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Aminoacetone, a Putative Endogenous Source of Methylglyoxal, Causes Oxidative Stress and Death to Insulin-Producing RINm5f Cells

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Aminoacetone (AA), triose phosphates, and acetone are putative endogenous sources of potentially cytotoxic and genotoxic methylglyoxal (MG), which has been reported to be augmented in the plasma of diabetic patients. In these patients, accumulation of MG derived from aminoacetone, a threonine and glycine catabolite, is inferred from the observed concomitant endothelial overexpression of circulating semicarbazide-sensitive amine oxidases. These copper-dependent enzymes catalyze the oxidation of primary amines, such as AA and methylamine, by molecular oxygen, to the corresponding aldehydes, NH_4^+ ion and H_2O_2. We recently reported that AA aerobic oxidation to MG also takes place immediately upon addition of catalytic amounts of copper and iron ions. Taking into account that (i) MG and H_2O_2 are reportedly cytotoxic to insulin-producing cell lineages such as RINm5f and that (ii) the metal-catalyzed oxidation of AA is propagated by $O_2^{\bullet-}$ radical anion, we decided to investigate the possible pro-oxidant action of AA on these cells taken here as a reliable model system for pancreatic β-cells. Indeed, we show that AA (0.10−5.0 mM) administration to RINm5f cultures induces cell death. Ferrous (50−300 μM) and Fe^{3+} ion (100 μM) addition to the cell cultures had no effect, whereas Cu^{2+} (5.0−100 μM) significantly increased cell death. Supplementation of the AA- and Cu^{2+}-containing culture medium with antioxidants, such as catalase (5.0 μM), superoxide dismutase (SOD, 50 U/mL), and *N*-acetylcysteine (NAC, 5.0 mM) led to partial protection. mRNA expression of MnSOD, CuZnSOD, glutathione peroxidase, and glutathione reductase, but not of catalase, is higher in cells treated with AA (0.50−1.0 mM) plus Cu^{2+} ions (10−50 μM) relative to control cultures. This may imply higher activity of antioxidant enzymes in RINm5f AA-treated cells. In addition, we have found that AA (0.50−1.0 mM) plus Cu^{2+} (100 μM) (i) increase RINm5f cytosolic calcium; (ii) promote DNA fragmentation; and (iii) increase the pro-apoptotic (Bax)/antiapoptotic (Bcl-2) ratio at the level of mRNA expression. In conclusion, although both normal and pathological concentrations of AA are probably much lower than those used here, it is tempting to propose that excess AA in diabetic patients may drive oxidative damage and eventually the death of pancreatic β-cells.

Introduction

Diabetes *mellitus* is a metabolic disorder characterized by hyperglycemia caused by insufficient insulin secretion or receptor insensitivity to endogenous insulin (*1*). High intracellular concentration of glucose can lead to accumulation of methylglyoxal (MG), a potent protein and DNA modifying agent, which has been implicated in the neuropathy, nephropathy, retinopathy, and atherosclerosis manifested in diabetes (*2*−*4*). Model studies with RINm5f cells, a lineage of pancreatic β-cells, challenged with MG (*5*) and H_2O_2 (*6*), have shed some light on the molecular mechanisms implicated in MG and H_2O_2 cytotoxicities. This includes oxidative damage to mitochondria, iron and copper overload, and increased cellular calcium (*7*−*10*).

Triose phosphate (resulting from glycolysis), acetone (from β-ketoacids), and aminoacetone (AA), a catabolite of threonine and glycine, have been suggested as putative endogenous sources of MG in diabetes (Scheme 1) (*2, 11*). Threonine dehydrogenase catalyzes the oxidation of threonine to glycine and acetyl-CoA by NAD^+ (*12*), but when the acetylCoA/CoASH ratio increases, during nutritional deprivation (e.g., in diabetes), the enzyme mainly produces AA (*13*). Aminoacetone is known to undergo enzymatic oxidation by a semicarbazide-sensitive amine oxidase (SSAO), which belongs to a family of copper- and quinone-dependent enzymes, that are capable of promoting oxidative deamination of primary amines, yielding the corresponding aldehydes (MG, from threonine), H_2O_2, and NH_4^+ (*14*). High levels of SSAO, produced by the vessel endothelium, have been found in the plasma of diabetic patients (*14, 15*). However, similar to 5-aminolevulinic acid (ALA) (*16*), a porphyrin precursor implicated as pro-oxidant in porphyric disorders, AA bears an amino group vicinal to the carbonyl function. This structural feature renders both AA and ALA prone to undergo phosphate catalyzed enolization and subsequent iron/copper-catalyzed oxidation to produce the corresponding α-oxoaldehyde, MG and 4,5-dioxovaleric acid, respectively, in addition to deleterious reactive oxygen species (*17, 18*) (Scheme 2).

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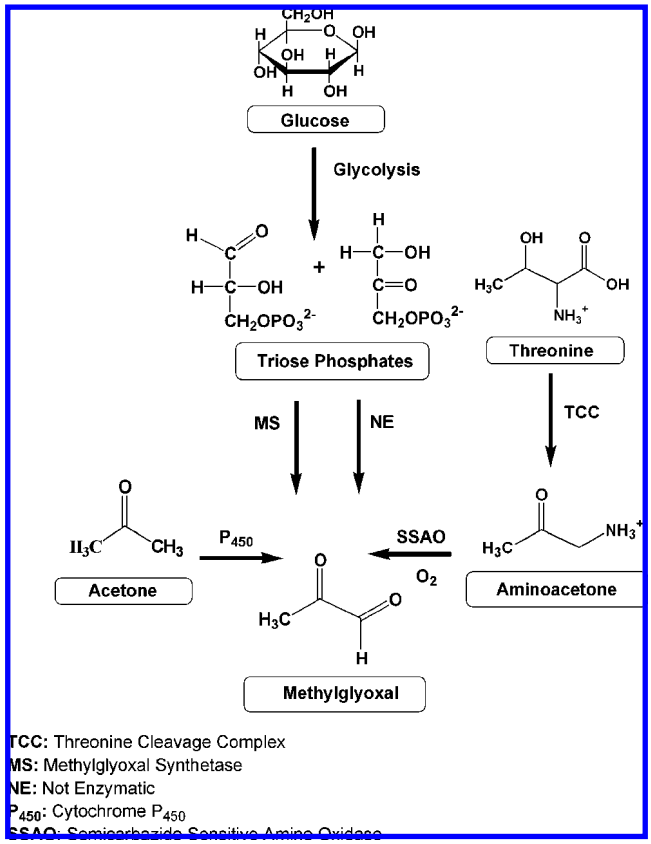
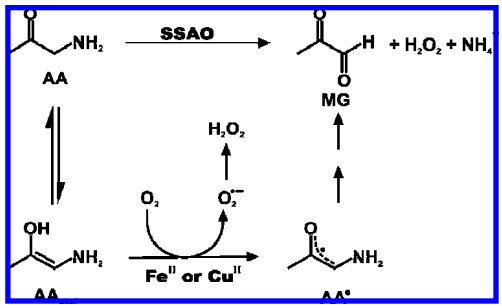
Scheme 1. Enzymatic Pathways for Methylglyoxal Formation, after Kalapos (*11*)



Scheme 2. Enzymatic and Metal-Catalyzed Pathways for Aminoacetone Oxidation to Methylglyoxal, Hydrogen Peroxide, and Ammonium Ion, after Dutra et al. (*21*)



Accordingly, *in vitro* studies revealed that AA (i) promotes DNA damage in HL60 cell cultures, in the presence of Cu^{2+} ions (*19*); (ii) readily consumes oxygen in the presence of horse spleen ferritin, resulting in iron release and decreased ferritin iron uptake and ferroxidase activities (*17*); (iii) causes calcium- and cyclosporine A-mediated transition pore opening, with consequent swelling of isolated rat liver mitochondria (*20*); and (iv) induces protein aggregation, copper release, and decreased ferroxidase activity of ceruloplasmin (*21*), a copper storage protein recently found to be elevated in diabetes (*22*).

Although Ekblom (*23*) mentions that AA and methylamine concentrations in plasma of diabetic patients are increased, the physiological concentrations of AA in tissues of diabetic patients are not yet known, probably by virtue of its conjugation with proteins and SSAO-catalyzed consumption, we decided to investigate the effects of direct treatment of insulin-producing cells with AA and the possible mechanism involved in its action. We show that millimolar AA or micromolar AA/Cu^{2+} are lethal to RINm5f cells by triggering apoptosis and, probably, necrosis (not studied here). This is supported by increased mRNA expression of antioxidant enzymes and apoptotic protein (Bax), DNA fragmentation patterns, and elevated cytosolic calcium.

Material and Methods

Chemical Reagents. Unless otherwise stated, reagents of the highest available purity were purchased from Sigma-Aldrich (St. Louis, MO) and HPLC quality solvents purchased from Merck (Darmstadt, GE). Aminoacetone·HCl was prepared according to Hepworth (*24*) and recrystallyzed from ethanol/ether (8:2). The AA crystals [34% yield; δ (ppm), in D_2O: 2.08 (3H, s), 3.88 (2H, s)] were weighed and sealed in Eppendorf vials into a nitrogen glovebox and stored at −20 °C. Stock solutions of AA were prepared in nitrogen purged Milli-Q purified water immediately before use. Stock solutions of EDTA·Fe^{n+} and citrate·Fe^{n+} were obtained by dissolving the corresponding iron salts in Milli-Q purified water, containing EDTA or citrate in a molar ratio 1:1.2. Concentration of H_2O_2 was determined according to Cotton and Dunford (*25*) and before experiments, its concentration was confirmed from absorbance at 240 nm, using an extinction coefficient of 42 $M^{-1}cm^{-1}$ (*26*). A stock solution of MG was prepared in water and concentration was calculated from its absorption at 286 nm after derivatization with semicarbazide hydrochloride ($\varepsilon = 32 \times 10^3$ $mol^{-1}L$ cm^{-1}) (*27*). All other stock solutions and buffers were prepared with Milli Q Millipore deionized water.

Cell Cultures. RINm5f and NIH-3T3 fibroblast cells were maintained, respectively, in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, MD) and Dulbecoo's modified Eagles's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Cultilab, Campinas, Brazil), 50 U/mL penicillin, and 50 U/mL streptomycin (Gibco, MD). Cells were grown in an atmosphere of 97.5% O_2/2,5% CO_2 at 37 °C and passaged before reaching confluence using trypsin/EDTA (Gibco, MD).

Cell Viability. RINm5f cells were seeded in 200 μL culture medium (FCS 10%) in 96-well microplates (10^4 cells/well) and allowed to attach to the plates for 48 h before the addition of AA. Cytotoxicity was monitored after AA treatment for 24 h in medium containing (or not) FCS using a microplate-treatment test with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (*28*). Viability was expressed in percentage of MTT (10 mg/mL) absorbance at 570 nm, measured in the absence and in the presence of AA. Enzymatic and chemical antioxidants, namely, catalase (5.0 μM), superoxide dismutase (50 U/mL), NAC (5.0 mM) and bathocuproine, a cuprous ion chelator (0.10 mM), were added to AA-treated cell cultures to provide protection against AA-induced oxidative stress. Pro-oxidant Fe^{2+} EDTA (5.0−300 μM), Fe^{2+} citrate (100−300 μM), Fe^{3+} EDTA (100 μM) and aqueous Cu^{2+} ions (5.0−300 μM) were added to the plates aiming to enhance the AA cytotoxicity.

mRNA Expression of Antioxidant Enzymes. RINm5f cells were seeded in 1 mL of medium in 12-well microplates (10^5 cells/well) and allowed to attach to the plates for 48 h before the addition of AA plus Cu^{2+} ions. The cells were exposed to AA (0.50−1.0 mM) plus Cu^{2+} ions (10−50 μM) in medium without FCS for 2 h, and after removal of AA/Cu^{2+} ions, for an additional period of 22 h at in RPMI-1640 supplemented with 10% FCS. Total RNA was isolated using the Trizol reagent (Invitrogen, Carisbad, CA). The RNA quality control was performed through the 280/260 and 230/260 ratios. The cDNA was synthesized by the use of Super Script III (Invitrogen). The amplification of the resulting cDNAs was performed using the SYBR GREEN Master Mix (Applied Biosystems, Foster City, CA) in a GeneAmp 7300 Sequence Detection System (Applied

Table 1. Gene Specific PCR Primers Used for Antioxidant Enzymes and Pro- and Anti-Apoptotic Proteins

gene	sequences	
	sense	antisense
Catalase	ATTGCCGTCCGATTCTCC	CCAGTTACCATCTTCAGTGTAG
GPx	GTTCGGACATCAGGAGAATGG	GGGTTCGATGTCGATGGTGC
MnSOD	GACCTGCCTTACGACTATG	TACTTCTCCTCGGTGACG
CuZnSOD	CCAGCGGATGAAGAGAGG	CCAATCACACCACAAGCC
GRd	CGGAAGTCAACGGGAAGAAG	CCACCCGTGGCGATCA
Bax	CAAGAAGCTGAGCGAGTGTC	GAAGTTGCCGTCTGCAAACA
Bcl-2	CTGGGATGCCTTTGTGGAA	CAGCCAGGAGAAATCAAACAGA

Biosystems) under the following conditions: 50 °C 2 min, 95 °C 10 min, 40 rounds of 95 °C for 15 s and 60 °C 1 min. A dissociation cycle was performed after each run to check for nonspecific amplification or contamination. The relative expression levels were estimated by utilizing the $2^{-\Delta\Delta Ct}$ formula (*30*), using HPRT as the housekeeping gene. The set of primers was designed using Primer Express 3 (Applied Biosystems) and validated through BLAST and BLAT. The primers used are listed in Table 1.

Cellular DNA Fragmentation. RINm5f cells were seeded in 1.0 mL medium in 12-well microplates (10^5 cells/well) and allowed to attach to the surface for 48 h before the addition of AA (1.0 mM) plus Cu^{2+} ions (100 μM). Aminoacetone plus Cu^{2+} ions were added to the culture medium in the absence of FCS for 24 h. The medium was then removed and the cells were harvested, rinsed twice with phosphate-buffered saline (PBS) and lysed in a hypotonic buffer, pH 7.4, containing 50 μL/mL propidium iodide (Invitrogen), sodium citrate 0.10% m/v, and Triton X −100 0.10% m/v. Cellular DNA fragmentation was measured by flow cytometry as described by Formichi (*29*).

mRNA Expression of Pro- and Anti-Apoptotic Proteins. RINm5f cells were seeded in 1 mL of medium in 12-well microplates (10^5 cells/well) and allowed to attach to the plates for 48 h before the addition of AA plus Cu^{2+} ions. The cells were exposed to AA plus Cu^{2+} ions in medium without FCS for 24 h. The medium was then removed, the cultures were rinsed twice with PBS and the cells were harvested. Total RNA was extracted from the cell cultures (10^5 cells/well approximately) using the Trizol reagent (Invitrogen), according to the manufacturer instructions. cDNA was synthesized from 2 μg of total RNA using the SuperScript III Reverse Transcriptase kit (Invitrogen), with a random and OligodT primers mixture. Real-time PCR was performed according to the SybrGreen assay protocol (Applied Biosystems), using the Sequence Detector ABI PRISM 5700, (Perkin-Elmer/Applied Biosystems). The gene-specific PCR primers, which are listed in Table 1, span an intron within the cDNA sequence target, making the cDNA amplification product easily distinguishable from the genomic product. We used a 2-step amplification protocol, with a denaturing temperature of 95 °C and an annealing-extension temperature of 60 °C. Relative gene expression levels were calculated from cycle threshold values (Ct) using the $2^{-(\Delta Ct - \Delta\Delta Ct)}$ formula (*30*). The rat GAPDH and HPRT genes were used as internal controls for each individual sample gene expression.

Intracellular Calcium. RINm5f cells were seeded in one mL medium in 12-well microplates (10^5 cells/well) and allowed to attach to the plates for 48 h before the addition of AA plus Cu^{2+} ions. The cells were exposed to AA (0.50−1.0 mM) plus Cu^{2+} ions (100 μM) in medium without FCS for 4 h. The medium was then removed and the cells were harvested and resuspended in one mL of fresh medium. One hundred nanomoles Fluo-4 AM (*31*). (Molecular Probes, Invitrogen) were added to the cultures, followed by incubation for 45 min in the dark at 37 °C prior to analysis by flow cytometry.

Statistical Analysis. The results were analyzed by Student's *t* test (to compare two groups) and One-Way ANOVA, using Bonferroni Test (GraphPad Prism version 4.02) (for analyses of three or more groups). Probability of $p < 0.05$ was used as the criterion for statistical significance. Each experimental datum represents at least three different wells subjected to the same treatment.

Results

Cell Viability upon Treatment with AA/Metal Ions. Treatment of RINm5f cells with AA (1.0−5.0 mM) in FCS-containing medium for 24 h, led to a concentration-dependent decrease in the number of viable cells (Figure 1). Cell death could also be induced by addition of the AA oxidation products to the cell cultures, namely, MG (0.10−5.0 mM) (Figure 1) and H_2O_2 (5.0−200 μM) (Figure 1). That millimolar MG and micromolar H_2O_2 are lethal to insulin-producing cells, including the RINm5f lineage, was previously reported by several authors (*32, 33*). Kalapos et al. (*34*) observed glutathione depletion upon administration of 20 mM MG to isolated mouse hepatocytes, which implies increased MG-triggered cell susceptibility to oxidative stress.

For comparison, NIH-3T3 fibroblast cultures were also challenged with millimolar AA but were found to be much less sensitive to the treatment than the insulin-producing cells (Figure 2). The much higher concentration of AA than those of MG and H_2O_2 required to cause significant cell death is only apparent, since the extent of AA oxidation medium to MG plus H_2O_2 in the culture is limited by the amount of dissolved oxygen (solubility of O_2 in water at room temperature is roughly 200 μM) and by the actual concentration of free MG in aqueous medium, which is less than 1% of the nominal title due to extensive hydration of MG to MG.H_2O and MG.2H_2O (*33*). In addition, AA is known to undergo dimerization in concentrated

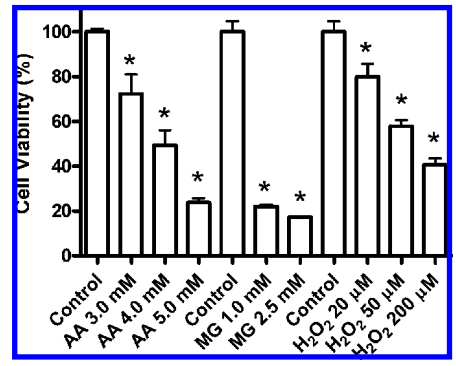


Figure 1. Aminoacetone, methylglyoxal, and hydrogen peroxide effect on the viability of RINm5f insulin-producing cells. Cells (10^4 cells/well) were treated in FCS 10% (v/v)-containing RPMI-1640 medium with 1.0−5.0 mM AA, 0.10−2.5 mM MG, or 5.0−200 μM H_2O_2 for 24 h. Each set of experiments, performed on different days, is referred to a distinct control sample. Viability was measured by the MTT assay and expressed as % of untreated cells. All values represent the mean ± SEM of 4 independent experiments. *$p < 0.05$ vs control.

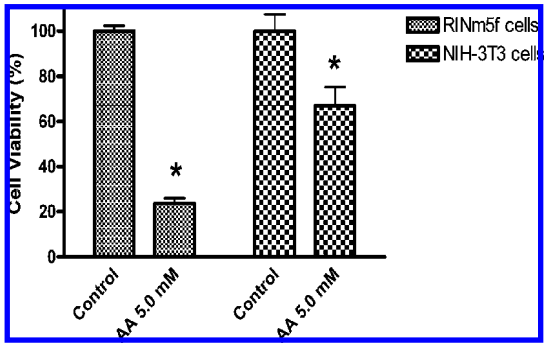


Figure 2. Viability of RINm5f cells and NIH-3T3 fibroblasts treated with AA. RINm5f (10^4 cells/well) and NIH-3T3 (10^4 cells/well) cells were cultured in FCS 10% (v/v)-containing RPMI-1640 and DMEN medium with AA (5.0 mM) for 24 h. Viability was measured by the MTT assay and expressed as % of untreated cells. All values represent the mean ± SEM of 3 independent experiments. *$p < 0.05$ vs control.

stock solutions at neutral pH (*19*) and oxidation by molecular oxygen, especially in the presence of adventitious transition metals (*17*). The scarce literature on lethal effects of AA directly administered to cells is probably due to the fact that only recently AA became commercially available because its preparation poses technical difficulties related to stability and recrystallization (*33*).

Similar to dihydroxyacetone phosphate (*35*) and ALA (*36*), chemically viewed as α-hydroxycarbonyl and α-aminocarbonyl metabolites, respectively, AA is rapidly enolized at physiological pH and further oxidized by molecular oxygen in the presence of transition metal ions (Fe^{n+} and Cu^{n+}). This reaction is propagated by the superoxide anion radical that could exert direct or indirect, cell signaling-mediated toxic effects to the RINm5f cells, including apoptosis (*37*).

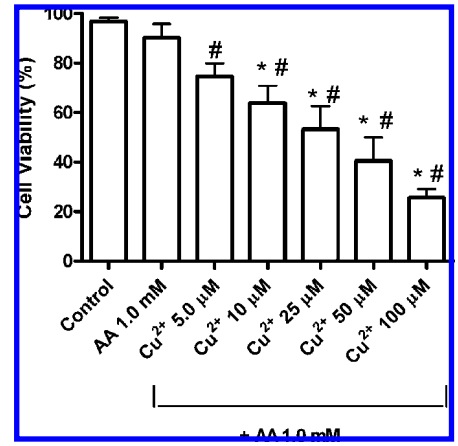


Figure 4. Concentration effect of Cu^{2+} ion on RINm5f cells treated with AA. RINm5f cells (10^4 cells/well) treated with 1.0 mM AA in the presence (or not) of 5.0−100 μM Cu^{2+} ion for 24 h in the absence of FCS. In the absence of AA, Cu^{2+} ion (5.0−100 μM) did not significantly affect cell viability (not shown). Viability was measured by the MTT assay and expressed as % of untreated cells. All values are the mean ± SEM of 4 individual experiments. *$p < 0.05$ vs control and # $p < 0.05$ vs AA control.

Taking into account that diabetes is clinically and biochemically a multifaceted disorder, including iron and copper discharge in blood and other tissues (*38*), we set out to examine a possible enhancing effect of aqueous Cu^{2+}, Fe^{2+}EDTA, Fe^{3+}EDTA, and Fe^{2+}citrate on the AA toxicity to RINm5f cells. Indeed, addition of micromolar Cu^{2+} to AA (0.50−1.0 mM)−containing RINm5f cultures increased the rate of cell death (Figure 3), whereas iron complexes were ineffective at the concentrations used here (not shown). One could argue that the cell plasmatic membrane is not fully permeable to the iron complexes (*39*). Interestingly, cells raised in FCS-supplemented

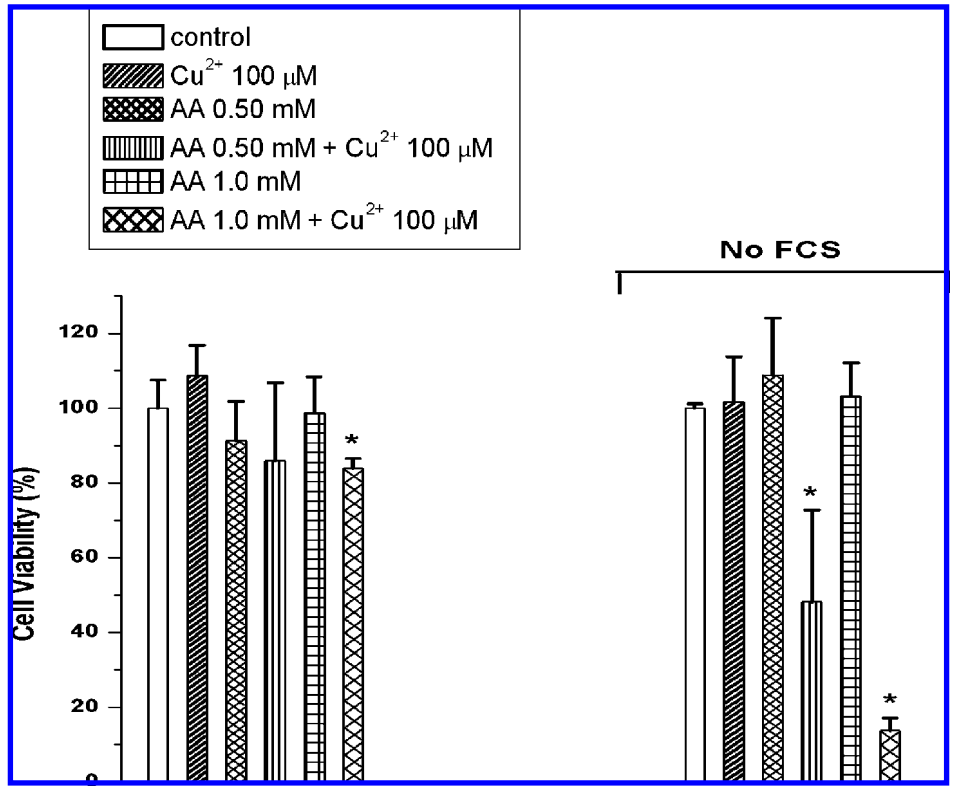


Figure 3. Protective effect of FCS on RINm5f cells treated with AA, in the presence (or absence) of Cu^{2+} ion. RINm5f cells (10^4 cells/well) were cultured in the absence and presence of FCS 10% (v/v)-containing RPMI-1640 medium and treated with (0.50−1.0) mM AA, 100 μM Cu^{2+} ion, or both for 24 h. Viability was measured by the MTT assay and expressed as % of untreated cells. All values represent the mean ± SEM of 4 independent experiments. *$p < 0.05$ vs control.

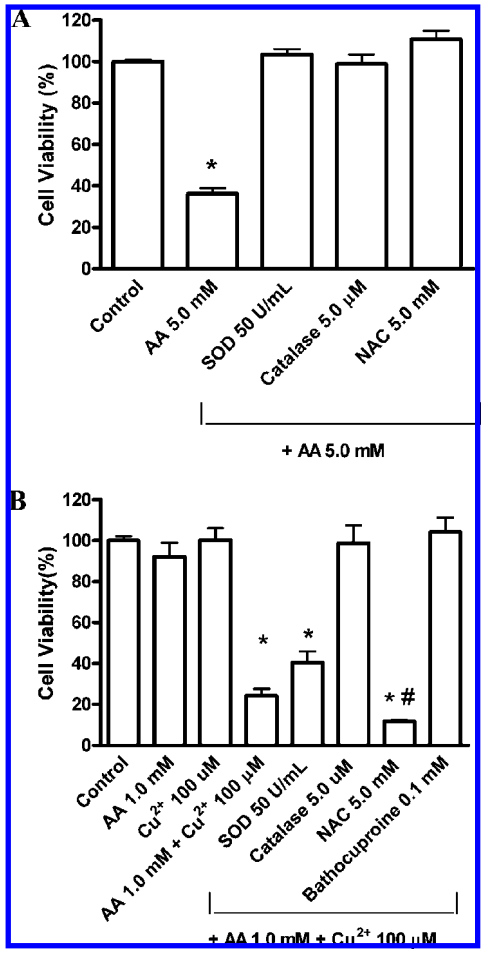


Figure 5. Protective effect of chemical (NAC) and enzymatic (SOD, catalase) antioxidants and of a cuprous chelator (bathocuproine) on RINm5f cell death triggered by AA. (A) Cells treated with 5.0 mM AA in the presence (or absence) of antioxidants for 24 h: 50 U/mL SOD, 5.0 μM catalase, and 5.0 mM NAC. (B) Cells treated with 1.0 mM AA and 100 μM Cu^{2+} ions, in the presence (or absence) of antioxidants for 24 h: 50 U/mL SOD, 5.0 μM catalase, 5.0 mM NAC, or 0.10 mM bathocuproine. Bathocuproine alone or bathocuproine/Cu^{2+} or bathocuproine/AA had no significant effect on cell death (not shown). All experiments were performed in the absence of FCS. Viability was measured by the MTT assay and expressed as % of untreated cells. All values represent the mean ± SEM of 3 individual experiments. *$p < 0.05$ vs control and $^{\#}p < 0.05$ vs AA/Cu^{2+} treatment.

medium were more resistant to AA in the presence or in the absence of Cu^{2+} than cultures treated in medium devoid of FCS (Figure 3). This can be attributed to oxygen radical and MG scavengers present in the serum, such as albumin, hemoglobin, creatinine, and metal chelators, which may artifactually minimize the lethality of the AA-generated deleterious products (*40*). Possible contribution of SSAO present in the FCS serum (*41, 42*) for RINm5f cell death was discarded at our experimental conditions as the serum did not augment the cell susceptibility to AA. Accordingly, when the experiments with AA and Cu^{2+} were performed in FCS-free medium, highest rates of RINm5f cell death were observed at low concentrations of both cytotoxicants: 100 μM Cu^{2+} and 0.50−1.0 mM AA (Figure 3) or 5.0−100 μM Cu^{2+} and 1.0 mM AA (Figure 4). A concentration-dependent pro-oxidant role of the AA/Cu^{2+} system in RINm5f cell viability is therefore demonstrated here during *in vitro* acute treatment. It is tempting to propose that, in diabetic patients, continuous exposure of pancreatic islets of Langerhans to lower concentrations of AA and Cu^{2+} may similarly cause impairment of the β-cells functions.

In this context, it is important to mention that the literature on the presence of SSAO in endocrine pancreas is controversial. Some reports document undetectable activity of SSAO in human endocrine pancreas (*43, 44*), whereas its presence has been found inconclusive in the same tissue of rats (*45, 46*). Even if SSAO is present in endocrine pancreatic cell, this activity may not be found in cell lines derived from it such as RINm5f. In order to verify whether or not AA-triggered RINm5f cell death can be attributable to membrane SSAO, we ran experiments with SSAO inhibitors such as semicarbazide, and 2-bromoethylamine (*23, 47, 48*), and with another SSAO substrate, namely benzylamine (*49*) (not shown). At concentrations ranging from 1.0 to 10 mM, semicarbazide did not affect significantly AA (5.0 mM)-induced cell death, but it became toxic above 20 mM even in the absence of AA. This may be partly due to the fact that superoxide anion radical is a normal, essential oxidizing and signaling cell metabolite highly reactive with semicarbazide (*17*). As 2-bromoethylamine alone caused cell death at concentrations above 0.10 mM, their letal effect in the presence of AA could not be properly explored. In turn, the SSAO-catalyzed oxidation of benzylamine would produce toxic hydrogen peroxide, but it was found to be innocuous to the cells at concentrations up to 0.5 mM. Ten milimolar benzylamine caused extensive cell death, both in presence (85%) and absence (70%) of FCS. This was not reversible upon addition of catalase, therefore excluding the intermediacy of H_2O_2 in cell viability. Altogether, these data strongly suggest absence of SSAO in the membrane of RINm5f cells, but these experiments should be viewed as preliminary. Thus we cannot preclude a contribution of membrane SSAO in the mechanism of AA-promoted RINm5f cells.

The mechanism of AA/Cu^{2+}-promoted cell death probably involves superoxide anion and hydroxyl radicals ultimately produced by oxidation of AA by Cu^{2+}, yielding the AA$^{\bullet}$ enoyl radical and Cu^{+}, whose consecutive reactions with oxygen leads to the formation of oxygen radicals (*17, 19*). An alternative route for superoxide production, possibility prevalent at AA concentrations above 10 mM, was suggested by Hiraku et al. (*19*): dimerization of AA to a pyrazine derivative, which can transfer one electron to oxygen yielding superoxide radical and pyrazine oxidation products. Obviously, this pathway precludes formation of cytotoxic MG, which has been previously shown to be the main AA oxidation product at our experimental conditions.

Cellular Protection Afforded by Added Antioxidants. Aiming to demonstrate that AA-promoted RINm5f cell death is mediated by the superoxide radical, hydroxyl radical, Cu^{+} ions and H_2O_2, adequate chemical (5.0 mM NAC) and enzymatic (50 U/mL SOD, 5.0 μM catalase) antioxidants were tested. Total protection against cell death was provided by these antioxidants when the RINm5f cultures were treated with 5.0 mM AA in the absence of copper (Figure 5A). These findings are consistent with previous reports that AA oxidation products causes chemical lesions to cell proteins, membranes, DNA, and mitochondria mainly through H_2O_2 and $O_2^{\bullet}$-intermediates (*17, 20, 21, 50*). Upon supplementation of Cu^{2+} ions (100 μM) to the AA-containing cell cultures, only partial inhibition of cell death was verified (Figure 5B). Catalase was the most efficient antioxidant, probably by eliminating AA-generated H_2O_2, thereby hampering production of HO$^{\bullet}$ through a Cu^{+}-dependent Fenton type reaction. Superoxide dismutase and NAC did not show a significant protective effect due to an expected prevailing pro-oxidative role of H_2O_2-generated HO$^{\bullet}$ radical in cell death. On the contrary, NAC behaves as a potent pro-oxidant in the presence of Cu^{2+} ions and H_2O_2 (*51*), since it can reduce Cu^{2+}

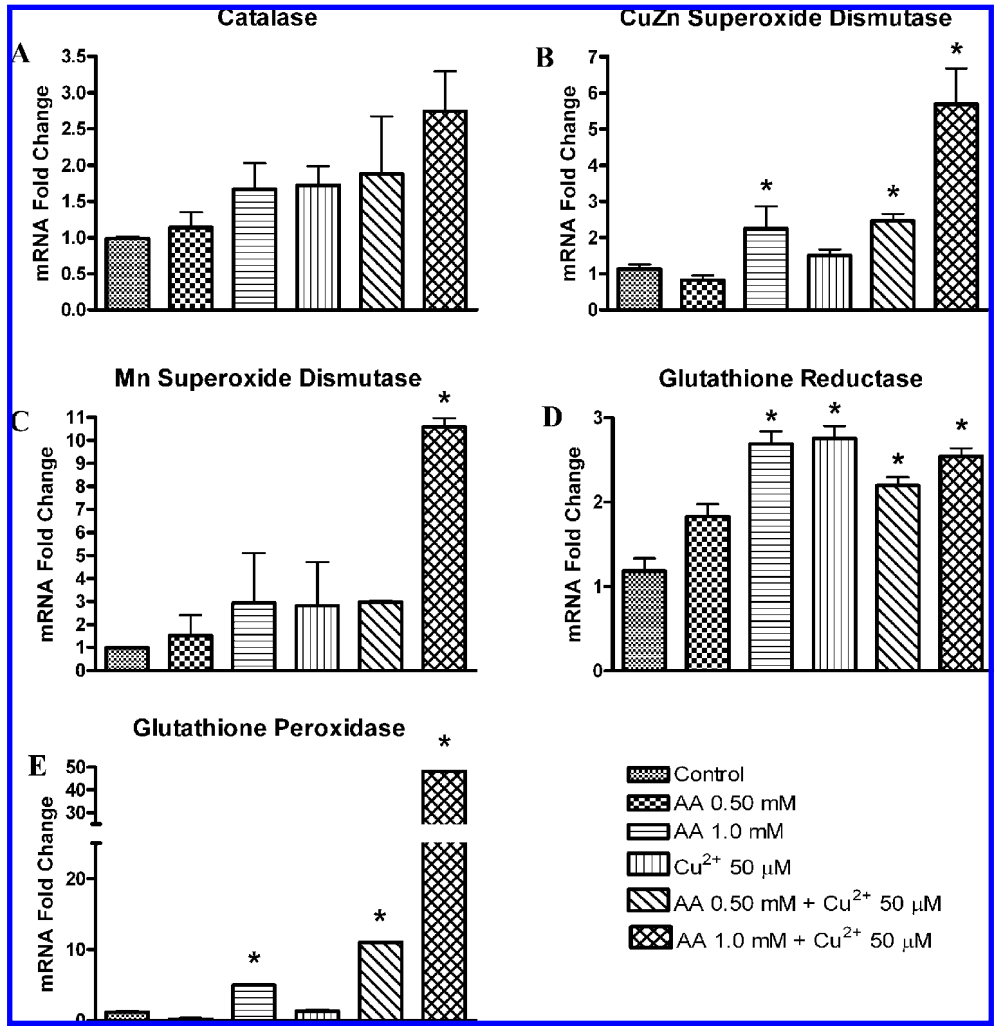


Figure 6. mRNA expression of antioxidant enzymes (catalase, CuZnSOD, MnSOD, GRd, and GPx) in RINm5f cells treated with AA and Cu^{2+} ions. Cells were treated with 0.50−1.0 mM AA, 50 μM Cu^{2+} ions, or both reagents for 2 h in the absence of FCS. The medium was then removed, fresh medium with FCS 10% (v/v) was added, and the cells were incubated for further 22 h. mRNA expression was measured by RT-PCR and expressed in fold change relative to untreated cells. All values represent the mean ± SEM of 3 independent experiments. *$p < 0.05$ vs control.

to Cu^{+} ions at the cost of thiol oxidation to the corresponding thiyl radical and thereby Cu^{+} can trigger the Fenton type reaction. Aiming to demonstrate formation of Cu^{+} ion and its participation in cell death, the cuprous ion chelator, bathocuproine (0.10 mM), was added to cell cultures treated with AA plus Cu^{2+} (Figure 5B). A significant protective effect was found, thus demonstrating the actual participation of Cu^{+} ion in cell damage.

Induction of Enzymatic Antioxidant Defenses by AA. Since AA may behave as an endogenous source of H_2O_2 and oxygen radicals, one should expect enhanced expression of antioxidant enzymes upon challenging the cultured cells with sublethal doses of the AA/Cu^{2+} system. Accordingly, treatment of RINm5f cells with 0.50−1.0 mM AA in the absence and in the presence of 10−50 μM Cu^{2+} for only 2 h resulted in striking mRNA expression of CuZnSOD, MnSOD, glutathione reductase (GRd) and glutathione peroxidase (GPx), whereas catalase mRNA expression was not significantly altered (Figure 6A−E). These data may reflect increased enzyme activities into the cells. Glutathione peroxidase, the key enzyme responsible for H_2O_2 and organic peroxide detoxification, displayed an astounding 50-fold increase in its corresponding mRNA (Figure 6E). Again these data are consistent with the hypothesis of intracellular AA-linked generation of reactive oxygen species and increased levels of inducible oxidant defenses.

DNA Fragmentation Induced by AA. With the objective of identifying the cellular mechanism of cell death triggered by AA, RINm5f cells cultures were treated with AA (1.0 mM) plus Cu^{2+} ions (100 μM) during 24 h (Figure 7A and B). DNA cell fragmentation was found to increase 4-fold when compared to the control. Similarly, Hiraku et al. (*19*) reported that HL 60 cells treated with AA/Cu^{2+} undergo DNA fragmentation. These findings are in agreement with a large number of data showing that MG, H_2O_2 and OH• radical species are able to react with DNA, leading to apoptosis and cell death (*52*−*54*). One can thus infer that extensive proteolysis associated with AA accumulation, as seems to be the case during β-cell destruction in diabetes, may be attributed to exacerbated production of MG and H_2O_2.

Effect of AA on Pro- and Anti-Apoptotic Protein mRNA Expression. Apoptosis or programmed cell death is a well established event triggered by intracellular chemical signals or stimulated by external agents (*55*). In both cases, apoptosis takes place by means of an ordered cascade of enzymatic reactions, initiated by the Bcl-2 protein family members (*56*). Some members of this family, such as Bcl-2 itself and Bcl-xL, inhibit apoptosis, however, another namely Bax, accelerates it (*57*). Bax is located in the cytoplasm, but, during apoptotic processes, it undergoes oligomerization and further insertion into the mitochondrial inner membrane, which, ultimately, releases

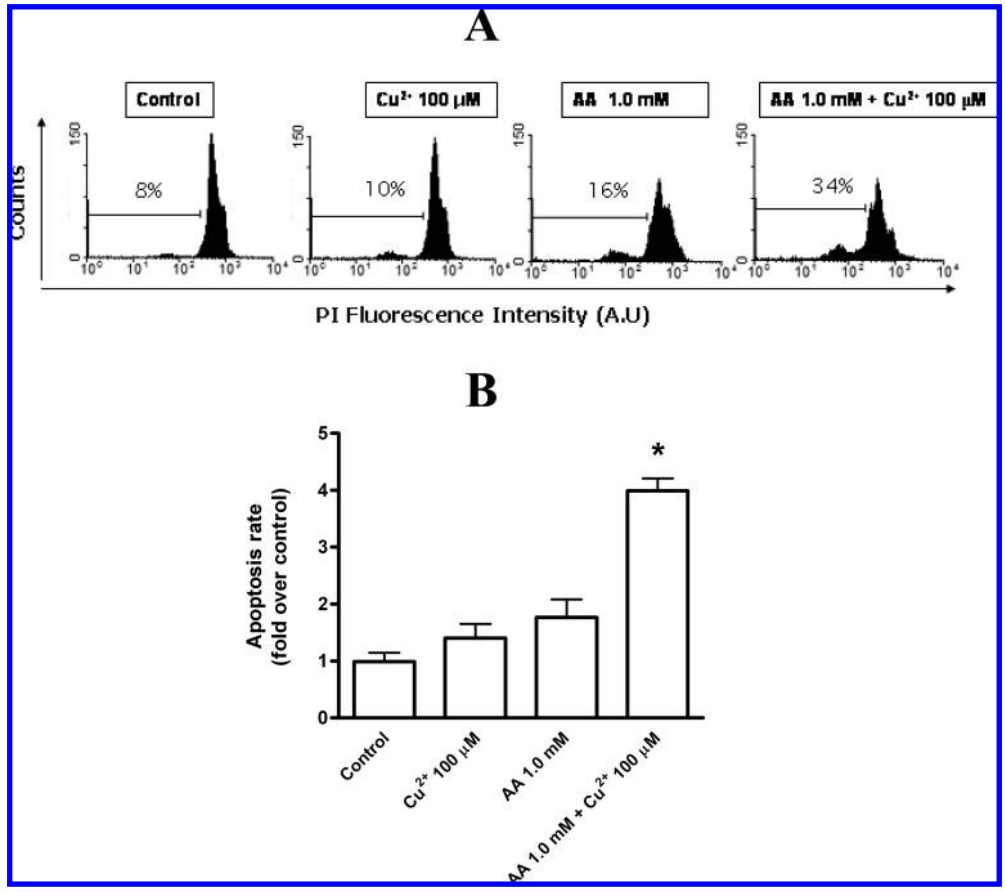


Figure 7. Apoptosis of RINm5f cells induced by the treatment with AA and Cu^{2+} ion. Cells were treated for 24 h in the presence of 1.0 mM AA, 100 μM Cu^{2+} ion, or both reagents in FCS free RPMI-1640 medium. The medium was then removed, the cells were harvested, ressuspended, and lyzed in a hypotonic buffer containing PI. Cellular DNA fragmentation was measured by flow cytometry. (A) RINm5f cell apoptosis representative histogram. (B) Quantitative analysis of apoptosis levels is expressed in bar graph form of the results shown in A. In B, apoptosis rate is expressed as fold over nontreated cells (control). All values represent the mean ± SEM of 3 independent experiments. *$p < 0.05$ vs control.

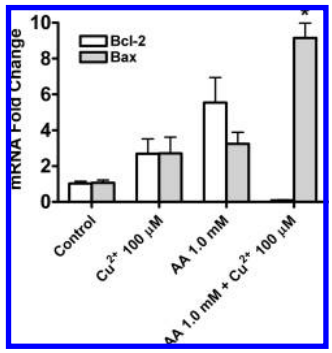


Figure 8. mRNA expression of pro (Bax)- and anti (Bcl-2)-apoptotic proteins of RINm5f cells treated with AA and Cu^{2+} ion. Cells were treated for 24 h in the presence of 1.0 mM AA, 100 μM Cu^{2+} ion, or both reagents. mRNA expression was measured by RT-PCR and is expressed in fold change relative to untreated cells. All values represent the ± SEM of 3 independent experiments. *$p < 0.05$ vs mRNA Bcl-2 expression.

cytochrome *c* into the intermembrane space, followed by caspase activation (*58, 59*) and ultimately cell death.

Aiming to better understand the process by which AA leads to RINm5f cell death, RINm5f cells were treated with 1.0 mM AA plus 100 μM Cu^{2+} ions and real time PCR was utilized to monitor Bcl-2 and Bax mRNA expression in these cells (Figure 8). As expected, expression of Bax was found to be much higher (10-fold) in AA- and copper-treated cells, as compared to the controls containing only AA or copper, suggesting increased cellular concentration of Bax. Curiously, Bcl-2 expression was down-regulated by the treatment, being 2-fold higher than Bax expression in the absence of copper. In this respect, noteworthy previously reported experiments showing that Bcl-2 overexpression in NT-2 (teratocarcinoma) and SK-N-MC (neuroblastoma) cell lines abolish chemical insult provoked by reactive species of oxygen and nitrogen (*60*). Down-regulation of Bcl-2, was demonstrated by Wang and co-workers (*61*) in human lung epithelial H460 cells treated with cisplatin, a ROS-producing chemotherapeutic agent used against several types of cancer. Bcl-2 down-regulation was suppressed by treatment with exogenous catalase or GPx overexpression, indicating that H_2O_2 is involved in cisplatin-driven H460 cell death. Our findings support the notion that AA-induced decrease of RINm5f cell viability requires copper to activate the protective antioxidant enzymatic machinery. The observed mRNA expression of pro- and antiapoptotic proteins in RINm5f cells induced by AA, with consequent DNA fragmentation, strengthens our hypothesis that AA may play an important coadjuvant role in the metabolism of diabetic patients by promoting β-cell lesions and, eventually, death by apoptosis.

Increase of Intracellular Calcium upon Treatment with AA. Intracellular [Ca^{2+}] homeostasis is controlled by both calcium influx/efflux throughout the plasma membrane and the capacity of intracellular organelles to sequester and store calcium. Disruption of this delicate balance may increase cytosol Ca^{2+} above nanomolar concentrations, leading to DNA fragmentation and cell death (*62*). Reactive species, such as MG, a protein cross-linker and adduct generator, and H_2O_2, the primer source of deleterious HO• radical, can damage the plasma

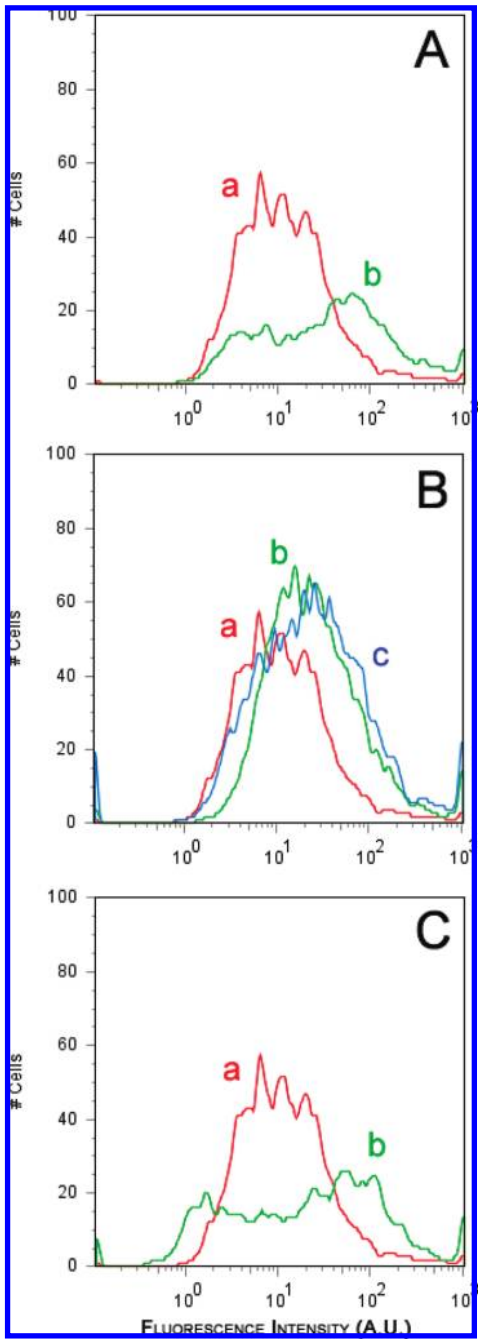


Figure 9. Representative FACS plots resulting of RINm5f cell $[Ca^{2+}]_i$ increase upon treatment with AA plus Cu^{2+} ion. Cells were treated for 4 h in the presence of 0.50−1.0 mM AA, 100 μM Cu^{2+} ion, or 0.50−1.0 mM AA plus 100 μM Cu^{2+} ion in FCS free medium. The medium was then removed, and the cells were trypsinized and ressuspended in fresh medium. The cells were then incubated with 100 nM Fluo4 AM in the dark for 45 min at 37 °C in 97.5% O_2/2.5% CO_2. (A) (a) control, (b) 100 μM Cu^{2+} ion. (B) (a) control, (b) 0.50 mM AA, (c) 1.0 mM AA. (C) (a) control, (b) 0.50 mM AA plus 100 μM Cu^{2+} ion. Figures are representative of 4 independent experiments.

membrane, creating pores that will allow abnormally high Ca^{2+} influx (*10, 63*).

Figure 9(A−C) represents flow fluorescence distribution of calcium in cells treated with Cu^{2+} ions (100 μM) (Figure 9A) or AA (0.50 − 1.0 mM) (Figure 9B) alone and AA 0.50 plus Cu^{2+} ions (100 μM) (9c). One can observe that Cu^{2+} ions and AA added separately promote little inner calcium increase even when significant cell death was not observed. In the presence of Cu^{2+}, 0.50 mM AA led to a considerably high increase of inner calcium, while the treatment with 1.0 mM AA at the same conditions led the majority of cells to death (not shown). This can be viewed as a result from oxidation of membrane proteins and fatty acids initiated by $HO^{\bullet}$ radicals generated in the Cu^{2+}-catalyzed reaction of AA with molecular oxygen, as previously demonstrated in isolated rat liver mitochondria treated with AA (*20*). On the other hand, Best and Thornalley (*64*) reported that 1.0 mM of MG can cause cellular membrane depolarization, with subsequent opening of Ca^{2+} channels. The authors suggested that the conversion of MG to *D*-lactate, and accumulation of the latter, cause β-cell swelling and activation of the volume-sensitive anion channel, resulting in generation of inward current (Ca^{2+}, Na^{+}), which leads to depolarization and electrical activity. This hypothesis is supported by the experiment depicted in Figure 9B, with MTT assays revealing that cell viability does not decrease in these conditions, nevertheless, significant, although little accumulation of internal Ca^{2+} occurs.

Discussion

Aminoacetone, in the milimolar range, is shown here to induce dose-dependent apoptosis in RINm5f insulin-producing cells, by means of oxygen radicals and MG produced during AA oxidation by molecular oxygen (Figures 1−2). Cells cultured in FCS-containing medium were more resistant to AA-triggered cell death, probably due the protein antioxidants present in the serum (Figure 3). In the absence of FCS, AA and copper ions, both at micromolar concentrations promote cell death (Figures 3−4), which can be prevented upon addition of antioxidants such as SOD, catalase, and NAC (Figure 5). However, only catalase can afford total protection of AA- and Cu^{2+}- treated cells (Figure 5B), indicating the pivotal role of Cu^{+}, H_2O_2 and $HO^{\bullet}$ in cell oxidative injury. Superoxide dismutase afforded only partial protection perhaps due to the enzyme inactivation by hydroxyl radical (*65*). On the other hand, the observed pro-oxidant activity of NAC in the presence of copper is reportedly attributed to NAC-driven one-electron reduction of Cu^{2+} to Cu^{+} ion, which acts as a Fenton reductant with H_2O_2 (*50*). Accordingly, bathocuproine, a Cu^{+} ion chelating agent, prevented cell death (Figure 5B). Noteworthy in this regard, Hamada et al. (*8*) demonstrated that MG and 3-deoxyglucosone levels were significantly higher in diabetic rats than in nondiabetic controls and that a metal chelating agent, namely trientine, significantly diminished the levels of these toxic α-dicarbonyl metabolites. These findings are revealing from the redox balance viewpoint, since diabetes is characterized by an overload of "free" transition metal ions and substantial production of free radicals (*66*).

It is tempting to confront the presently observed RINm5f susceptibility to AA and copper treatment, mediated by reactive oxygen species, with the reported low levels of antioxidant proteins expression by pancreatic β-cells (*67*). Perhaps this also explains why RINm5f cells were more sensitive to treatment with AA than NIH-3T3 fibroblastic cells (Figure 2). Accordingly, we found here that the RINm5f mRNA levels of enzymes which constitute the antioxidant cell machinery, namely CuZn-SOD, MnSOD, GPx, GRd, and catalase, were considerably augmented upon addition of AA and Cu^{2+} to the cell cultures. Outstanding was the 50-fold increase of GPx mRNA (Figure 6A−E), since this enzyme is admittedly inducible by H_2O_2 and responds for the fine control of deleterious cellular peroxides. Similarly, the expression of antioxidant enzymes mRNAs of astroglyal cells and hepatocytes was found to be elevated upon cell exposure to H_2O_2 (*68, 69*). Not negligible was the fact that RINm5f treatment with AA (0.50−1.0 mM) plus Cu^{2+} ions (100 μM) for only 2 h was revealed to be significantly effective to

promote cell death (not shown), when compared with a longer (24 h) period suggesting that continuous, chronic exposure of pancreatic cells to low concentrations of AA and copper may indeed contribute to impairment of insulin production in diabetes.

Also noteworthy was the observed high expression of MnSOD mRNA. Induction of mitochondrial MnSOD by AA, accompanied by increased intracellular calcium (Figure 9A−C) and DNA cell fragmentation (Figure 7A and B), concomitant with augmented Bax and decreased Bcl-2 mRNA expression (Figure 8), strongly suggest that apoptosis is the main path of AA-induced cell death. Many investigators point pancreatic β-cell mitochondria as the main target of hyperglycemia in diabetes (*70, 71*). Dysfunction of these organelles would directly contribute to decreased capacity of insulin production (*72*).

The literature lacks information on the actual AA concentrations in plasma of normal and diabetic patients, perhaps due to its chemical instability in the tissues (e.g., easy oxidation, dimerization, conjugation with proteins), rendering the MG concentration and SSAO activity the only parameters available to infer AA levels in diabetes. Recently, a reliable HPLC method for detection and quantification of AA in biological samples has been described (*73*), and MG and copper were found to be elevated in diabetic patients (*74*−*76*). Intraperitoneal injection of AA in rats resulted in roughly the same concentration of MG in urine of these animals (*77*). In addition, SSAO, the enzyme that catalyzes the oxidation of primary amines (benzylamine, methylamine, AA), was found to be elevated in the plasma of diabetics (*14*), and to exhibit high affinity for AA. The K_m value reported for SSAO-AA present in homogenates of human umbilical artery is 125.9 ± 20.5 μM (*78*), and in membrane fractions isolated from rat aorta, 19 ± 3 μM (*79*). Altogether, our data support the hypothesis that AA may significantly contribute to impairment and, eventually, cell death of β-cells in diabetes mellitus.

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