International
Journal of Toxicology
Volume 28 Number 1
January/February 2009 33-42
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10.1177/1091581809333139
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Menadione Reduction by Pharmacological Doses of Ascorbate Induces an Oxidative Stress That Kills Breast Cancer Cells

Raphaël Beck, Julien Verrax, Nicolas Dejeans, Henryk Taper, and Pedro Buc Calderon

Oxidative stress generated by ascorbate-driven menadione redox cycling kills MCF7 cells by a concerted mechanism including glycolysis inhibition, loss of calcium homeostasis, DNA damage and changes in mitogen activated protein kinases (MAPK) activities. Cell death is mediated by necrosis rather than apoptosis or macroautophagy. Neither 3-methyladenine nor Z-VAD affects cytotoxicity by ascorbate/menadione (Asc/Men). BAPTA-AM, by restoring cellular capacity to reduce MTT, underlines the role of calcium in the necrotic process. Oxidative stress-mediated cell death is shown by the opposite effects of N-acetylcysteine and 3-aminotriazole. Moreover, oxidative stress induces DNA damage (protein poly-ADP-ribosylation and γ-H2AX phosphorylation) and inhibits glycolysis. Asc/Men deactivates extracellular signal-regulated kinase (ERK) while activating p38, suggesting an additional mechanism to kill MCF7 cells. Since ascorbate is taken up by cancer cells and, due to their antioxidant enzyme deficiency, oxidative stress should affect cancer cells to a greater extent than normal cells. This differential sensitivity may have clinical applications.

Keywords: ascorbate-menadione redox cycling; calcium homeostasis; glycolysis; mitogen-activated protein kinase; MCF7 cells; oxidative stress

Among several strategies to induce cancer cell death, the generation of oxidative stress is an interesting approach because cancer cells are particularly vulnerable to any treatment interfering with the maintenance of redox homeostasis. The rationale is the existence of a differential redox control of proliferation and viability in nontransformed versus malignant cells because the latter usually exhibit high levels of reactive oxygen species (ROS) that stimulate cell proliferation and promote genetic instability.[1] Thus, we and others have reported that the exposure of cancer cells to oxidative stress generated by redox cycling between ascorbate (vitamin C) and menadione (2-methyl-1, 4-naphthoquinone, also called vitamin K_3) leads to their death, as has been shown under in vitro and in vivo conditions.[2-10] As shown in Figure 1, a redox cycle occurs by electron transfer from ascorbate to menadione. In agreement with a previous report,[11] we have demonstrated that the rapid reoxidation of the semiquinone to its quinone form leads to the generation of ROS (mainly hydrogen peroxide) and to oxidative stress.[12] Indeed, we have shown that the cytotoxicity induced by the association of ascorbate and menadione (Asc/Men) in several cell lines is mediated by an oxidative stress and leads to a necrotic-like cell death.[9,12-14] Recently, we reported that the toxicity by Asc/Men against K562 cells (chronic myeloid leukemia) may be the consequence of a glycolysis arrest leading to a decrease in adenosine triphosphate (ATP).[10] Because cancer cells, even in the presence of oxygen, use glycolysis to transform glucose into ATP (Warburg effect), the arrest of

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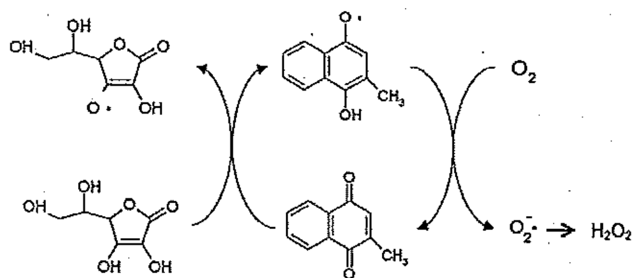

Figure 1. Ascorbate-Driven Menadione Redox Cycling.
Note: Ascorbate reduces the quinone to its semiquinone free radical form. In the presence of molecular oxygen, the semiquinone is oxidized back to the quinone form and oxygen is reduced to superoxide anion, which generates other reactive oxygen species such as hydrogen peroxide.

such a critical metabolic pathway by ROS may have dramatic consequences for the survival of these cells.

Oxygen radicals have diverse effects on cells: in some situations, exposure to ROS can induce cell death, but conversely, ROS may activate signaling pathways that are thought to prevent cell death. Thus, the cell's decision to survive or die upon oxidative stress is regulated by a complex network in which the mitogen-activated protein kinases (MAPKs) are often implicated. Because the regulation of MAPK pathways is of critical importance for cancer cells, exploring the potential effect of oxidative stress on MAPK activity in these cells would be of particular interest. For instance, activation of the extracellular signal-regulated kinase (ERK) and other protein kinases of the MAPK pathway has often been associated with tumor promotion.[15,16] Moreover, ERK activation has been associated with a decreased survival in patients with breast cancer,[17] and its inhibition is known to enhance the effects of chemotherapy.[18]

Because ERK is 5- to 20-fold overexpressed in breast cancer cells compared with normal cells,[19] the aim of this work was to study the effects of oxidative stress on these cells. Our hypothesis is that oxidative stress induced by Asc/Men provokes ATP depletion, disrupts signal transduction pathways, and kills MCF7 cells (a human breast cancer cell line). To address this question, we used the following experimental approach: first, we assessed the capacity of MCF7 cells to reduce 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) dye in the absence and in the presence of Asc/Men, and we recorded the morphology of these stressed cells. Second, we evaluated the effect of oxidative stress on glycolysis by measuring the intracellular contents of both ATP and nicotinamide adenine dinucleotide (NAD) as well as the rate of lactate formation. Third, we assessed some markers of oxidative DNA damage (eg, γ-H2AX and the formation of poly-ADP-ribosylated proteins). Finally, we investigated the effects of oxidative stress on the activity of the ERK/MAPK proliferation pathway and the JNK/p38 stress kinases.

Methods

Chemicals

Menadione bisulfite (Men), sodium ascorbate (Asc), N-acetylcysteine (NAC), 3-aminotriazole (ATA), SB202190, 3-methyladenine (3-MA), BAPTA-AM and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, Mo). UO126 was purchased from Cell Signaling Technology (Danvers, Mass). Z-VAD-fmk was purchased from R&D Systems (Minneapolis, Minn). Complete mini protease inhibitor cocktail was purchased from Roche Applied Science (Mannheim, Germany). All other chemicals were ACS reagent grade.

Cell Culture and Treatments

The MCF7 cell line was a gift of Dr F. Brasseur (Ludwig Institute for Cancer Research, LICR-Brussels). The cells were cultured in Dulbecco's modified eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum, penicillin (10 000 U/mL), streptomycin (10 mg/mL), gentamycin (50 mg/mL), and 1.2% glutamine. The cultures were maintained at a density of about 50.10^3 cells/cm^2. The medium was changed at 48- to 72-hour intervals. All cultures were maintained at 37°C in a 95% air/5% CO_2 atmosphere with 100% humidity. For each passage, cells were washed twice with phosphate-buffered saline (PBS; Gibco) and then incubated at 37°C with trypsin-EDTA at 0.25% (Sigma-Aldrich). For each experiment, cell viability was assessed by trypan blue exclusion and should be at least 95%. Cultures were treated with 1 mM sodium ascorbate and 10 μM menadione bisulfite to generate a constant level of H_2O_2. When indicated, the antioxidant NAC was added to cell cultures at a concentration of 3 mM, whereas ATA, a catalase inhibitor,[20] and 3-methyladenine, an autophagy inhibitor,[21] were added to cell cultures at 5 mM and 10 mM, respectively, both 1 hour prior to the

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addition of ascorbate and menadione. BAPTA-AM (5 or 10 μM) and Z-VAD-fmk (50 μM) were added 15 minutes and 2 hours, respectively, before the addition of ascorbate and menadione.

Cell Cytotoxicity Assay

The cytotoxicity by ascorbate/menadione was assessed by following the reduction of MTT to blue formazan.[22] Briefly, after 24 hours of incubation in the absence or in the presence of ascorbate/menadione, aliquots of cell suspension (100 μL) were taken and incubated with MTT (0.5 mg/mL) for 2 hours at 37°C. Blue formazan crystals were solubilized by adding DMSO, and the colored solution was subsequently read at 550 nm. Results are expressed as percentage of MTT reduction compared with control untreated conditions. In addition, to monitor cell morphology during treatment, cells were maintained at 37°C in an atmosphere of 100% humidity and 5% CO_2 using an incubator coupled to an Axiovert S100 microscope (Zeiss, Oberkochen, Germany). One photograph was taken every 15 minutes during 24 hours using an AxioCam Hrm camera (Zeiss).

ATP, NAD, and Lactate Formation Measurements

We assessed ATP content by using the bioluminescence kit ATPLite (PerkinElmer, Waltham, Mass) according to the procedure given by the manufacturer. The results are expressed as nmol/10^6 cells. The amount of NAD was determined according to Klingenberg,[23] and the results are expressed as nmol/mg protein. The formation of lactate was determined by measuring its conversion into pyruvate hydrazone while NAD is converted into NADH.[24] The NADH formation may be quantified by measuring the absorbance at 340 nm. The results are expressed as nmol lactate/10^6 cells · per minute. The amount of protein was measured by the Bradford method[25] using bovine serum albumin as standard.

Immunoblotting Assays

At the indicated times, cells were washed twice with ice-cold PBS and then resuspended in a RIPA lysis buffer supplemented with 1 tablet of complete mini protease inhibitor cocktail. The samples were kept on ice for 20 minutes. They were then either centrifuged at 13 000 *g* for 20 minutes at 4°C or sonicated for 15 seconds for the detection of γ-H2AX. Supernatants or sonicated samples were collected and then stored at −80°C. Equal amounts of proteins were subjected to SDS-PAGE (6%-15% separating gel) followed by electroblot to nitrocellulose membranes. The membranes were blocked during 1 hour in TBS buffer (pH 7.4) containing 5% powdered milk protein and then incubated overnight at 4°C with the appropriate antibody. Rabbit polyclonal antibodies against ERK, phospho-ERK, phospho-RAF1, p38, phospho-p38, JNK, and phospho-JNK were from Cell Signaling Technology (Danvers, Mass). Antibodies against poly-(ADPribose) (PAR) were from BD Biosciences Pharmingen (Franklin Lakes, NJ). Rabbit polyclonal antibody against phospho-H2AX (γ-H2AX) was from Upstate (Billerica, Mass). After washing, membranes were exposed for 60 minutes at room temperature to a secondary antibody from Chemicon International (Temecula, Calif) linked to HRP or alkaline phosphatase. Finally, the protein bands were detected by chemiluminescence or colorimetric reaction.

Data Analysis

Results are expressed as mean ± standard errors of the mean (SEM). Differences between the experimental groups were analyzed using 1-way or 2-way analysis of variance followed, where appropriate, by a Bonferroni post hoc test. These tests were performed using GraphPad Prism software (GraphPad, San Diego, Calif). We considered $P < .05$ to be statistically significant.

Results

Cytotoxicity of Asc/Men on MCF7 Cells

Figure 2A shows that the oxidative stress induced by Asc/Men (1 mM/10 μM) decreases by more than 70% the capacity of MCF7 cells to reduce the MTT dye after 24 hours of incubation. This cytotoxic effect is not observed when these compounds are added separately, indicating a synergistic effect of both compounds. The addition of NAC to cell cultures exposed to an oxidative stress restores to a significant degree their capacity to reduce MTT. Conversely, by preincubating the cells with ATA, a well-known catalase inhibitor, a more profound depression in the capacity to reduce MTT was observed in MCF7-treated cells.

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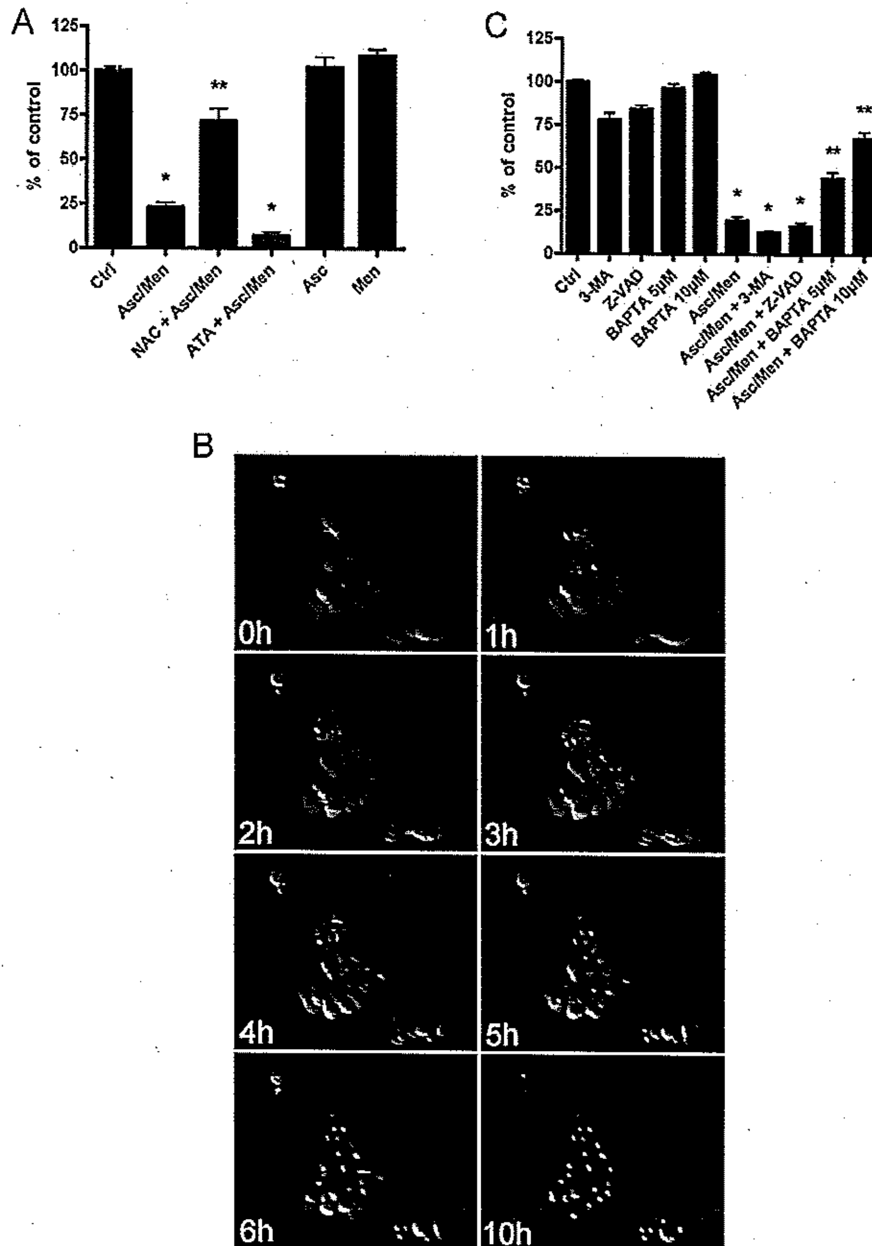

Figure 2. Cytotoxicity of Asc/Men on MCF7 Cells.

Note: (A) Cells were incubated for 24 hours in the presence of medium alone (Ctrl), 1 mM ascorbate (Asc), 10 µM menadione (Men), 1 mM ascorbate plus 10 µM menadione (Asc/Men), Asc/Men (1 mM/10 µM) supplemented with 3 mM N-acetylcysteine (Asc/Men + NAC), or Asc/Men (1 mM/10 µM) supplemented with 3-aminotriazole (Asc/Men + ATA). In this last case, ATA was preincubated for 1 hour before adding Asc/Men. At the end of the incubation, aliquots of cell suspension were taken and the MTT test was performed as described in the methods. Results are expressed as percentages of control and represent mean ± SEM (n = 3). *Statistically different from control condition. **Statistically different from Asc/Men condition. (B) Cells were incubated in the presence of ascorbate (1 mM) plus menadione (10 µM). A photograph of the cells is taken every 15 minutes as indicated in the methods. The picture shows the morphology at the indicated times. (C) Cells were incubated for 24 hours in the absence (Ctrl) or the presence of Asc/Men (1 mM/10 µM) supplemented or not with 10 mM 3-MA, 50 µM Z-VAD, or BAPTA-AM (at either 5 or 10 µM). Z-VAD and BAPTA-AM were preincubated for 2 hours and 15 minutes, respectively. The MTT test was performed as described in the methods. Results are expressed as percentages of control and represent mean ± SEM (n = 3). *Statistically different from Ctrl condition. **Statistically different from Ctrl and from Asc/Men conditions.

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Figure 2B shows the change of the morphology of MCF7 cells exposed to Asc/Men (1 mM/10 μM). During the first hours of incubation, the volume of cells is diminishing while blebs start to appear on the plasma membrane. After 5 hours of incubation, the plasma membranes of the cells begin to disrupt. This indicates that cell lysis begins after 5 hours of incubation with Asc/Men. This timing was confirmed by measurement of the release of cellular lactate dehydrogenase into the incubation medium (data not shown).

Figure 2C shows that cytotoxicity induced by oxidative stress may be modulated by some metabolic inhibitors. The strong decrease in MTT reduction induced by Asc/Men (1 mM/10 μM) was not modified by the addition of both the macroautophagy inhibitor 3-MA (10 mM) and the pan caspase inhibitor Z-VAD-fmk (50 μM) to cell cultures. However, the preincubation of cells in the presence of the calcium chelator BAPTA-AM (at either 5 or 10 μM) restores to a significant degree the capacity of stressed cells to reduce MTT.

Effects of Oxidative Stress Induced by Asc/Men on Some Glycolytic Markers

Regarding the effect of oxidative stress on glycolysis, Figure 3A shows the rate of lactate formation by MCF7 cells during the first 3 hours of incubation: compared with control cells (1.13 μmol lactate/10^6 cells × hour), cells incubated with Asc/Men reduced the formation of lactate by 76% (0.27 μmol lactate/10^6 × hour). Moreover, as soon as 1 hour after the beginning of the incubation, oxidative stress reduced by about 78% and 82% the intracellular contents of NAD (Figure 3B) and ATP (Figure 3C), respectively. Given the fast onset of these cellular events, they should be considered the cause rather than the consequence of cell death.

Effects of an Oxidative Stress Induced by Asc/Men on DNA Integrity

In previous reports we have shown that among ROS formed during menadione redox cycling, hydrogen peroxide was the main oxidizing agent.[8] This ROS is stable and is able to diffuse across different cell compartments. Most probably in this way it reaches the nucleus and causes DNA lesions as shown by the phosphorylation of histone H2AX, namely γ-H2AX, a marker of DNA strand breaks, as well as by the increased amount of PAR proteins. These latter indicate the activation of the enzyme PAR polymerase (PARP), which is required for the activity of DNA repair enzymes. Once again, no effects on DNA were observed when ascorbate and menadione were tested separately (Figure 4).

Are MAPK Signaling Cascades Affected by the Oxidative Stress Induced by Asc/Men?

Figure 5 shows that MCF7 cells incubated under control conditions displayed phosphorylated p44/42 MAPK protein bands, whereas in cells exposed to Asc/Men, a progressive loss of these phosphorylated protein bands is observed. Indeed, the phosphorylation of ERK strongly decreased between 2 and 3 hours of incubation, whereas the amount of phospho-RAF is decreased between 1 and 2 hours of incubation. The constitutive forms of ERK1/2 are not modified in cells incubated with Asc/Men. Regarding the stress kinases, the phosphorylation of JNK/SAPK remained unaffected by oxidative stress whereas the stress-induced p38 MAPK was activated. The incubation of cells with UO126 and SB202190, 2 well-known MAPKs inhibitors, did not modify cell death by Asc/Men (data not shown). This suggests that both ERK inhibition and p38 activation represent secondary pathways in mechanisms leading to cell death by Asc/Men.

Discussion

Increasing lines of evidence show that the induction of an oxidant injury may be an interesting strategy to kill cancer cells. Indeed, constitutively high levels of cellular oxidative stress and dependence on ROS signaling may represent a redox vulnerability of malignancy that can be targeted by chemotherapeutic intervention using redox modulators.[1] Thus, prooxidant pharmacological agents that substantially increase cellular ROS would preferentially impair redox homeostasis in cancer cells because they lack antioxidant enzymes.[14,26] Among prooxidant agents, the combination of ascorbate and menadione has the advantage that ascorbate is preferentially taken up by cancer cells,[27] most probably because of their overexpression of GLUT1 transporters,[28] which favors the in situ ascorbate-driven menadione redox cycling. In agreement with our previous results obtained in other cancer cell lines of both human and murine origin, we show here that ascorbate

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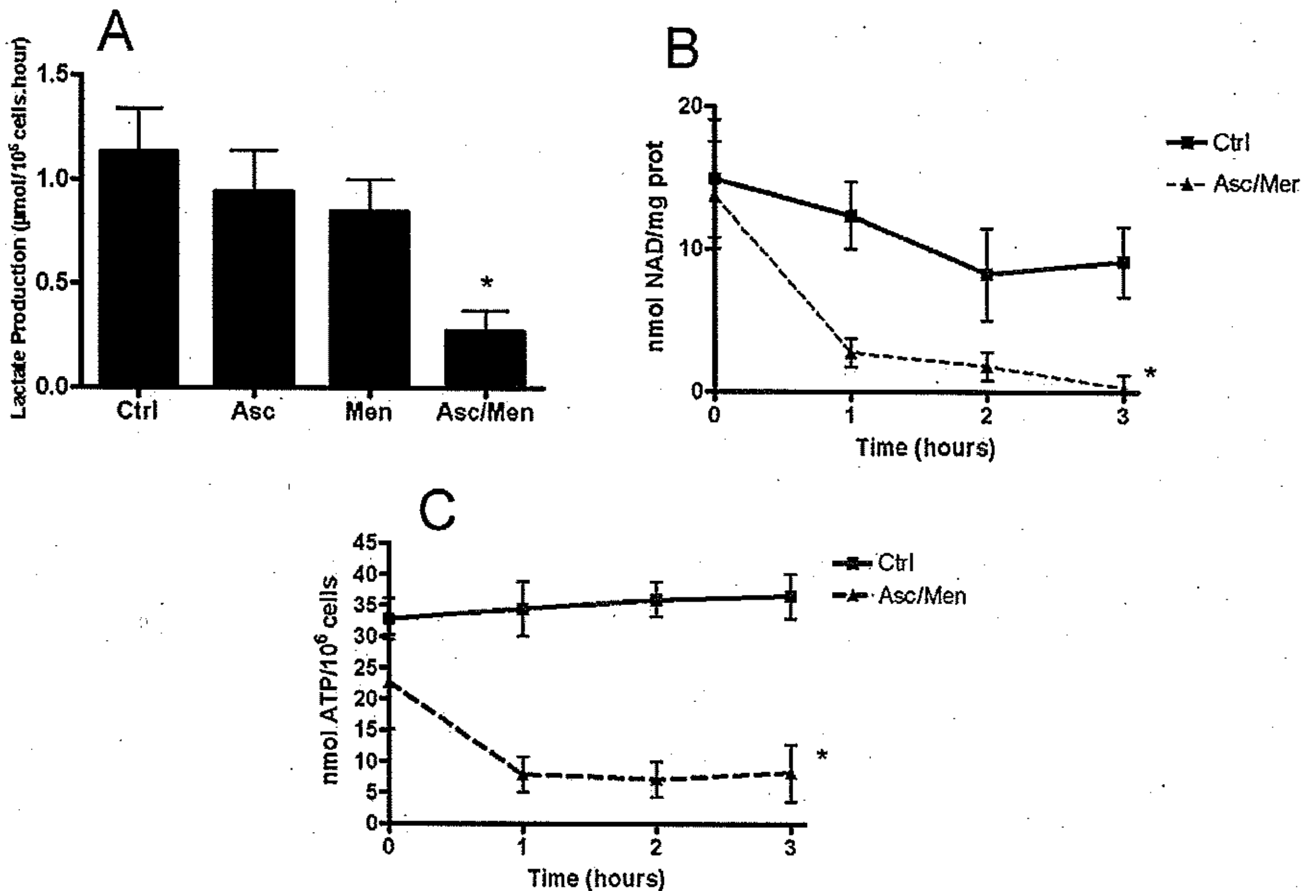

Figure 3. Effect of Oxidative Stress on Some Glycolytic Markers.
Note: Cells were incubated in the presence of medium alone (Ctrl), ascorbate (1 mM), menadione (10 µM), or ascorbate (1 mM) plus menadione (10 µM) (Asc/Men). (A) Lactate production rate of the MCF7 cells during the first 4 hours of incubation. Results represent mean $\pm$ SEM (n = 5). (B) NAD content is expressed as nmol/mg protein and the results represent mean $\pm$ SEM (n = 3). (C) ATP content is expressed as nmol/10^6 cells and the results represent mean $\pm$ SEM (n = 4). *Statistically different from control condition.

increases the cycle of oxidation-reduction of menadione, leading to ROS formation and subsequently cell death. This latter is likely induced by a concerted mechanism including glycolysis inhibition, calcium homeostasis deregulation, DNA damage, and changes in MAPK activities.

This is not the first report describing the effects of an H_2O_2-generating system on MCF7 cell survival; other authors have already reported that enhanced H_2O_2 production is a major pathway for menadione-induced DNA damage and cytotoxicity.[29] On the other hand, H_2O_2 may affect calcium homeostasis by elevating intracellular Ca^{2+} concentration, triggering several pathways associated with calcium-dependent hydrolases that play important roles in cellular injury and ultimately cause cell death. Our results show that Asc/Men deregulates calcium homeostasis as shown by the protective effect of the intracellular calcium chelator BAPTA-AM. Indeed, common players in necrotic cell death, irrespective of the stimulus, are calcium deregulation and ROS. During necrosis, elevated cytosolic calcium levels typically lead to mitochondrial calcium overload, bioenergetics effects, and activation of proteases and phospholipases. ROS initiate damage to lipids, proteins, and DNA, which consequently results in mitochondrial dysfunction, ion balance deregulation, and loss of membrane integrity.[30] Studies are now in progress to elucidate a potential role of calcium homeostasis and endoplasmic reticulum stress on cytotoxicity by Asc/Men as well as to identify the origin of such a calcium flux.

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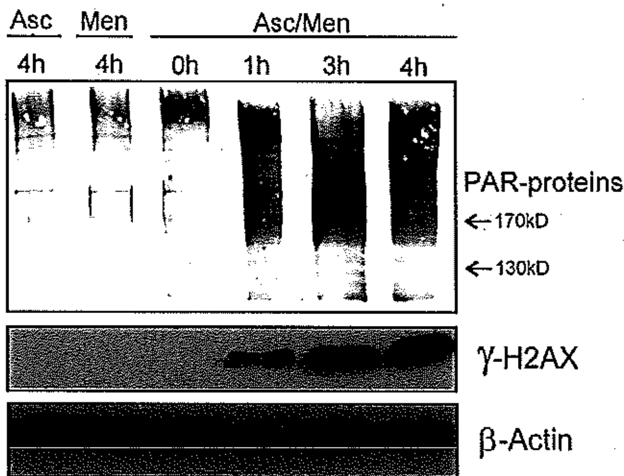

Figure 4. Effect of Oxidative Stress on DNA Integrity.
Note: Cells were incubated in the presence of medium alone (Ctrl), ascorbate (1 mM), menadione (10 μM), or ascorbate (1 mM) plus menadione (10 μM) (Asc/Men). At indicated times, cells were washed 2 times with PBS and lysed. The presence of γ-H2AX and the amount of poly-ADP-ribosylated (PAR) proteins were measured by Western blot as described in the methods. β-actin was used as a loading control for each lane. Blots are representatives of 1 of 3 independent experiments.

The loss of cell viability induced by Asc/Men is a likely (but not the only) consequence of glycolysis impairment because depletion of both ATP and NAD is occurring before cells start to die. These results are in agreement with previous reports obtained with chronic myeloid leukemia (K562) cells.[9,10] The NAD depletion can be explained by the activation of PARP. Indeed, DNA strand breaks lead to PARP activation, which results in the cleavage of NAD to yield nicotinamide and monomers of ADP-ribose. The poly-ADP-ribosylation of proteins (mainly histones) by PARP is required to allow DNA repair enzymes to enter into the DNA compacted structure. Therefore, the formation of PAR monomers at the expense of NAD leads to glycolysis arrest, ATP depletion, and necrotic cell death.[10,31]

The oxidative stress induced by ascorbate-driven menadione redox cycling inhibits phosphorylation of ERK and increases phosphorylation of p38 but does not affect JNK activity. This represents an interesting additional mechanism to induce cancer cell death because several new approaches are designed to target specifically the members of the ERK MAPK pathway, which is associated with tumor proliferation and poor prognosis. The MAPK pathway can be divided into 2

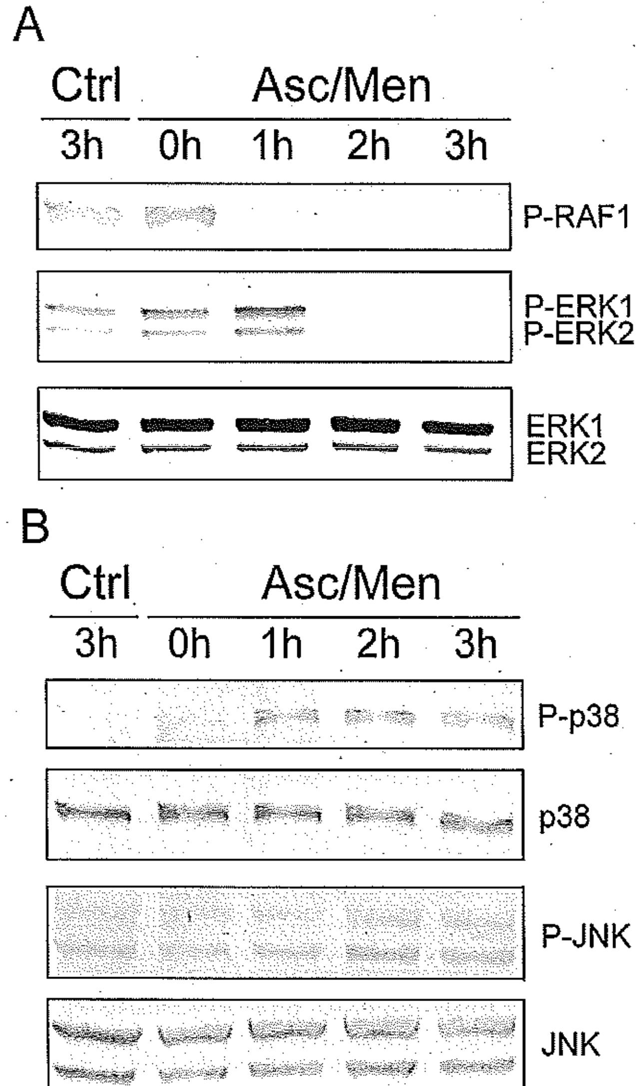

Figure 5. Effect of Oxidative Stress on the Activation of Raf-1, ERK, JNK, and p38 Proteins.
Note: Cells were incubated in the presence of medium alone (Ctrl) or ascorbate (1 mM) plus menadione (10 μM) (Asc/Men). At indicated times, cells were washed 2 times with PBS and lysed. Phospho-Raf1 (P-RAF1), phospho-ERK1/2 (P-ERK1, P-ERK2), ERK1/2, phospho-p38 (P-p38), p38, phospho-JNK (P-JNK), and JNK were detected by Western blot as indicated in the methods. Blots are representatives of 1 of 3 independent experiments.

independent parts: the ERK MAPK pathway, which includes Ras, Raf, MEK, and ERK and is involved in tumor proliferation; and the p38/JNK pathway, also called the stress MAPK pathway, which is associated with cell death and apoptosis.[32-34] Certainly, the inhibition of ERK1/2 phosphorylation observed in MCF7 cells under oxidative stress agrees with previous

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findings that demonstrate the responsiveness of the MAPK cascade to H_2O_2.[35] Interestingly, it has been reported that H_2O_2 is able to activate DUSP1 phosphatase, which inactivates ERK1/2.[36] Because in preliminary studies we have observed an increased expression of DUSP1 in cells stressed by Asc/Men (unpublished results), it is tempting to postulate that the inhibition of ERK phosphorylation in stressed cells may occur via DUSP1 activation by H_2O_2 generated during ascorbate-driven menadione redox cycling. Unlike the ERK response, the stress-activated p38 signal was rapidly induced by Asc/Men. If the regulation of JNK and p38 seems to be independent,[37] the p38 protein is known to be responsive to oxidative stress[38,39] and its activation may lead to cell death.[34,40] However, both the inhibition of ERK and the activation of p38 appear only as secondary pathways in mechanisms leading to cell death by Asc/Men given the lack of effect of MAPK inhibitors.

Figure 6 summarizes the main conclusions of this work: ascorbate-driven menadione redox cycling generates ROS. Among them, we have shown a main role of H_2O_2 as oxidizing agent. Hydrogen peroxide, either in a direct way or via a Fenton reaction, is able to impair glycolysis, cause oxidative DNA damage, deregulate calcium homeostasis, and affect components of various MAPK cascades. These impaired processes lead to MCF7 cell death. We postulate that oxidative stress induced by Asc/Men should affect cancer cells rather than normal cells, because they accumulate ascorbate and lack antioxidant enzymes. The consequences of this sensitivity may have important clinical applications. Within this frame, a similar approach for treatment of metastatic or locally advanced, inoperable transitional cell carcinoma of the urothelium (stage III and IV bladder cancer) has been granted by the FDA, and the results of a first clinical trial have been recently published.[41]

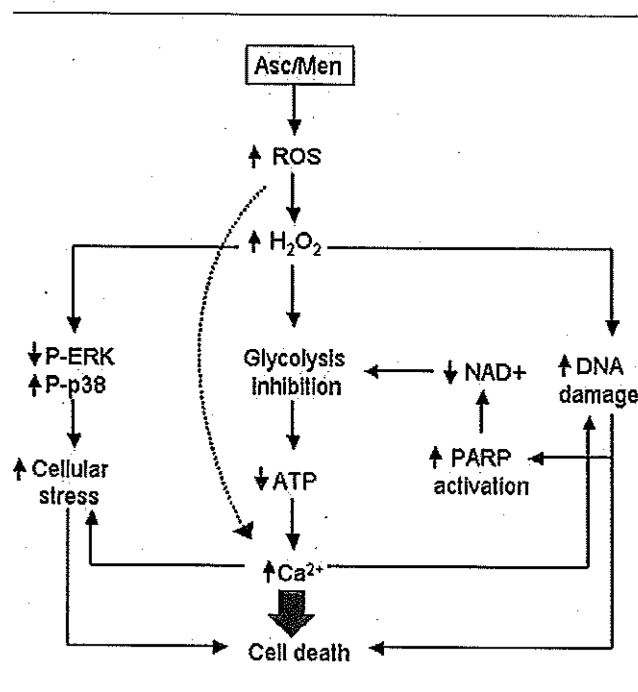

Figure 6. Hypothetical Scheme of the Mechanisms Involved in the Cytotoxicity by Asc/Men.
Note: Ascorbate-driven menadione redox cycling generates ROS, among which H_2O_2 plays a major role as oxidizing agent. It may act either in a direct way or via a Fenton reaction, and it impairs glycolysis, causes oxidative damage within DNA, deregulates calcium homeostasis, and affects components of various MAPK cascades. These concerted mechanisms lead to MCF7 cell death.

Acknowledgments

The authors thank Isabelle Blave for her excellent technical assistance and Dr Rosette Liderau for her valuable contribution by performing the PCR assays of stressed cells. This work was supported by grants from the Belgian Fonds National de la Recherche Scientifique (FNRS) (3.4594.04) and by the Fonds Spéciaux de Recherche (FSR) de l'Université Catholique de Louvain. Raphaël Beck is an FRIA recipient, Julien Verrax is an FNRS postdoctoral researcher, and Nicolas Dejeans is a Télévie-FNRS postdoctoral researcher.

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