Anti-apoptotic effects of pan-caspase inhibitor (Z-VAD), SOD or catalase on antimycin A-induced HeLa cell death

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Abstract. Antimycin A (AMA) is an inhibitor of the electron transport chain in mitochondria. In this study, we investigated the anti-apoptotic effects of pan-caspase inhibitor (Z-VAD), superoxide dismutase (SOD) or catalase on AMA-induced HeLa cell death in relation to the cell cycle. Treatment with Z-VAD, SOD or catalase rescued some HeLa cells from AMA-induced apoptosis, but did not prevent the growth inhibition of HeLa cells by AMA. DNA flow cytometric analysis indicated that treatment with AMA significantly induced an S-phase arrest of the cell cycle at 72 h. Interestingly, Z-VAD, SOD and catalase intensified S-phase arrest in AMA-treated cells. In conclusion, treatment with Z-VAD, SOD or catalase decreased apoptotic levels in AMA-treated cells, which was associated with the enhancement of the S-phase arrest of the cell cycle in these cells.

Introduction

Antimycin A (AMA) is a mixture primarily composed of antimycin A1 and A3 derived from *Streptomyces kitazawensis* (1). AMA inhibits succinate and NADH oxidase, and specifically inhibits mitochondrial electron transport between cytochromes b and c (2-4). Inhibition of electron transport causes a collapse of the proton gradient across the mitochondrial inner membrane, thereby collapsing mitochondrial membrane potential ($\Delta\Psi_m$) (2,4,5). This inhibition can produce reactive oxygen species (ROS) (5,6), which include hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and peroxynitrite ($ONOO^-$). A change in the redox state of the tissue implies a change in ROS generation or metabolism. Principal metabolic pathways include superoxide dismutase (SOD), which is expressed as extracellular, intracellular and mitochondrial isoforms. These isoforms metabolize $O_2^{\bullet-}$ to H_2O_2. Further metabolism by peroxidases, including catalase and glutathione peroxidase, yields O_2 and H_2O (7). Cells possess antioxidant systems to control the redox state, which is important for their survival. Excessive ROS production results in the activation of events that lead to death and survival in several types of cells (8-10). In addition, some evidence indicates that either ROS or the collapse of mitochondrial membrane potential ($\Delta\Psi_m$) opens the mitochondrial permeability transition pores. This occurrence is accompanied by the release of proapoptotic molecules such as cytochrome c into the cytoplasm (11), triggering apoptosis. Since AMA acts directly on the mitochondria, AMA-induced apoptosis has been reported in many experiments, including our recent report on HeLa and As4.1 juxtaglomerular cells (12-18).

The cell cycle in eukaryotes is regulated by cyclin-dependent kinases (CDKs). The cyclins, members of the cell cycle regulators, bind to and activate CDKs. The sequential formation, activation and subsequent inactivation of CDKs and cyclins is critical to the control of the cell cycle (19,20). The cyclin-dependent kinase inhibitors (CDKIs) p21 and p27 also play key roles in controlling cell cycle progression by negatively regulating cyclin-dependent kinase (CDK) activity (19-21). Previously, it was noted that less traditional factors, as well as traditional cell cycle regulators such as nutrients, growth factors and hormones, may have an important impact on cell cycle regulation. Of the less traditional factors, ROS was commonly thought to be entirely toxic to cells, leading to cell death. Evidence shows that ROS may also act as a signaling molecule (22,23) and, as such, may have a role in cell cycle progression.

We recently demonstrated that AMA produced ROS in HeLa cells and efficiently induced apoptosis, as evidenced by the flow cytometric detection of sub-G1 DNA content, annexin V binding assay and DAPI staining (13,18). This apoptotic process was accompanied by the loss of mitochondrial membrane potential ($\Delta\Psi_m$), the down-regulation of Bcl-2, the up-regulation of Bax, and the degradation of the PARP protein. In the present study, we investigated the anti-apoptotic effects of Z-VAD, SOD or catalase in relation to the cell cycle in AMA-treated HeLa cells.

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Abbreviations: AMA, antimycin A; ROS, reactive oxygen species; NADPH, nicotine adenine diphosphate; XO, xanthine oxidase; SOD, superoxide dismutase; FBS, fetal bovine serum; PBS, phosphate buffer saline; FITC, fluorescein isothiocyanate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; CDKI, cyclin-dependent kinase inhibitor; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

Key words: antimycin A, reactive oxygen species, Z-VAD, superoxide dismutase, catalase, apoptosis, cell cycle

Materials and methods

Cell culture. Human cervical adenocarcinoma HeLa cells were maintained in a humidified room atmosphere containing 5% CO_2 at 37˚C. HeLa cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY). Cells were routinely grown in 100-mm plastic tissue culture dishes (Nunc, Roskilde, Denmark) and harvested with a solution of trypsin-EDTA when they were in a logarithmic growth phase. Cells were maintained under culture conditions as described above for all experiments.

Reagents. AMA (Sigma-Aldrich Chemical Company, St. Louis, MO) was dissolved in ethanol at $2x10^{-2}$ M as a stock solution. Pan-caspase inhibitor (Z-VAD-FMK) was obtained from R&D Systems Inc. (Minneapolis, MN) and dissolved in DMSO (Sigma) at $1x10^{-2}$ M as a stock solution, and was also used as a control vehicle. SOD and catalase were obtained from Sigma and dissolved in 50 mM potassium phosphate buffer at 4733 U/ml. The stock solutions were wrapped in foil and kept at 4˚C or -20˚C.

Growth inhibition assay. The *in vitro* growth inhibition effect of AMA with or without Z-VAD, SOD or catalase on HeLa cells was determined by measuring the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance of living cells as previously described (24). In brief, $2x10^5$ cells/well were seeded onto 96-well microtiter plates (Nunc). After exposure to the indicated AMA concentration with or without Z-VAD, SOD or catalase for 72 h, 50 μl of MTT solution (Sigma) (2 mg/ml in PBS) were added to each well, and the plates were incubated for 3 or 4 additional hours at 37˚C. MTT solution in medium was withdrawn by pipetting. To achieve the solubilization of the formazan crystals that formed in viable cells, 100 or 200 μl of DMSO were added to each well. The optical density of each well was measured at 570 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co., Sunnyvale, CA). Each plate contained multiple wells of a given experimental condition and multiple control wells. This procedure was replicated in 2-4 plates/condition.

Cell cycle and sub-G1 analysis. The distribution of the cell cycle and of sub-G1 was determined by staining DNA with propidium iodide (PI; Sigma-Aldrich), a fluorescent biomolecule that can be used to stain DNA, as previously described (25). In brief, $1x10^6$ cells were incubated with 20 μM AMA with or without Z-VAD, SOD or catalase for 72 h. Cells were then washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol. Cells were again washed with PBS and incubated with propidium iodine (PI; 10 μg/ml) with the simultaneous treatment of RNase at 37˚C for 30 min. The percentage of cells in the different phases of the cell cycle or those with sub-G1 DNA content were measured with a FACStar flow cytometer (Becton-Dickinson, San Jose, CA) and analyzed using Lysis II and Cellfit software (Becton-Dickinson) or ModFit software (Verity Software House Inc., ME).

Detection of intracellular ROS. Intracellular ROS levels were detected by means of an oxidation-sensitive fluorescent probe dye, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen Molecular Probes, Eugene, OR). H_2DCFDA was intracellularly deacetylated by non-specific esterase, which was further oxidized by cellular peroxides to the fluorescent compound, 2,7-dichlorofluorescein (DCF) (Ex/Em = 485/535 nm). In brief, cells were incubated with AMA with or without Z-VAD, SOD or catalase for 72 h. The cells were then washed in PBS and incubated with 20 μM H_2DCFDA at 37˚C for 30 min according to the manufacturer's instructions. DCF fluorescence was detected using a FACStar flow cytometer (Becton-Dickinson). For each sample, 5,000 or 10,000 events were collected. ROS levels were expressed as the mean fluorescence intensity (MFI), which was calculated by CellQuest software.

Statistical analysis. Results represent the mean of two or three independent experiments; bar, SD. Microsoft Excel or Instat software (GraphPad Prism4, San Diego, CA) was used to analyze the data. The student's t-test or one-way analysis of variance (ANOVA) with post hoc analysis using Tukey's multiple comparison test was used for parametric data. Statistical significance was defined as $p<0.05$.

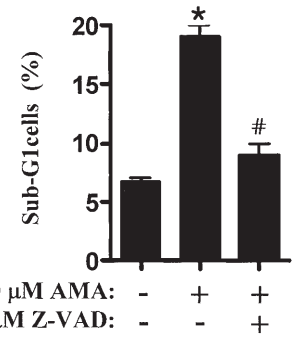

Figure 1. Effects of Z-VAD on cell death in AMA-treated HeLa cells. Exponentially growing cells were treated with the indicated AMA concentrations with or without Z-VAD (12.5 μM) for 72 h. The number of sub-G1 cells was assessed by DNA flow cytometric analysis. The percentage of sub-G1 cells is shown. *P<0.05 compared to the HeLa control group. #P<0.05 compared to cells treated with AMA alone.

Results

Effects of the pan-caspase inhibitor Z-VAD on apoptosis, cell growth and cell cycle distribution in AMA-treated HeLa cells. We examined the effects of the pan-caspase inhibitor Z-VAD on apoptosis, cell growth and cell cycle distribution in AMA-treated HeLa cells. As shown in Fig. 1, DNA flow cytometric analysis indicated that, at 72 h, 20 μM of AMA significantly increased the number of sub-G1 cells compared with the control cells. Treatment with Z-VAD resulted in a marked rescue of HeLa cells from AMA-induced apoptosis in view of sub-G1 cells and annexin V exposure (data not shown). AMA also inhibited HeLa cell growth (Fig. 2A). Notably, treatment with Z-VAD did not significantly prevent the growth inhibition of HeLa cells by AMA (Fig. 2A). Treatment with AMA for 72 h induced S-phase arrest of the cell cycle in HeLa cells (Fig. 2B). The S-phase arrest by AMA was observed in a time (24-72 h)- and dose (0.5-50 μM)-dependent manner (data

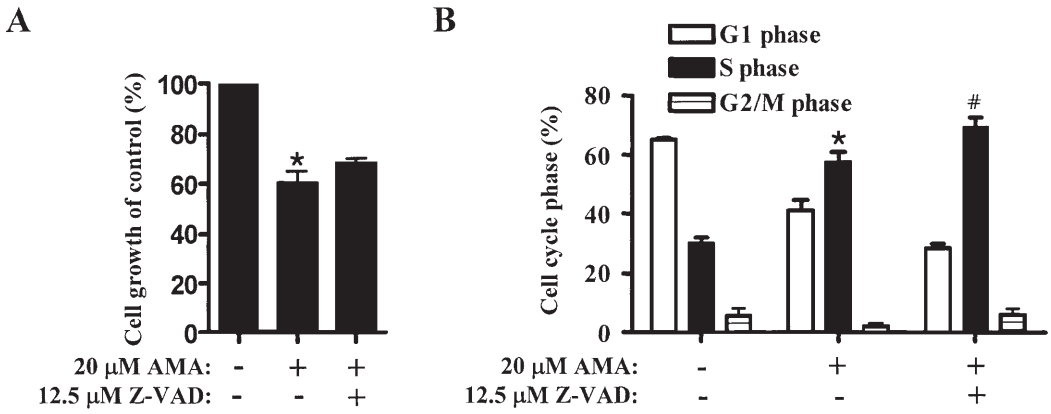

Figure 2. Effects of Z-VAD on cell growth and the cell cycle in AMA-treated HeLa cells. Exponentially growing cells were treated with the indicated AMA concentrations with or without Z-VAD (12.5 μM) for 72 h. (A) The growth of AMA- and/or Z-VAD-treated HeLa cells, assessed by MTT assay. (B) Cell cycle distribution in AMA- and/or Z-VAD-treated HeLa cells. *P<0.05 compared to the HeLa control group. #P<0.05 compared to cells treated with AMA alone.

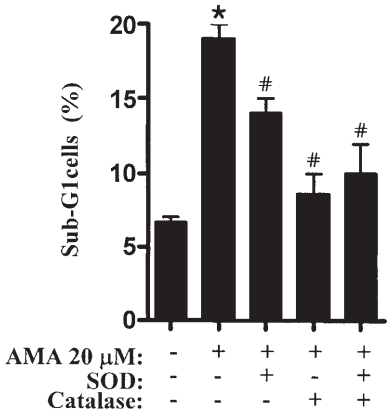

Figure 3. Effects of SOD and catalase on cell death in AMA-treated HeLa cells. Exponentially growing cells were treated with the indicated AMA concentrations with or without SOD and/or catalase for 72 h. The number of sub-G1 cells was assessed by DNA flow cytometric analysis. The sub-G1 cell percentage is shown. *P<0.05 compared to the HeLa control group. #P<0.05 compared to cells treated with AMA alone.

not shown). In particular, following treatment with Z-VAD, S-phase arrest of the cell cycle in AMA-treated cells was significantly intensified (Fig. 2B). Treatment with Z-VAD (12.5 μM) alone did not change cell growth inhibition and cell cycle distribution in control HeLa cells (data not shown).

Effects of exogenous SOD and catalase on apoptosis, cell growth and cell cycle distribution in AMA-treated HeLa cells. We examined whether exogenous SOD and catalase changed apoptosis and cell growth levels and cell cycle distribution in AMA-treated HeLa cells. As shown in Fig. 3, treatment with SOD (30 U/ml) and/or catalase (30 U/ml) significantly prevented apoptosis in AMA-treated HeLa cells at 72 h following the inspection of sub-G1 and annexin V-stained cells (data not shown). Treatment with SOD and/or catalase did not reduce the inhibition of growth in AMA-treated HeLa cells (Fig. 4A). In particular, S-phase arrest of the cell cycle in AMA-treated cells was enhanced by treatment with SOD and/or catalase (Fig. 4B). Neither synergistic nor additive effects of SOD

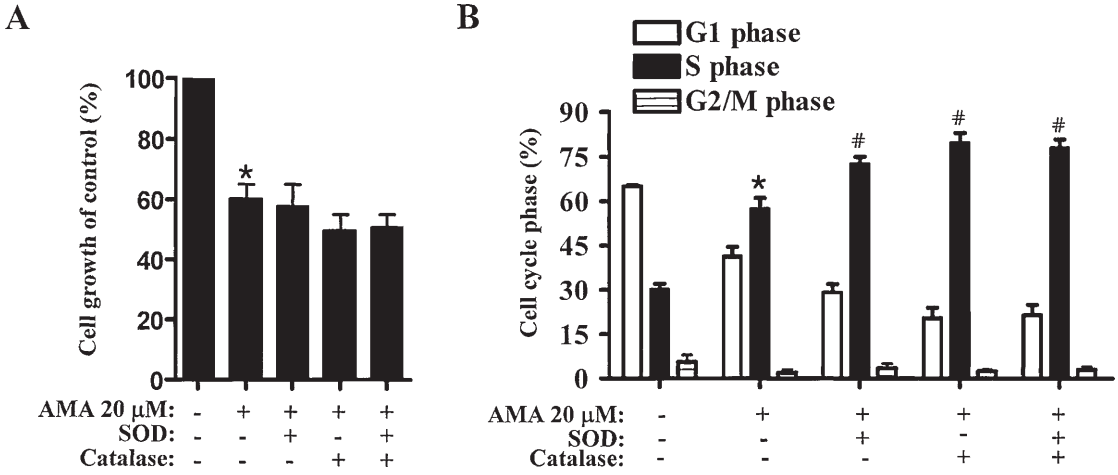

Figure 4. Effects of SOD and catalase on cell growth and the cell cycle in AMA-treated HeLa cells. Exponentially growing cells were treated with the indicated AMA concentrations with or without SOD and/or catalase for 72 h. (A) The growth of AMA- or SOD- and/or catalase-treated HeLa cells, which was assessed by MTT assay. (B) Cell cycle distribution in AMA- or SOD- and/or catalase-treated HeLa cells. *P<0.05 compared to the HeLa control group. #P<0.05 compared to cells treated with AMA alone.

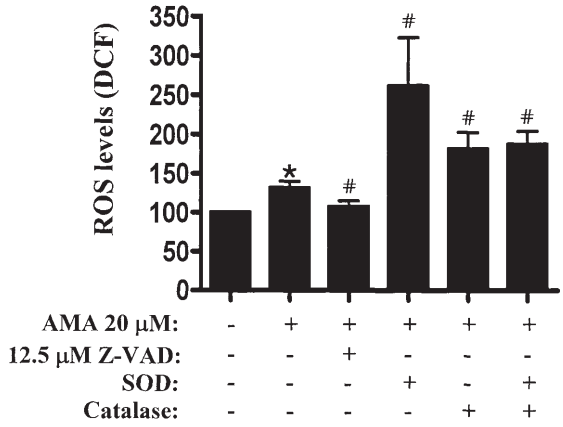

Figure 5. Effects of Z-VAD, SOD or catalase on ROS levels in AMA-treated HeLa cells. Exponentially growing cells were treated with the indicated AMA concentrations with or without Z-VAD, SOD and/or catalase for 72 h. Intracellular ROS (DCF fluorescence) levels in HeLa cells were determined by a FACStar flow cytometer. *P<0.05 compared to the HeLa control group. #P<0.05 compared to cells treated with AMA alone.

and catalase on the levels of apoptosis, growth inhibition and S-phase arrest in AMA-treated HeLa cells were observed (Figs. 3 and 4). Treatment with SOD and/or catalase alone did not significantly change cell growth inhibition and cell cycle distribution in control HeLa cells (data not shown).

Effects of Z-VAD, SOD and catalase on ROS levels in AMA-treated HeLa cells. To elucidate the involvement of ROS in cell cycle changes in HeLa cells, we assessed ROS levels using H_2DCFDA fluorescence. As shown in Fig. 5, ROS levels were increased in HeLa cells exposed to AMA for 72 h. Z-VAD, which showed anti-apoptotic and enhanced S-phase arrest effects in AMA-treated HeLa cells, significantly reduced ROS levels. However, exogenous treatment with SOD and/or catalase showed a similar effect as Z-VAD, which increased ROS levels in AMA-treated HeLa cells. Neither synergistic nor additive effects of SOD and catalase on ROS levels in these cells were observed (Fig. 5). Treatment with Z-VAD, SOD or catalase alone did not significantly change ROS levels (data not shown).

Discussion

In the present study, we focused on evaluating the anti-apoptotic effects of Z-VAD, SOD or catalase in relation to the cell cycle in AMA-treated HeLa cells, having demonstrated that AMA efficiently induces apoptosis (13). Cell cycle analysis has revealed that treatment with AMA significantly induces an S-phase arrest of the cell cycle for 72 h. The S-phase checkpoint, termed the replication checkpoint, has previously been described (26-28). This checkpoint monitors S-phase progression and slows the rate of ongoing DNA synthesis. The S-phase checkpoint is thought to involve the activation of the ATM and ATR kinases, with the subsequent activation of Chk1 and Chk2 in response to DNA damage (26,29). Since AMA acts directly on the mitochondria and produces ROS in HeLa cells, it is possible that ROS readily damages biological molecules, especially DNA, inducing S-phase arrest of the cell cycle in HeLa cells. However, we recently reported that AMA did not induce any specific phase arrest of the cell cycle in As4.1 juxtaglomerular cells (12), and induced a G1-phase arrest in Calu-6 lung cancer cells (unpublished data). These results suggest that the specificity of cell cycle arrest by AMA depends on differences in cell type, and that AMA-induced ROS cannot always trigger the S-phase checkpoint, depending on the type of cell.

According to the current data, treatment with Z-VAD, SOD or catalase prevented AMA-induced apoptosis, but did not significantly reduce the inhibition of growth in AMA-treated HeLa cells. The anti-apoptotic effect of Z-VAD was associated with the down-regulation of ROS levels in AMA-treated HeLa cells, whereas that of SOD or catalase was correlated with the up-regulation of ROS levels in the cells. These results suggest that ROS changes by AMA, Z-VAD, SOD or catalase are not directly related to the induction of apoptosis. Interestingly, following treatment with Z-VAD, SOD or catalase, an intensified S-phase arrest of the cell cycle was observed in AMA-treated cells. The enhancement of S-phase arrest by these agents was not closely related to ROS levels. In AMA-treated HeLa cells, the S-phase arrest intensified by Z-VAD, SOD or catalase appeared to increase the resistance of cells to apoptosis. It is possible that the S-phase checkpoint prolonged/increased by Z-VAD, SOD or catalase in AMA-treated HeLa cells did not trigger the apoptotic events in the cells. However, the augmentation of S-phase arrest by these agents makes cells more resistant to apoptosis, and is in need of further study. We did not observe a synergistic or additive effect of SOD and catalase on cell death (sub-G1 cells), cell growth and S-phase arrest of the cell cycle. It is possible that catalase functions at the downstream region of SOD, and/or that SOD and catalase negatively regulate each other's entrance into cells.

In summary, we have demonstrated that AMA, an inhibitor of electron transport in mitochondria, induces the S-phase arrest of the cell cycle in HeLa cells. Treatment with Z-VAD, SOD or catalase decreased apoptotic levels in AMA-treated cells, which was accompanied by the enhancement of S-phase arrest of the cell cycle in these cells.

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