and pellets were lysed in 5 mM Tris (pH 7.0), 20 mM EDTA, 0.5% Triton X-100. LDH activity was assayed spectrophotometrically following the decrease in absorbance of NADH at 340 nm. The percentage of LDH released from cells to culture medium was calculated according to the following formula: $\%\,\text{LDH relaesed} = (\text{LDH in culture medium}/\text{LDH in culture medium} + \text{LDH in cell lysates}) \times 100$.

2.8. Other analyses

MDA and 4-HNE were measured using the LPO-586 method [33]. Protein concentration was determined by Bradford's technique (Bio-Rad). Data are presented as means ± SD of at least three independent experiments, except where results of blots, in which case a representative experiment is depicted in the figures. Comparisons between group values were analyzed using ANOVA. Differences were considered significant when $P \leq 0.05$.

3. Results

3.1. Effect of NO on lipid peroxidation and necrotic cell death in RINm5Fcells

Figure 1A shows that exposure of cells to NO donors and to $ONOO^-$ leads to an increase in the generation of final lipid peroxidation products MDA and 4-HNE. At the concentration tested (100 μM), $ONOO^-$ showed the strongest action. Generation of NO following induction of NOS2 with IL-1β (50 U/mL) also leads to an increase in the generation of MDA and 4-HNE; inhibition of NOS2 with the L-form of NMMA (500 μM) cancels out this action (Fig. 1B). Figure 1C shows that $FeSO_4$ (200 μM) enhances lipid peroxidation induced by NO. This effect was more important when SNAP was tested. Figure 1D shows that necrotic cell death, measured as percentage of LDH released to medium, induced by NO donor, $ONOO^-$ and IL-1β was not affected when lipid peroxidation was enhanced with Fe^{2+}.

3.2. Effect of antioxidant enzymes on IL-1β-induced lipid peroxidation, DNA fragmentation and LDH release

Figure 2A shows that addition of SOD and catalase to cells decreases MDA and 4-HNE production induced by IL-1β. A 50% decrease in lipid peroxide production was observed when both enzymes were added. Over-expression of Mn-SOD in cells confers a remarkable decrease (75%) in IL-1β-induced lipid peroxidation. Figure 2B shows that IL-1β-induced DNA fragmentation was significantly reduced (45%) in Mn-SOD transfected cells. Figure 2C shows that NOS2 protein level was not altered by Mn-SOD over-expression, ruling out a putative interference of prcCMV expression system on NOS2 expression.

The effect of antioxidant enzymes on NO-induced LDH release is shown in Table 1. Exposure of cells to IL-1β for

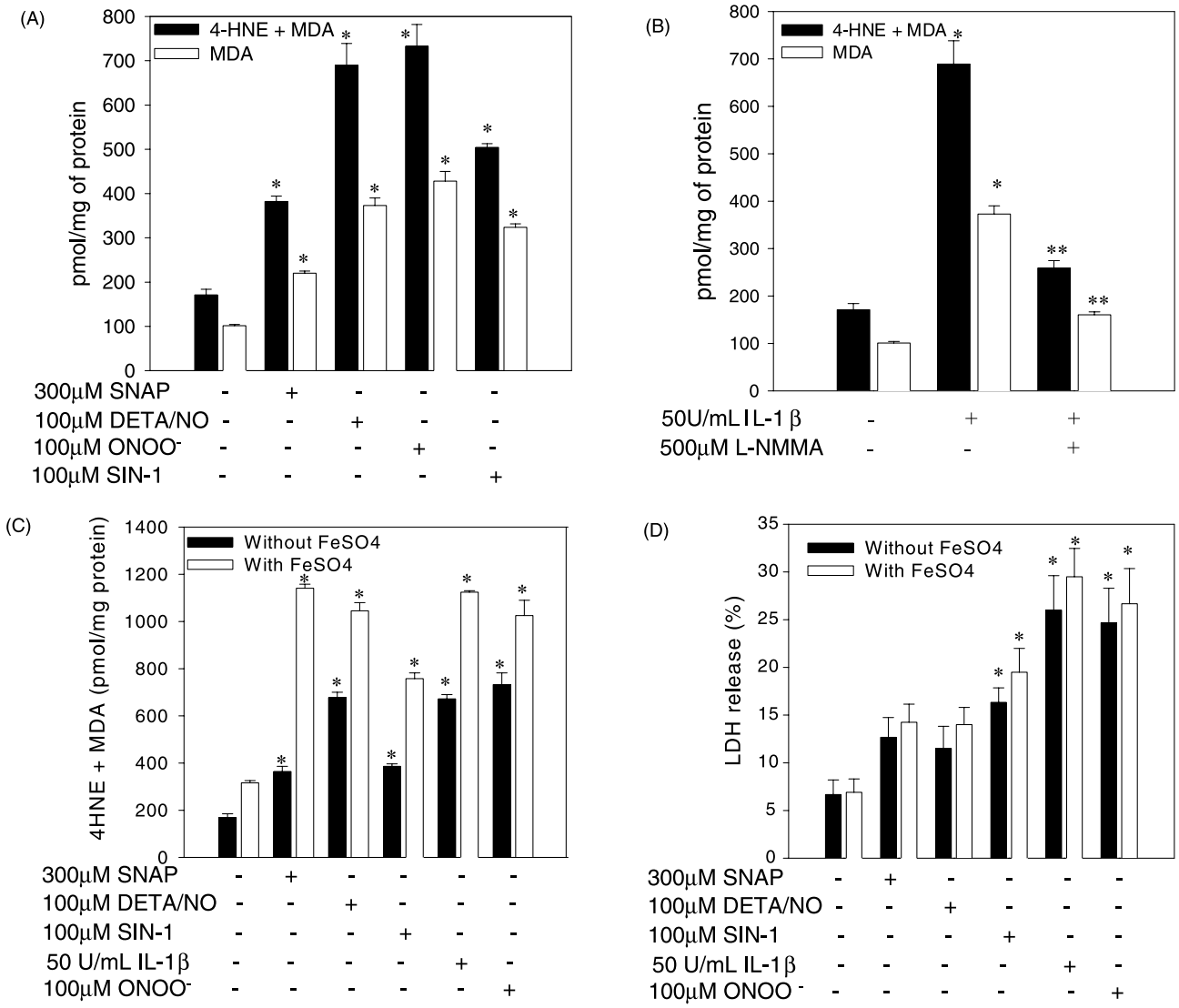

Fig. 1. Effect of NO on lipid peroxidation in RINm5F cells. (A) Lipid peroxidation induced by NO donors and $ONOO^-$. 4-HNE and MDA were measured in supernatants from cells incubated with 100 μM SIN-1, 100 μM SNAP, 100 μM $ONOO^-$ and 100 μM DETA/NO for 1 hr. (B) Lipid peroxidation induced by endogenous generation of NO. Cells were cultured for 24 hr with 50 U/mL IL-1β in the presence or in the absence of 500 μM of the NOS inhibitor L-NMMA. (C) Effect of Fe ions on NO-induced lipid peroxidation. Cells were cultured in the same conditions as in A and B in the absence or in the presence of 200 μM $FeSO_4$. (D) LDH release to medium. Cells were exposed to SNAP, DETA/NO or SIN-1 for 12 hr, to IL-1β for 24 hr and to $ONOO^-$ for 6 hr in the presence or in the absence of $FeSO_4$ (200 μM). Lipid peroxidation and LDH release were measured as described in Section 2. Data are means ± SD from three independent experiments. $^*P \leq 0.005$ vs. control cells; $^{**}P \leq 0.005$ vs. IL-1β-treated cells.

24 hr led to significant release of LDH enzyme to media. Neither addition of SOD and catalase to medium nor over-expression of Mn-SOD were able to modify significantly IL-1β action.

3.3. Effect of ALEs inhibitors on DETA/NO-induced cell death

Figure 3 shows concentration dependency studies of AG and PM on LDH release induced by DETA/NO. No protective action of ALEs inhibitors against DETA/NO-induced LDH release was observed at the concentrations tested (1, 10 mM).

3.4. Effect of ALEs inhibitors on NO-dependent fragmentation and carbonylation of PARP

Panel A in Fig. 4 shows that following exposure of cells to 100 μM DETA/NO for 15 hr, an 85 kDa fragment that cross-react with anti-PARP was observed. The addition of either PM or AG counteracted partially the effect DETA/NO on PARP degradation in a concentration-dependent manner (panel B). Panel C shows that exposure of cells to DETA/NO (100 μM) for 6 hr, led to a strong carbonylation of precipitated PARP that was canceled by AG and PM in a concentration-dependent manner.

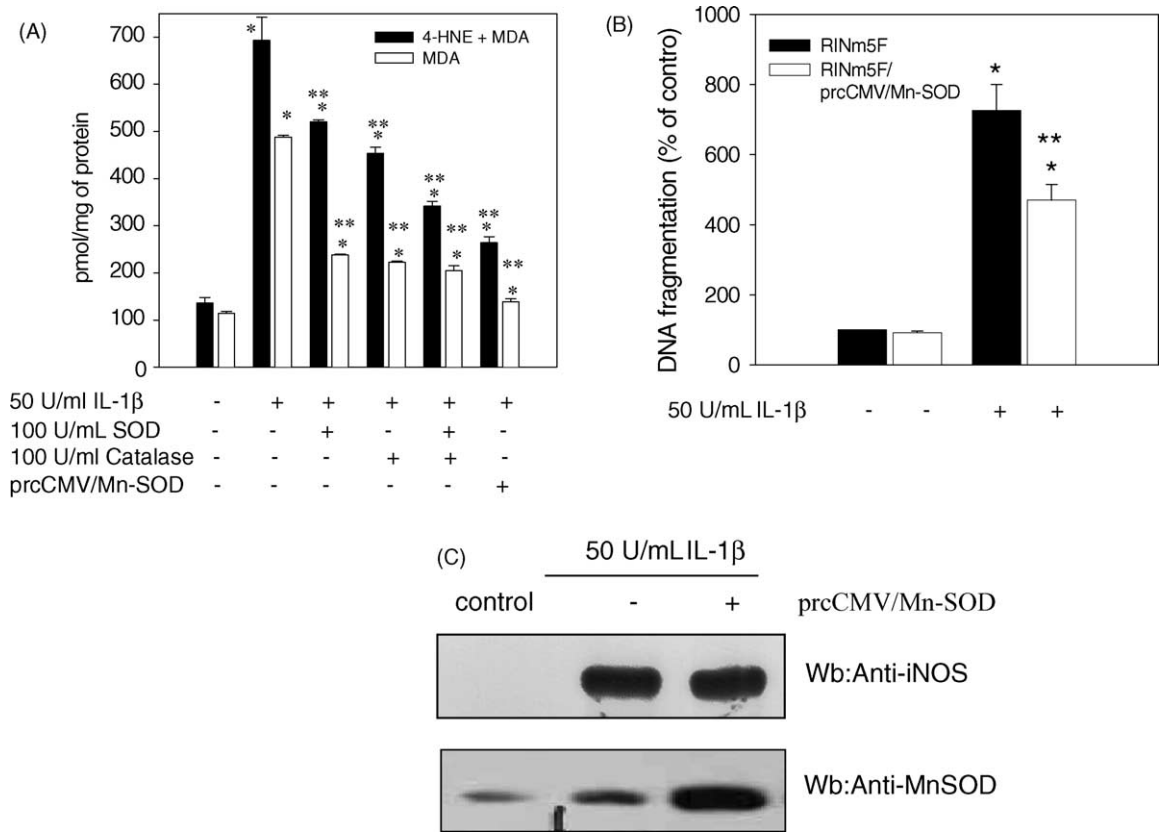

Fig. 2. Effect of antioxidants enzymes on NO-induced lipid peroxidation and DNA fragmentation. (A) Effect of antioxidant enzymes on lipid peroxidation. Non-transfected cells were incubated with IL-1β for 24 hr or in the presence of SOD and catalase; Mn-SOD-transfected cells were cultured in the absence or in the presence of IL-1β. Lipid peroxidation was measured as described in Section 2. (B) Effect of transfection of Mn-SOD on DNA fragmentation. Cells stably transfected with Mn-SOD and non-transfected cells were exposed to 50 U/mL IL-1β for 24 hr. Analysis of DNA fragmentation was performed as indicated in Section 2. (C) Effect of transfection of Mn-SOD on expression of NOS2. Cells stably transfected with SOD and non-transfected cells were exposed to IL-1β for 24 hr. NOS2 and Mn-SOD were detected by immunoblot as described in Section 2. (A, B) Data are means ± SD from three independent experiments. $^{*}P \leq 0.005$ vs. control cells; $^{**}P \leq 0.005$ vs. IL-1β-treated cells. (C) The figure is representative from three independent experiments.

3.5. Effect of ALEs inhibitors on NO-induced activation of apoptotic parameters

Table 2 shows that exposure of cells to 100 μM DETA/NO for 12 hr led to enhanced DNA fragmentation and activation of caspase-3. AG and PM counteracted the effect of DETA/NO on DNA fragmentation in a concentration-dependent manner. NO exposure also leads to 4-fold increase in degradation of the fluorogenic caspase-3 substrate DEVD-AFC. DEVDase activity decreases 63% when cells were cultured in the presence of 1 mM AG and 1 mM PM; no further inhibition was observed at higher inhibitor concentration. Neither AG nor PM by himself significantly induces DNA fragmentation or caspase-3 activation at the concentrations tested (data not shown). When cells were exposed to 100 μM DETA/NO for 12 hr, release of cytochrome *c* from mitochondria to cytosol is observed (Fig. 5). PM and AG blocked NO-induced release of cytochrome *c* in a concentration-dependent manner (panels A and B), the former being the most potent inhibitor.

Table 1
Effect of antioxidants enzymes on IL-1β-induced LDH release

Conditions	LDH release (%)
Control	6.67 ± 1.53
50 U/mL of IL-1β	26.00 ± 3.61*
50 U/mL of IL-1β + 100 U/mL SOD	24.00 ± 2.64*
50 U/mL of IL-1β + 100 U/mL catalase	22.35 ± 3.51*
50 U/mL of IL-1β + 100 U/mL SOD + 100 U/mL catalase	21.33 ± 5.51*
50 U/mL of IL-1β, cells with prcCMV/Mn-SOD	19.52 ± 2.52*

Non-transfected cells were incubated with 50 U/mL IL-1β for 24 hr in absence or in the presence of 100 U/mL SOD, and 100 U/mL catalase (Cat). Cells stably transfected with prcCMV/Mn-SOD were cultured in the absence or in the presence of 50 U/mL IL-1β for 24 hr. LDH release was measured as described in Section 2. Data are means ± SD from three independent experiments.

* $P \leq 0.005$ vs. control cells.

3.6. Discussion

Type I diabetes results from autoimmune destruction of insulin-producing pancreatic β-cells [34]. Although we remain ignorant about fundamental aspects of the pathogenesis of this disease in humans, murine models have been instrumental in deciphering the cellular and molecular

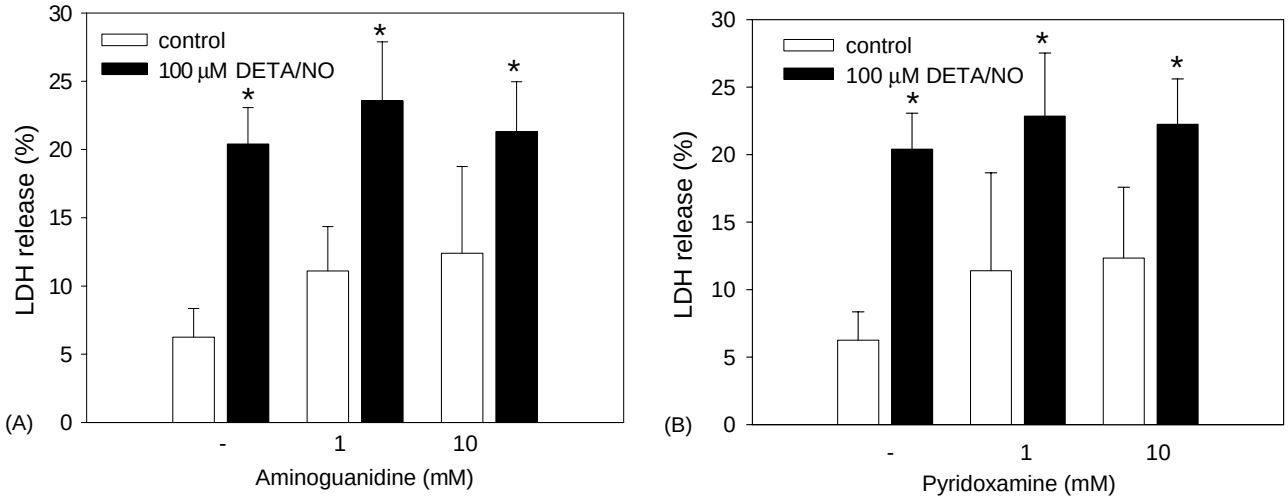

Fig. 3. Effect of DETA/NO on LDH release in RINm5F cells. Effect of AG (A) and PM (B) on LDH release induced by DETA/NO. Cells were exposed to DETA/NO for 12 hr in the absence or in the presence of AG or PM. LDH release was measured as described in Section 2. Data are means ± SD from three independent experiments. $^{*}P \leq 0.005$ vs. control cells.

mechanisms of β-cell death. Thus, a role for NO in the apoptotic death of β-cells has been substantiated with cellular and molecular studies [35–40]. High output of NO generated following induction of NOS2 by activates an apoptotic program that involves cytochrome *c* release, Bcl-2 degradation and caspase-3 activation in insulin-secreting cells [5,11,12,36,40–42]. These phenomena are independent of GC/PKG system, suggesting that additional mechanisms are involved.

It has been established that NO promotes the generation of reactive products such as MDA and 4-HNE from lipids [8,13,17–21,43–45]; we have thus tested the hypothesis that lipid peroxidation play a role in the apoptotic process triggered by NO.

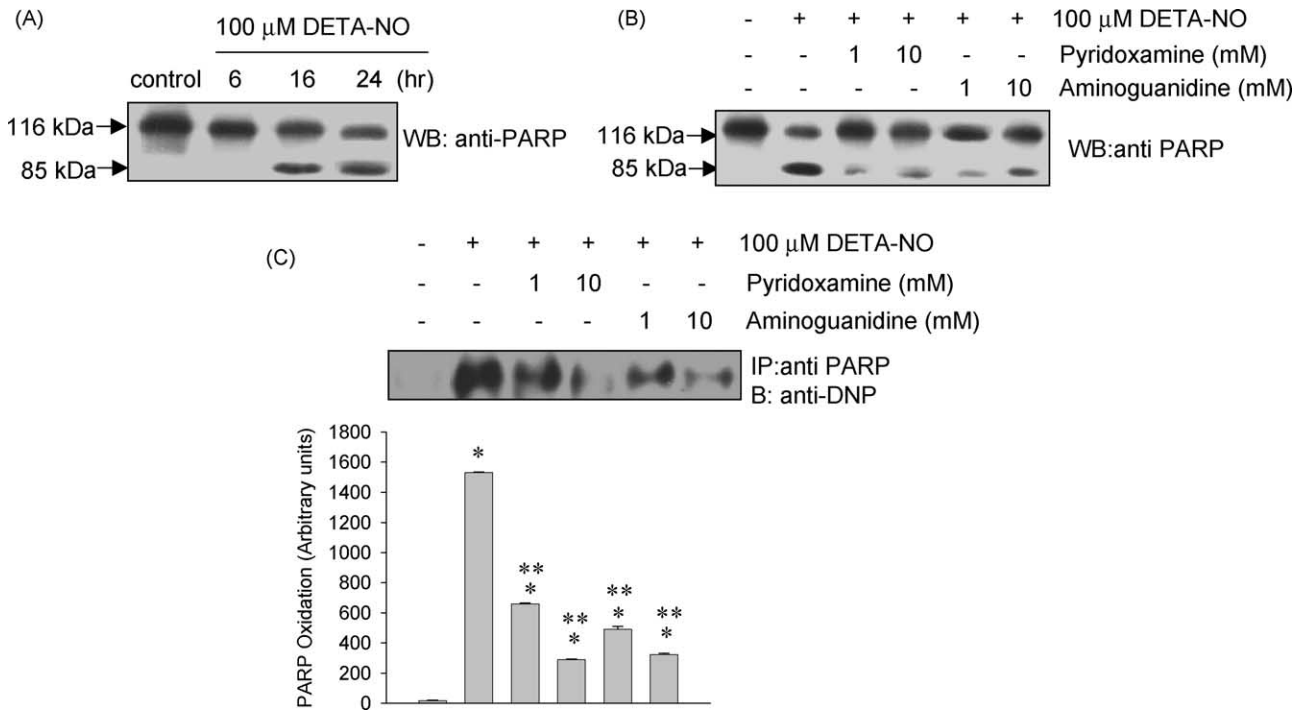

Fig. 4. Effect of ALEs inhibitors on NO-induced PARP degradation and carbonylation. (A) Time dependency study of DETA/NO effect on PARP degradation. Cell were exposed to DETA/NO for the indicated times and PARP was then detected by immunoblot as in Section 2. (B) ALEs inhibitors prevent PARP cleavage induced by NO. Cells were exposed to DETA/NO for 15 hr in the absence or presence of AG and PM. PARP was then detected by immunoblot as described in Section 2. (C) ALEs inhibitors prevent NO-induced PARP carbonylation. Cells were exposed for 6 hr to 100 μM DETA/NO in the absence or presence of AG and PM as indicated. PARP protein was then precipitated from cell homogenates and subsequently submitted to derivatization protocol as in Section 2. Values shown are means ± SD and are expressed as densitometry arbitrary units. Carbonylated PARP was then detected by blot of derivatized protein with anti-DNP as described in Section 2. Figures are representative from three independent experiments. $^{*}P \leq 0.005$ vs. control cells, $^{**}P \leq 0.005$ vs. DETA/NO condition.

Table 2
Effect of ALEs inhibitors on DETA/NO-induced DNA fragmentation and caspase-3 activity

Conditions	DNA fragmentation (% of control)[a]	DEVDase activity (pmol/min/mg protein)[b]
Control	100	55.00 ± 2.00
100 μM DETA/NO	163.00 ± 9.17*	200.00 ± 26.91*
100 μM DETA/NO + 1 mM AG	109.00 ± 4.51**	88.00 ± 2.00*,**
100 μM DETA/NO + 10 mM AG	102.00 ± 3.10**	74.00 ± 4.58**
100 μM DETA/NO + 1 mM PM	90.67 ± 6.81**	85.67 ± 3.63*,**
100 μM DETA/NO + 10 mM PM	97.67 ± 4.04**	75.33 ± 2.52**

[a] 1×10^5 cells were exposed to 100 μM DETA/NO for 12 hr in the absence or in the presence of 1, 10 mM of PM and 1, 10 mM AG as indicated here. Analysis of DNA fragmentation was performed as indicated in Section 2.

[b] Cells were exposed to 100 μM DETA/NO for 12 hr in the absence or in the presence of AG and PM. DEVDase activity was measured as described in Section 2. Data are mean ± SD from three independent experiments.

* $P \leq 0.05$ vs. control cells.

** $P \leq 0.05$ vs. DETA/NO condition.

When insulin-secreting RINm5F cells are exposed to NO donors and to the proinflammatory cytokine IL-1β, a significant increase in MDA/4-HNE production and LDH release was observed (Fig. 1). Final products of lipid oxidation have been have been implicated in several models of cell damage and apoptosis, although the mechanisms involved have not been fully elucidated. We have found that addition of antioxidant enzymes SOD and catalase to cells substantially reduced IL-1β-dependent generation of lipooxidation products; over-expression of Mn-SOD in cells reduced more effectively IL-1β-induced MDA/4-HNE production without interfering with NOS2 protein expression (Fig. 2A and B). On the other hand, IL-1β-dependent release of LDH from necrotic cells was not suppressed by these manipulations, thus suggesting that necrotic death of cells is not dependent of the generation of lipooxidation products (Table 1). Previous work in our laboratory has shown that activation of GC/PKG system mediates both NO and IL-1β-induced cell necrosis [11]. Over-expression of antioxidant enzymes protects against toxicity of a combination of proinflammatory cytokines and of reactive oxygen species but not against that of IL-1β alone in RINm5F cells [46,47]. These findings emphasize the participation of reactive oxygen species (ROS) in the process of pancreatic β-cell destruction, but also indicate that ROS-independent mechanisms are relevant to β-cell death. Determining the contribution of both mechanisms is important for designing preventive strategies. It has been reported that AG inhibits NOS2 enzyme activity and expression [48,49]. This is consistent with the finding that pretreatment with AG attenuated NO-dependent rat intestinal apoptosis after ischemia-reperfusion [50]. An additional action of AG as scavenger of fatty oxidation products has been reported [29]. We thus explored the possibility that AG also protects cells from damage induced by NO generated from chemical donors. We found that AG and PM, an effective scavenger of carbonyl intermediates from carbohydrate and lipid degradation, significantly reduced DNA fragmentation, caspase-3 activation, cytochrome *c* release from

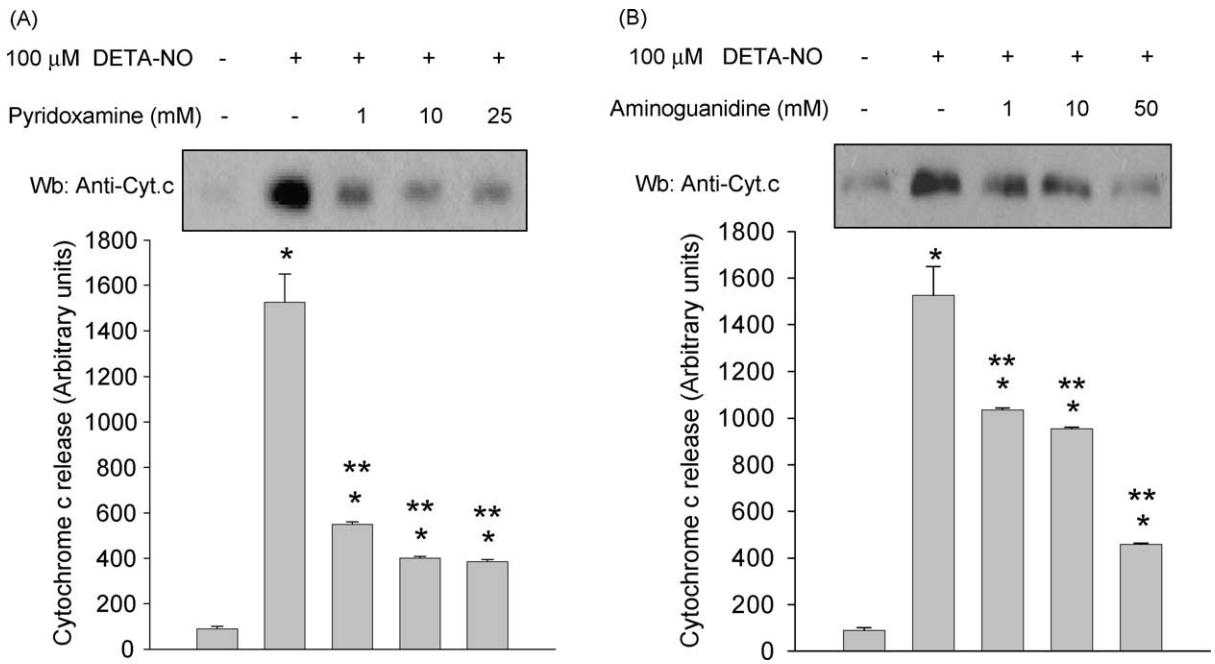

Fig. 5. Effect of ALEs inhibitors on NO-induced cytochrome *c* release in RINm5F cells. (A) PM prevents cytochrome *c* release induced by DETA/NO. Cells were exposed to DETA/NO for 12 hr in the absence or in the presence of PM (1–25 mM). (B) AG prevents cytochrome *c* release induced by DETA/NO. Cells were exposed to 100 μM of DETA/NO for 12 hr in the absence or presence of AG (1–50 mM). Cytosolic cytochrome *c* was then detected by immunoblot as in Section 2. Figures are representative from three experiments. Values shown are means ± SD and are expressed as densitometry arbitrary units. *$P \leq 0.05$ vs. control cells, **$P \leq 0.05$ vs. DETA/NO condition.

mitochondria and PARP degradation induced by NO (Table 2, Figs. 4 and 5) without affecting significantly LDH release (Fig. 3), lipid peroxidation or NO production from donors (data not shown) [30]. We conclude that the mode of cell death is differentially regulated by NO in RINm5 cells. Carbonylation-dependent processes seem to be involved in the apoptotic mode of death, but plays little role in necrotic death. Interestingly, NO-induced carbonylation of PARP protein preceded its apoptotic degradation. AG and PM prevented this actions, thus suggesting that carbonylation of PARP could be mechanistically involved in its degradation during apoptosis (Fig. 4).

The evidence collected in the present paper is consistent with the notion that ALEs generation is involved in NO-triggered apoptotic events in insulin-secreting cells. It is entirely possible that ALEs-dependent carbonylation could control the fate of apoptotic proteins. Identification of targets for carbonylation and elucidation of its relevance in the control of apoptosis in β-cells are thus warranted.

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References

[1] Murphy MP. Nitric oxide and cell death. Biochim Biophys Acta 1999;1411:401–14.
[2] Bredt DS. Endogenous nitric oxide synthesis: biological functions and pathophysiology. Free Radic Res 1999;31:577–96.
[3] Brockhaus F, Brune B. U937 apoptotic cell death by nitric oxide: Bcl-2 downregulation and caspase activation. Exp Cell Res 1998;238:33–41.
[4] Hortelano S, Álvarez AM, Bosca L. Nitric oxide induces tyrosine nitration and release of cytochrome *c* preceding and increase of mitochondrial transmembrane potential in macrophages. FASEB J 1999;13:2311–7.
[5] Tejedo J, Ramírez R, Cahuana G, Rincón P, Sobrino F, Bedoya FJ. Evidence for involvement of c-Src in the anti-apoptotic action of nitric oxide in serum-deprived RINm5F cells. Cell Signal 2001;13:809–17.
[6] LaFevre-Bernt M, Corbin JD, Francis SH, Miller WT. Phosphorylation and activation of cGMP-dependent protein kinase by Src. Biochim Biophys Acta 1998;1386:97–105.
[7] Gow AJ, Ischiropoulos H. Nitric oxide chemistry and cellular signaling. J Cell Physiol 2001;187:277–82.
[8] Wei T, Chen C, Hou J, Xin W, Mori A. Nitric oxide induces oxidative stress and apoptosis in neuronal cells. Biochim Biophys Acta 2000; 1498:72–9.
[9] Eu JP, Liu L, Zeng M, Stamler JS. An apoptotic model for nitrosative stress. Biochemistry 2000;39:1040–7.
[10] Boyd CS, Cadenas E. Nitric oxide and cell signaling patways in mitochondrial-dependent apoptosis. J Biol Chem 2002;383: 411–23.
[11] Tejedo J, Bernabé JC, Ramírez R, Sobrino F, Bedoya FJ. NO induces a cGMP-independent release of cytochrome *c* from mitochondria which precedes caspase-3 activation in insulin producing RINm5F cells. FEBS Lett 1999;459:238–43.
[12] Bernabé JC, Tejedo J, Rincón P, Cahuana G, Ramírez R, Sobrino F, Bedoya FJ. Sodium nitroprusside-induced mitochondrial apoptotic events in insulin-secreting RINm5F cells are associated with MAP kinases activation. Exp Cell Res 2001;269:222–9.
[13] Hughes MN. Relationships between nitric oxide, nitroxyl ion, nitrosonium cation and peroxynitrite. Biochim Biophys Acta 1999;1411: 263–72.
[14] Patel RP, McAndrew J, Sellak H, White CR, Jo H, Freeman BA, Darley-Usmar VM. Biological aspects of reactive nitrogen species. Biochim Biophys Acta 1999;1411:385–400.
[15] Murphy ME, Sies H. Reversible conversion of nitroxyl anion to nitric oxide by superoxide dismutase. Proc Natl Acad Sci USA 1991;88: 10860–4.
[16] Espey MG, Miranda KM, Feelisch M, Fukuto J, Grisham MB, Vitek MP, Wink DA. Mechanisms of cell death governed by the balance between nitrosative and oxidative stress. Ann NY Acad Sci 2000;899: 209–21.
[17] Fabisiak JP, Tyurin VA, Tyurina YY, Sedlov A, Lazo JS, Kagan VE. Nitric oxide dissociates lipid oxidation from apoptosis and phosphatidylserine externalization during oxidative stress. Biochemistry 2000;39:127–38.
[18] Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. Arch Biochem Biophys 1991;288:481–7.
[19] Hogg N, Kalyanaraman B. Nitric oxide and lipid peroxidation. Biochim Biophys Acta 1999;1411:378–84.
[20] Violi F, Marino R, Milite MT, Loffredo L. Nitric oxide and its role in lipid peroxidation. Diabetes Metab Res Rev 1999;15:283–8.
[21] Rubbo H, Radi R, Trujillo M, Telleri R, Kalyanaraman B, Barnes S, Kirk M, Freeman BA. Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. J Biol Chem 1994;269: 26066–75.
[22] Linton S, Davies MJ, Dean RT. Protein oxidation and aging. Exp Gerontol 2001;36:1503–18.
[23] Levine RL, Stadtman ER. Oxidative modification of proteins during aging. Exp Gerontol 2001;36:1495–502.
[24] Stadtman ER. Protein oxidation in aging and age-related diseases. Ann NY Acad Sci 2001;928:22–38.
[25] Hawkins CL, Davies MJ. Generation and propagation of radical reactions on proteins. Biochim Biophys Acta 2001;1504:196–219.
[26] Leeuwenburgh C, Hansen P, Shaish A, Holloszy JO, Heinecke JW. Markers of protein oxidation by hydroxyl radical and reactive nitrogen species in tissues of aging rats. Am J Physiol 1998;274: R453–61.
[27] Adams S, Green P, Claxton R, Simcox S, Williams MV, Walsh K, Leeuwenburgh C. Reactive carbonyl formation by oxidative and non-oxidative pathways. Front Biosci 2001;6:A17–24.
[28] Onorato JM, Jenkins AJ, Thorpe SR, Baynes JW. Pyridoxamine, an inhibitor of advanced glycation reactions, also inhibits advanced lipoxidation reactions. Mechanism of action of pyridoxamine. J Biol Chem 2000;275:21177–84.
[29] Al-Abed Y, Bucala R. Efficient scavenging of fatty acid oxidation products by aminoguanidine. Chem Res Toxicol 1997;10:875–9.
[30] Voziyan PA, Metz TO, Baynes JW, Hudson BG. A post-Amadori inhibitor pyridoxamine also inhibits chemical modification of proteins by scavenging carbonyl intermediates of carbohydrate and lipid degradation. J Biol Chem 2002;277:3397–403.
[31] Unlucerci Y, Bekpinar S, Kocak H. Testis glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase activities in aminoguanidine-treated diabetic rats. Arch Biochem Biophys 2000;379: 217–20.

[32] Carballo M, Vilaplana R, Márquez G, Conde M, Bedoya FJ, González-Vilchez F, Sobrino F. A newly synthesized molecule derived from ruthenium cation, with antitumour activity, activates NADPH oxidase in human neutrophils. Biochem J 1997;328:559–64.
[33] Esterbauer H, Cheeseman KH. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxinonenal. Meth Enzymol 1990;186:407–21.
[34] Atkinson MA, Eisenbarth GS. Type I diabetes: new perspective on disease pathogenesis and treatment. Lancet 2001;358:221–9.
[35] Pipeleers D, Hoorens A, Marichal-Pipeleers M, Van de Casteele M, Bouwens L, Ling Z. Role of pancreatic β-cells in the process of β-cells death. Diabetes 2001;50:S52–7.
[36] Barbu A, Welsh N, Saldeen J. Cytokine-induced apoptosis and necrosis are preceded by disruption of the mitochondrial membrane potential (Deltapsi(m)) in pancreatic RINm5F cells: prevention by Bcl-2. Mol Cell Endocrinol 2002;190:75–82.
[37] Lortz S, Tiedge M, Nachtwey T, Karlsen AE, Nerup J, Lenzen S. Protection of insulin-producing RINm5F cells against cytokine-mediated toxicity through over-expression of antioxidant enzymes. Diabetes 2000;49:1123–30.
[38] Cunningham JM, Green IC. Cytokines, nitric oxide and insulin secreting cells. Growth Regul 1994;4:173–80.
[39] Dimmeler S, Ankarcrona M, Nicotera P, Brüne B. Exogenous nitric oxide (NO) generation or IL-1β-induced intracellular NO production stimulates inhibitory auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase in RINm5F cells. J Immunol 1993;150: 2964–71.
[40] Bergmann L, Kroncke KD, Suschek C, Kolb H, Kolb-Bachofern V. Cytotoxic action of IL-1β against pancreatic islets is mediated via nitric oxide formation and is inhibited by NG-monomethyl-L-arginine. FEBS Lett 1992;299:103–6.
[41] Eizirik DL, Mandrup-Poulsen T. A choice of death—the signal-transduction of immune-mediated beta-cell apoptosis. Diabetologia 2001;44:2115–33.
[42] Tiedge M, Lortz S, Munday R, Lenzen S. Protection against the co-operative toxicity of nitric oxide and oxygen free radicals by over-expression of antioxidant enzymes in bioengineered insulin-producing RINm5F cells. Diabetologia 1999;42:849–55.
[43] Rubbo H. Nitric oxide and peroxynitrite in lipid peroxidation. Medicina (B Aires) 1998;58:361–6.
[44] Bloodsworth A, O'Donnell VB, Freeman BA. Nitric oxide regulation of free radical and enzyme-mediated lipid and lipoprotein oxidation. Arterioscler Thromb Vasc Biol 2000;20:1707–15.
[45] Goss SP, Singh RJ, Hogg N, Kalyanaraman B. Reactions of *NO, *NO2 and peroxynitrite in membranes: physiological implications. Free Radic Res 1999;31:597–606.
[46] Lortz S, Tiedge M, Nachtwey T, Karlssen AE, Nerup J, Lenzen S. Protection of insulin-producing RINm5F cells against cytokine-mediated toxicity through overexpression of antioxidant enzymes. Diabetes 2000;49:1123–30.
[47] Lortz S, Tiedge M. Sequential inactivation of reactive oxygen species by combined overexpression of SOD isoforms and catalase in insulin-producing cells. Free Radic Biol Med 2003;34:683–8.
[48] Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J 2001;357:593–615.
[49] Ruetten H, Thiemermann C. Prevention of the expression of inducible nitric oxide synthase by aminoguanidine or aminoethyl-isothiourea in macrophage and in the rat. Biochem Biophys Res Commun 1996;225: 525–30.
[50] Wu B, Iwakiri R, Tsunada S, Utsumi H, Kojima M, Fujise T, Ootani A, Fujimoto K. iNOS enhances rat intestinal apoptosis after ischemia-reperfusion. Free Radic Biol Med 2002;33:649–58.