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Role of glycolysis inhibition and poly(ADP-ribose) polymerase activation in necrotic-like cell death caused by ascorbate/menadione-induced oxidative stress in K562 human chronic myelogenous leukemic cells

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Among different features of cancer cells, two of them have retained our interest: their nearly universal glycolytic phenotype and their sensitivity towards an oxidative stress. Therefore, we took advantage of these features to develop an experimental approach by selectively exposing cancer cells to an oxidant insult induced by the combination of menadione (vitamin K_3) and ascorbate (vitamin C). Ascorbate enhances the menadione redox cycling, increases the formation of reactive oxygen species and kills K562 cells as shown by more than 65% of LDH leakage after 24 hr of incubation. Since both lactate formation and ATP content are depressed by about 80% following ascorbate/menadione exposure, we suggest that the major intracellular event involved in such a cytotoxicity is related to the impairment of glycolysis. Indeed, NAD^+ is rapidly and severely depleted, a fact most probably related to a strong Poly(ADP-ribose) polymerase (PARP) activation, as shown by the high amount of poly-ADP-ribosylated proteins. The addition of *N*-acetylcysteine (NAC) restores most of the ATP content and the production of lactate as well. The PARP inhibitor dihydroxyisoquinoline (DiQ) was able to partially restore both parameters as well as cell death induced by ascorbate/menadione. These results suggest that the PARP activation induced by the oxidative stress is a major but not the only intracellular event involved in cell death by ascorbate/menadione. Due to the high energetic dependence of cancer cells on glycolysis, the impairment of such an essential pathway may explain the effectiveness of this combination to kill cancer cells.

Key words: ascorbate/menadione; oxidative stress; glycolysis; PARP activation; NAD^+ depletion

Cancer cells are known to present a large genetic heterogeneity. Indeed, despite some classical mutations such as p53, no typical cancer cell genotype exists and each invasive cancer appears as the consequence of a particular genetic pathway traveled during carcinogenesis.[1,2] However, this genetic diversity does not correlate with the clinical observations where a common invasive behavior including uncontrolled growth and destruction of normal tissues, are noted. Interestingly, such an evolutionary process leads to the acquisition of particular phenotypes, among them the upregulation of glycolysis is probably the oldest described.[3] In addition to this high energetic dependence, cancer cells generally exhibit a poor antioxidant status.[4–6] Therefore, we developed a novel strategy consisting of the generation of an oxidative stress by the use of a combination of sodium ascorbate (vitamin C) and menadione (vitamin K_3).[7] In this strategy, a redox cycle is initiated by electron transfer from ascorbate ($AscH^-$) to quinone (Q) as shown in the next equations:

$$AscH^- + Q \rightarrow SQ^{\cdot -} + Asc^{\cdot -} + H^+ \quad (1)$$

$$SQ^{\cdot -} + O_2 \rightarrow Q + O_2^{\cdot -} \quad (2)$$

The rapid reoxidation of the semiquinone ($SQ^{\cdot -}$) to its quinone (Q) form by molecular oxygen leads to generation of reactive oxygen species derived from superoxide anion ($O_2^{\cdot -}$), such as hydrogen peroxide (H_2O_2) or hydroxyl radicals ($HO^{\cdot}$). Among these reactive species we have reported that H_2O_2 is the key mediator of the cytolytic effect caused by ascorbate/menadione in TLT cells, a murine hepatoma-derived cell line.[8,9] Up to now, we have reported that the cytotoxicity induced by the combination of ascorbate and menadione is time- and dose-dependent. The cell death is only observed if the compounds are simultaneously added, at least at the concentrations they have been employed.[9] In addition, the cytotoxicity of an combination between ascorbate and a given quinone relies on the half-redox potential of the quinone.[10] Otherwise, not every quinone derivative may replace the menadione. Aminotriazole, a catalase inhibitor, increases the cytotoxicity of ascorbate/menadione, reinforcing the major role of hydrogen peroxide in this process.[11] Moreover, the antioxidant enzyme catalase and the GSH-precursor NAC as well as tyrosine phosphatase inhibitors (*e.g.,* vanadate), suppress the cytotoxic effect induced by ascorbate/menadione.[8–10] The transcription factor NF-kB, constitutively active in TLT cells, is inhibited by ascorbate/menadione.[7] Finally, the cancer cell death is caspase independent, and on the basis of morphological observations, this type of cell death has been called ''autoschizis''.[7–13]

Since cancer cells are strongly dependent on glycolysis, we postulate that cell death by ascorbate/menadione occurs by ATP depletion due to glycolysis arrest. Therefore, the aim of this study was to analyse the mechanisms by which ascorbate/menadione may affect glycolysis thus conditioning cell death or cell survival. To support this hypothesis, several parameters were recorded during the incubation of K562 cells (a human chronic myelogenous leukemic cell line) in the absence or in the presence of ascorbate and menadione either alone or in combination. They include intracellular contents of ATP and NAD^+ as well as cell survival. Cellular uptake of radiolabelled deoxyglucose was recorded during 10 min to exclude any artefact concerning glucose depletion through a putative nutrient transport impairment. The intracellular amounts of glucose-6-phosphate, fructose-1,6-diphosphate and dihydroxyacetone-phosphate and the activity of hexokinase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were measured to assess the glycolytic pathway. The amount of poly-ADP-ribosylated proteins was estimated by Western blots. Finally, a poly(ADP-ribose) polymerase (PARP) inhibitor, namely 1,5-Dihydroxyisoquinoline (DiQ), was used to check the role of NAD^+ depletion in cell death caused by ascorbate/menadione-induced oxidative stress.

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Material and methods

Cell line and cell culture conditions

The K562 cell line was a gift of Dr. F. Brasseur (Ludwig Institute for Cancer Research-LICR-Brussels). They were cultured in DMEM/F12 (Dulbecco's Modified Eagle Medium, Gibco) supplemented with 10% foetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamicin (50 μg/ml). The cultures were maintained at a density of 1–2 $\times$ 10^5 cells/ml. The medium was changed at 48–72 hr intervals. All cultures were maintained at 37°C in a 95% air/5% CO_2 atmosphere with 100% humidity.

Chemicals

Menadione sodium bisulfite, sodium ascorbate, 1,5-dihydroxyisoquinoline (DiQ), 2-deoxyglucose and dimethylsulfoxide (DMSO) were purchased from Sigma (St Louis, MO). [^{3}H]-2-Deoxy-D-glucose (13.0 Ci/mmol) was obtained from Amersham (Little Chalfont, Bucks., UK). Complete Mini protease inhibitor cocktail was purchased from Roche Applied Science (Mannheim, Germany). All other chemicals were ACS reagent grade. Ascorbate and menadione were used at 2 mM and 5 μM respectively (ratio = 400/1) in all the experiments. Fresh solutions were extemporaneously prepared in sterile water before use.

Assays

Cell death assay. Cellular viability was estimated by measuring the activity of lactate dehydrogenase (LDH), according to the procedure of Wrobleski and Ladue, both in the culture medium and in the cell pellet obtained after centrifugation.[14] The results are expressed as a ratio of released activity to the total activity.

Lactate production. The formation of lactate was recorded during 3 hr according to the method described by Hohorst.[15] Briefly, every hour, 1 ml of cellular suspension (containing 1.0 $\times$ 10^6 cells) was added to 100 μl of 70% perchloric acid (PCA). Samples were kept on ice for 30 min before storage at −20°C. After centrifugation (15,000*g*, 1 min) to remove the precipitated proteins, supernatants were isolated and neutralized with a solution of $KOH/KHCO_3$ 3 M. A second centrifugation was then performed to remove the $KClO_4$ precipitate. One hundred microliters of the supernatant were placed in a reaction mixture containing 2 mM of β-nicotinamide adenine dinucleotide (NAD^+), 0.6 M glycine, 3.2 mM ethylenediaminetetraacetatic acid (EDTA) and 0.24 M hydrazine. The reaction was started by the addition of 20 μl of pure LDH and changes in absorbance were recorded at 340 nm.

Determination of NAD^+ and ATP contents. ATP content was determined by using the Roche ATP Bioluminescence Assay Kit CLS II (Mannheim, Germany) and the results are expressed as nmol ATP/mg proteins. The amount of protein content was determined by the method of Lowry using BSA as reference.[16]

NAD^+ content was determined following the method described by Klingenberg.[17] Briefly, 3.0 $\times$ 10^7 cells were washed twice with ice-cold potassium phosphate buffered saline (PBS) and resuspended in 0.6 M perchloric acid. Cell extracts were neutralized with 3 M KOH and placed in glycylglycine buffer (125 mM). After centrifugation to remove the $KClO_4$ precipitate, 20 μl of sample or NAD^+ standard were placed in a reaction mixture containing 1 M ethanol, 0.1 mM 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide [MTT], 0.9 mM phenazine methosulfate, 14 U alcohol dehydrogenase and 0.1 M nicotinamide in 60 mM glycylglycine at pH 7.4. Changes in absorbance were recorded at 560 nm.

Glucose uptake. Glucose uptake was measured by using [^{3}H]-2-deoxy-D-glucose (2-DOG). Briefly, exponentially growing cells were incubated at 1.0 $\times$ 10^6 cells. Initial rates of glucose uptake were measured by adding 1 μCi/ml of [^{3}H]-2-deoxy-D-glucose. Glucose uptake was determined in the absence and in the presence of ascorbate/menadione during 4 min under conditions where the uptake was linear for 10 min. Cells were washed with ice-cold PBS and then treated with Triton X-100 (2%). Radioactivity was determined by liquid-scintillation counting. The kinetic analysis of 2-DOG uptake were performed in the presence of a range of concentrations varying from 0.01 to 0.1 mM 2-DOG. The results were normalized towards protein contents.

Glycolytic enzyme activities and metabolites quantification. For the determination of hexokinase activity, 1.0 $\times$ 10^7 cells were washed twice in ice-cold PBS, resuspended in PBS and sonicated for 15 sec at 100 W with a Labsonic U (Braun, Melsungen, Germany). Samples were kept on ice and the dosage was immediately performed. Twenty microliters of each sample was added to a reaction mixture containing 0.2 mM β-nicotinamide adenine dinucleotide phosphate (NADP), 5 mM adenosine 5′-triphosphate magnesium salt (Mg^{++}-ATP), 1 mM dithiothreitol (DTT), 1.5 U glucose-6-phosphate dehydrogenase (G-6-PDH) and 50 mM Hepes buffer. Reaction was started by the addition of 5 mM glucose and the O.D. was read at 340 nm during 5 min.

For the determination of glyceraldehyde-3-phosphate dehydrogenase activity, 1.0 $\times$ 10^6 cells were washed twice in ice-cold PBS, resuspended in PBS and sonicated for 15 sec (100 W). Samples were kept on ice and the assay was immediately performed. Twenty microliters of each sample was added to a reaction mixture containing 0.15 mM β-nicotinamide adenine dinucleotide reduced (NADH), 1 mM adenosine 5′-triphosphate magnesium salt (Mg^{++}-ATP), 1 mM ethylenediaminetetraacetatic acid (EDTA), 6.5 mM 3-phosphoglyceric acid, 15 U phosphoglycerate kinase (PGK) and 85 mM triethanolamine buffer (TEA). The O.D. was read at 340 nm during 5 min.

Glycolytic phosphosugar intermediates were quantified in 0.5 M PCA deproteinized cell extracts with coupled assays according to procedures described elsewhere.[18,19]

Immunoblotting. At the indicated times, cells were washed twice with ice-cold PBS and then resuspended in a lysis buffer containing 0.1% phenylmethylsulfonyl fluoride, 0.1% NP40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 100 mM sodium vanadate in PBS supplemented with one tablet of Complete Mini protease inhibitor cocktail. The samples were kept on ice for 20 min before sonication (15 sec, 100 W) and storage at −20°C. Equal amounts of proteins (20 μg) were subjected to SDS-PAGE (4–20 % separating gel) followed by electroblot to nitrocellulose membranes. The membranes were blocked 1 hr in TBS buffer (pH 7.4) containing 5% powdered milk protein followed by an overnight incubation with diluted antibodies in a fresh solution of powdered milk protein (1%, w/v) in TBS buffer. The membranes were washed and incubated for 60 min with a dilution of secondary antibody coupled to horseradish peroxidase or alkaline phosphatase. Anti-PAR and anti-γ-H2AX were rabbit polyclonal antibodies diluted 1:1,000. They were purchased respectively from BD Biosciences (San Diego, CA) and Cell Signalling (Beverly, MA). Anti-β-actin was a mouse monoclonal antibody used at 1:10,000 and purchased from Abcam (Cambridge, UK). Goat anti-rabbit antibody and rabbit anti-mouse polyclonal antibody were purchased respectively from Chemicon International (Temecula, CA) and DakoCytomation (Glostrup, Denmark).

Statistical analysis

Data were analysed using 1-way or 2-way analysis of variance (ANOVA) followed by Scheffé test for significant differences between means. For statistical comparison of results at a given time point, data were analysed using Student's *t* test.

Results

Glycolysis is rapidly inhibited by oxidative stress induced by ascorbate/menadione, leading to a cellular energetic impairment

The results presented in Figure 1*a* show that after 3 hr of incubation the lactate production decreases by about 80% in the ascorbate/menadione-treated group as compared to control value. Such

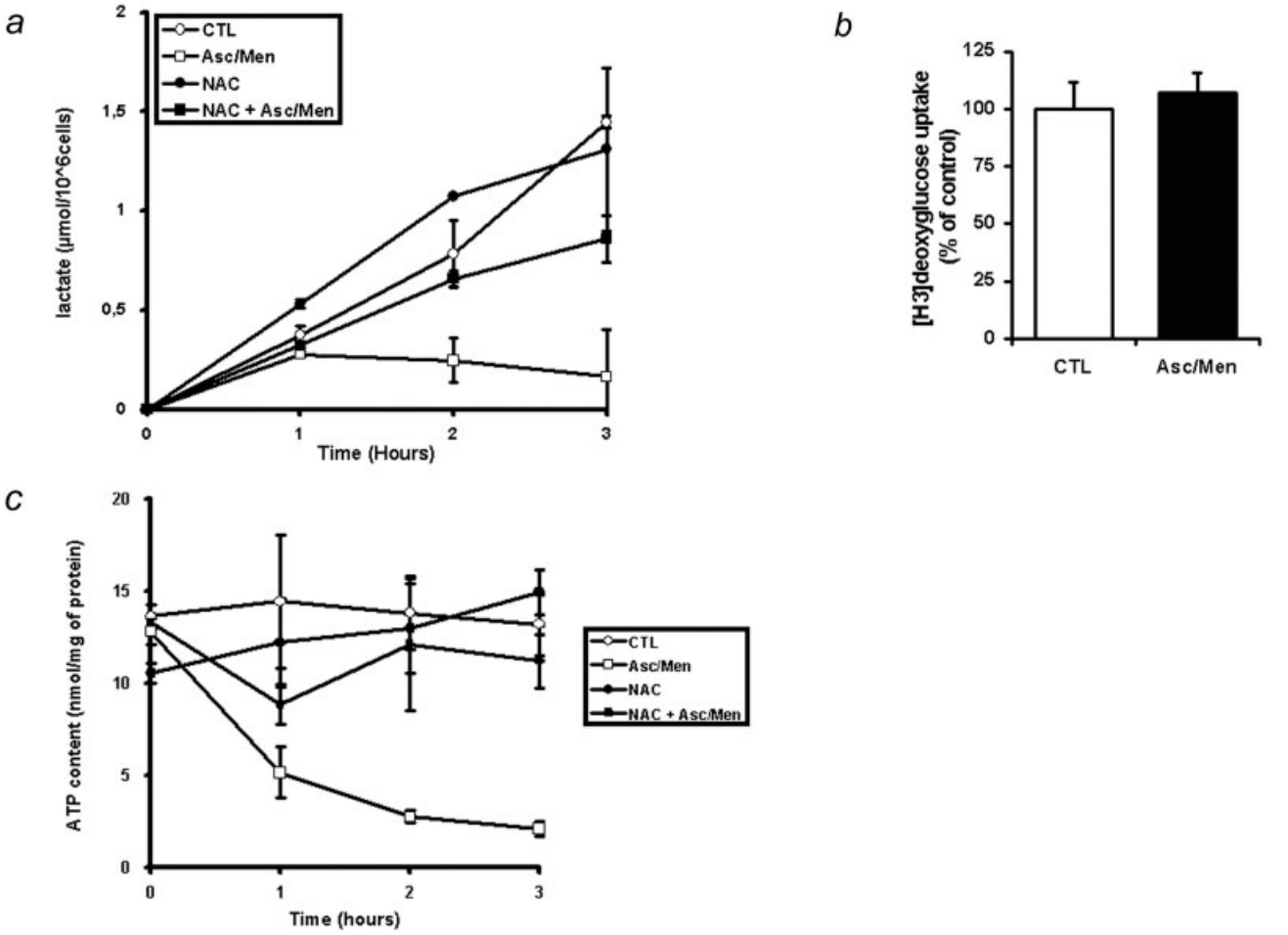

Figure 1 – Effect of ascorbate/menadione on lactate formation, glucose uptake and ATP content in K562 cells. (*a*) K562 Cells were incubated for 3 hr at 37°C in the absence (control) or in the presence of a mixture of both compounds (2 mM ascorbate and 5 µM menadione); 3 mM NAC was used without preincubation. Aliquots of cell suspension were taken every hour and the rate of lactate formation was measured as indicated in the Material and Methods section. The results represent the mean values ± SEM of at least 3 separate experiments. The ascorbate/menadione group was significantly different from the control ($p < 0.01$, using 2-way ANOVA). (*b*) K562 Cells were incubated at 37°C in the absence (control) or in the presence of the combination between ascorbate (2 mM) and menadione (5 µM). [^{3}H]-2-Deoxy-D-glucose uptake was determined after 4 min as indicated in the Material and Methods section. The results represent the mean values ± SEM of at least 6 separate experiments. (*c*) K562 Cells were incubated for 3 hr at 37°C in the absence (control) or in the presence of a mixture of both compounds (2 mM ascorbate and 5 µM menadione); 3 mM NAC was used without preincubation. Aliquots of cell suspension were taken and the ATP content was measured as indicated in the Material and Methods section. The results represent the mean values ± SEM of at least 3 separate experiments. The ascorbate/menadione group was significantly different from the control ($p < 0.05$, using 2-way ANOVA).

a glycolysis inhibition occurs only when ascorbate and menadione are added simultaneously while the addition of ascorbate and menadione separately did not affect the formation of lactate (data not shown). To rule out an early putative impairment of the glucose transport in the abolishment of lactate generation, the initial rates of glucose uptake were measured in the absence and in the presence of ascorbate/menadione. The results show in Figure 1*b* (expressed as % of radioactivity incorporated in cells), indicate that glucose uptake is similar in both control and ascorbate/menadione-treated cells. This result shows that the glycolytic flow is inhibited rather than the glucose uptake. As a consequence of this glycolysis arrest, the intracellular ATP level in the ascorbate/menadione-treated cells strongly decreases after 1 hr of incubation (Fig. 1*c*).

Since *N*-acetyl-cysteine (NAC) is a very potent inhibitor of ascorbate/menadione cytotoxicity,(10) we tested whether NAC may restore the glycolytic flow. Underlining the major role of oxidative stress, the incubation of K562 cells in the presence of ascorbate/menadione and NAC, resulted in the maintenance of both lactate (Fig. 1*a*) and ATP (Fig. 1*c*). It should be noted that during that time of incubation no cell death is observed at such concentrations of ascorbate and menadione (data not shown).

Glycolysis is inhibited through poly(ADP-ribose) polymerase activation leading to NAD$^+$ depletion

By measuring the amount of phospho-metabolites, we concluded that glycolysis was arrested at the step catalysed by GAPDH. Indeed, as shown in Table I, there is a strong increase in both fructose-1,6-bisphosphate (10-fold) and dihydroxyacetone phosphate (6.5-fold). Such an accumulation suggests an impairment of GAPDH but its enzyme activity was slightly inhibited by about 30% which is not sufficient to explain the strong accumulation of both metabolites.

Table I also shows that the concentration of glucose-6-phosphate decreases by about 50% in ascorbate/menadione-treated cells. Since the hexokinase activity is not modified under these conditions, such a reduction is probably related to the activation of PFK1 and/or the activation, due to the oxidative stress, of the phosphopentose pathway.

TABLE I – QUANTIFICATION OF GLYCOLYTIC METABOLITES IN K562 CELLS

Glycolysis Intermediates and enzyme activities	Control	Ascorbate	Menadione	Ascorbate + Menadione
Glucose-6-phosphate	4.5 ± 0.4	4.7 ± 0.5	4.6 ± 0.3	2.2 ± 0.4[1]
Fructose-1,6-bisphosphate	14.9 ± 2.4	35 ± 8.9	14.6 ± 2.4	147.9 ± 16.3[2]
Dihydroxyacetone-phosphate	2.9 ± 1.5	5.1 ± 2.9	2.9 ± 1.9	18.2 ± 3.9[2]
Hexokinase	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
GAPDH	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	0.8 ± 0.1

K562 cells were incubated for 90 min at 37°C in the absence (control) or in the presence of ascorbate (2 mM), menadione (5 μM) and a mixture of both compounds. Aliquots of cell suspension were taken and the content of intermediates and the enzyme activities were measured as indicated in the Material and Methods section. The results are expressed as nmol/mg prot (intermediates) and U/mg prot (enzymes). They represent the mean values ± S.E.M of at least three separate experiments.
[1] $p < 0.05$ as compared with that of control cells.–[2] $p < 0.01$ as compared with that of control cells.

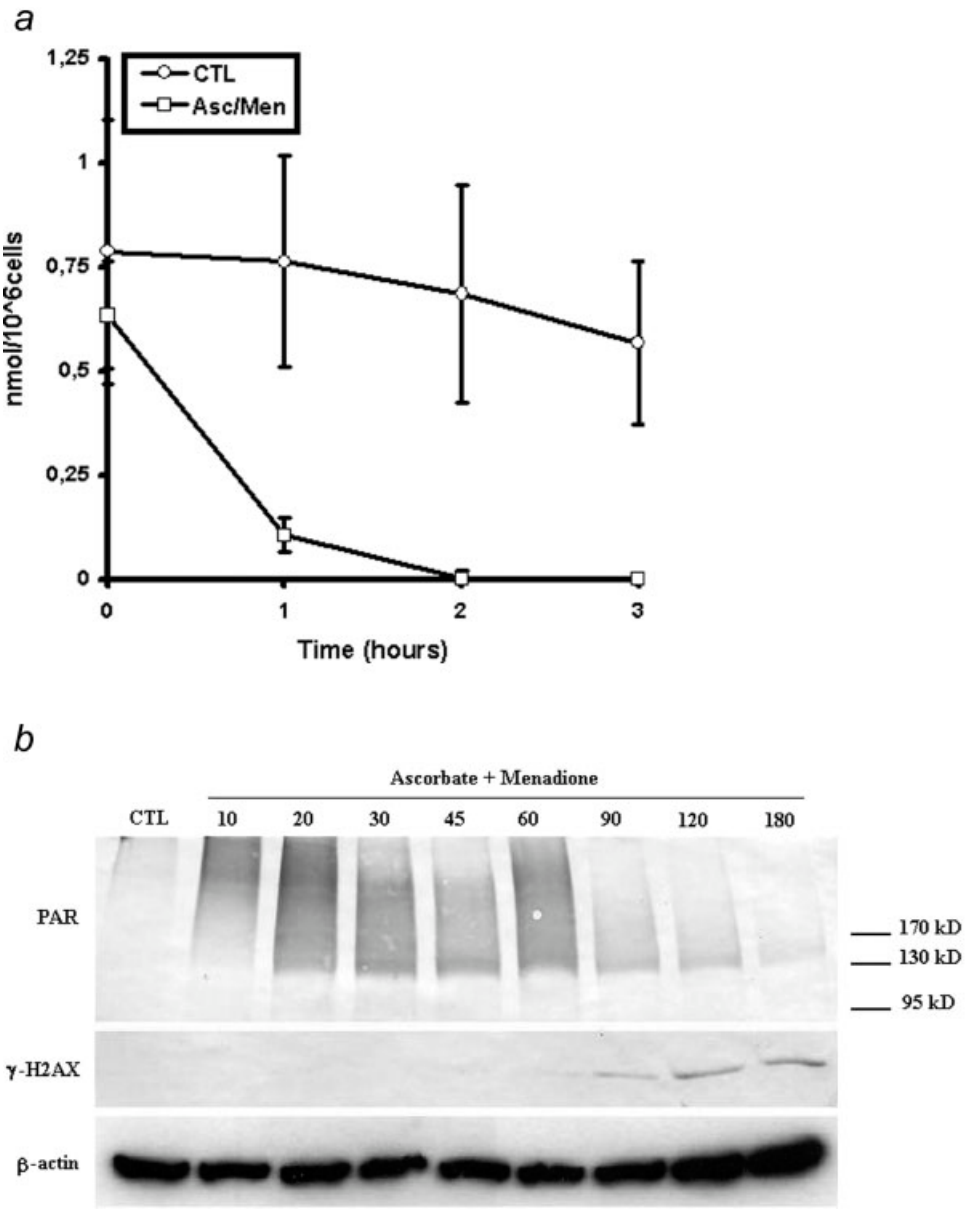

FIGURE 2 – Effect of ascorbate/menadione on NAD^+ content and on protein poly(ADP)ribosylation in K562 cells. (*a*) Changes of the NAD^+ content inside ascorbate/menadione-treated K562 cells. Ascorbate and menadione were used at 2 mM and 5 μM, respectively. Aliquots of cell suspension were taken at the indicated times and the NAD^+ content was measured as mentioned in the Material and Methods section. The results represent the mean values ± SEM of at least 3 separate experiments. (*b*) K562 Cells were incubated at 37°C in the absence or in the presence of a mixture of both compounds (2 mM ascorbate and 5 μM menadione). At the indicated times (minutes), cells were harvested and immunoblotting was performed using antibodies against poly (ADP-ribose) (PAR), β-actin and γ-H2AX as described in the Material and Methods section. Typical result out of 3 separate experiments is represented.

If the decrease in GAPDH activity cannot fully explain the inhibition of glycolysis by ascorbate/menadione, another critical target should be the intracellular level of NAD^+. Figure 2*a* shows that NAD^+ levels are strongly depleted when cells were incubated in the presence of ascorbate/menadione. Such a depletion appears rapidly and reaches nearly 100% after only 2 hr.

Such a drop in NAD^+ may be correlated with PARP activation since in ascorbate/menadione-treated cells, increased amounts of poly(ADP-ribosylated)-proteins were observed up to 60 min (Fig. 2*b*). Thereafter, a decrease in protein ADP-ribosylation was detected between 60 and 180 min of incubation. Similarly to control conditions, no ADP-ribosylation was observed in both

TABLE II – EFFECT OF A PARP INHIBITOR (DiQ) ON ASCORBATE/MENADIONE-TREATED CELLS

Ascorbate/menadione	Control		DiQ	
	–	+	–	+
LDH leakage (%)	8.5 ± 0.5	68.4 ± 7.1[1]	13.0 ± 1.0	27.2 ± 6.0
Lactate production (nmol/10^6 cells/h)	481 ± 19	120 ± 37[2]	454 ± 37	242 ± 55[3]
NAD^+ (nmol/mg protein)	0.96 ± 0.23	0.02 ± 0.02[2]	0.68 ± 0.14	0.26 ± 0.04[3]

K562 cells were incubated at 37°C in the absence (control) or in the presence of a mixture of both compounds (2 mM ascorbate and 5 μM menadione). DiQ was used at 100 μM and preincubated for 1 h. Aliquots of cell suspension were taken after 90 min and 24 h for the determination of the NAD^+ content and LDH leakage, respectively. The mean lactate production was determined over a period of 3 h by measuring the NAD^+ content as described in the Material and Methods section. Results represent the mean values ± S.E.M of at least three separate experiments.

[1] $p < 0.001$ as compared with that of control cells.–[2] $p < 0.01$ as compared with that of control cells.–[3] $p < 0.05$ as compared with that of DiQ alone.

ascorbate- and menadione-treated cells (data not shown). Interestingly, the presence of the phosphorylated form of the histone H2AX (namely γ-H2AX) was detected after 90 min of incubation, indicating the occurrence of DNA damage.

Supporting a major role of PARP activation, we demonstrated that cell viability, lactate formation and NAD^+ content were partially restored by inhibiting the activation of PARP (Table II). In the presence of ascorbate/menadione cell death is enhanced by 8-fold in the absence of PARP inhibitor while in the presence of DiQ, cell death only increases 2-fold. The production of lactate was decreased by 75% but in the presence of DiQ such a decrease was of 45%. Finally, the NAD^+ content was almost completely depleted while in the presence of DiQ it was only of 60%.

Discussion

As previously mentioned, cancer cells have a high energetic dependence towards glycolysis and a poor antioxidant status as well. Therefore, inhibition of glycolysis is an interesting intracellular target and appears to be a new possibility to induce cancer cell death.[20–22] Our hypothesis is that ascorbate enhances the menadione redox-cycle, increases the formation of reactive oxygen species generating oxidative stress.[23] Since glycolysis is inhibited during oxidative stress, we postulate that cancer cell death would be facilitated by this energetic impairment.

Since a severe depletion of ATP is observed when cells were incubated in the presence of ascorbate/menadione, we suggest that glycolysis should be the intracellular target of ascorbate/menadione (Fig. 3). The arrest of glycolysis was located at the step catalysed by GAPDH. Indeed, no changes were observed in glucose uptake whereas fructose-1,6-bisphosphate and dihydroxyacetone phosphate were strongly enhanced. However, a decrease in the amount of glucose-6-phosphate was observed. One possibility to explain this, is that oxidative stress by ascorbate/menadione decreases the amount of reduced glutathione (GSH).[10] To regenerate it, the oxidized form GSSG, is reduced back to GSH by GSSG reductase that uses NADPH as cofactor. The NADPH is regenerated then by the hexose monophosphate shunt that utilizes glucose-6-phosphate.[24] This metabolic pathway should be enhanced in case of NAD^+ depletion because cells will displace the NAD(P)/NAD(P)H equilibrium to restore the NAD^+ levels.

The strong depletion of ATP should therefore be the reflection of glycolysis impairment, explaining in this way how the combination ascorbate/menadione is killing cancer cells. Supporting this hypothesis, it has been reported that H_2O_2 stopped the glycolytic flow and inactivated GAPDH.[25,26] These effects may be the consequence of the depletion of intracellular NAD^+ *via* PARP activation and/or the inactivation of GAPDH by S-nitrosylation and formation of sulfenic acid.[27] Our results show that inhibition of GAPDH is not enough to explain the dramatic drop in ATP content and the inhibition in the formation of lactate caused by ascorbate/menadione. Actually, while GAPDH activity is inhibited by about 25%, both the ATP content and the formation of lactate are inhibited by about 80%. Since NAD^+ is also depleted to a similar extent, these results pointed out a critical role of PARP

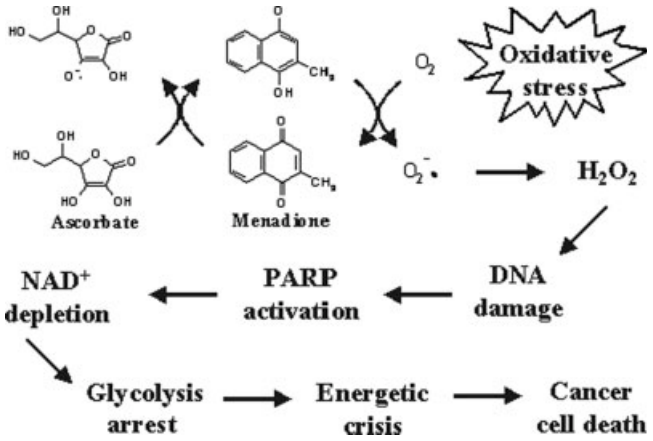

FIGURE 3 – Schematic summary of the proposed mechanism by which the ascorbate/menadione combination leads to cancer cell death. Menadione is nonenzymatically reduced by ascorbate to form semidehydroascorbate and the semiquinone free radical. Such a semiquinone is rapidly reoxidized to its quinone form by molecular oxygen. This redox-cycling generates various reactive oxygen species (ROS) among them H_2O_2 appears as the key mediator of cytotoxicity. The oxidative stress generated by the ascorbate/menadione combination leads to the occurrence of DNA damage and provokes a strong PARP activation. This latter consumes NAD^+ thus leading to a glycolysis arrest that is responsible for the rapid depletion of ATP observed in ascorbate/menadione-treated cells. Due to the lack of ATP, cancer cells will ultimately dye through a necrotic process.

activation as a mechanism involved in the cytotoxicity by ascorbate/menadione. This activation of PARP is likely the consequence of DNA damages that appear following the exposure towards the ascorbate/menadione combination, a fact supported here by the presence of γ-H2AX which is a well-known marker of early DNA strand breaks.[8,11,28]

Indeed, poly(ADP-ribosylation) is a post-translational modification consisting in the synthesis of ADP-ribose polymers on target proteins, a mechanism that regulates different functions such as DNA repair, replication, transcription and cell death.[29] The enzymes catalysing this reaction, namely PARPs, are members of a family of 7 members enzymes among which PARP-1 is the most abundant and the best known. This PARP-1 enzyme is an ubiquitous zinc-finger nuclear protein of 113 kDa that is present at high levels in each cell.[30] PARP-1 activity is rapidly enhanced both by single and double strand breaks, this leading to the synthesis of poly(ADP-ribose) (PAR) at the expense of β-NAD^+, cleaved in nicotinamide and ADP-ribose.[31] PAR has a fast turnover rate due to rapid degradation by the poly(ADP-ribose) glycohydrolase (PARG) that displays both endo- and exo-glycosidic activities.[32]

Activated PARP consumes cytosolic NAD^+, and because NAD^+ is required for glycolysis, the H_2O_2-induced PARP activation may render cells unable to use glucose as a metabolic substrate. Therefore, when an oxidative stress induces the activation of PARP, several proteins are poly-ADP-ribosylated (a mech-

anism of cell protection), including GAPDH (thus explaining why only 25% of its activity is lost), but since NAD^+ continues to be depleted, one of the main consequences for such a drop in NAD^+ is an arrest of the glycolysis, as well as several other oxido-reductase dependent processes. These metabolic events are particularly deleterious for cancer cells that are dependent on glycolysis for their energetic needs. Thus, when glycolysis is finally blocked, ATP depletion will follow, culminating 8–12 hr later in cell death. Since NAC totally protects against cell death by ascorbate/menadione but a PARP inhibitor did not, despite its use at doses much greater than its respective IC_{50} value (concentration leading to 50% activity inhibition), it appears that PARP activation is a major but probably not the only intracellular event leading to cell death.[33] The impairment caused by an oxidative stress (namely H_2O_2), indicates that critical events are occurring upstream in the cascade leading to cell death by ascorbate/menadione.

These results prompted us to again suggest that an ascorbate/menadione combination can be used as adjuvant therapy in cancer treatment. The rationale to use this combination is supported by 3 lines of evidences: first, because of their lack of antioxidant defenses, cancer cells are more sensitive towards an oxidative injury[4–6]; second, ascorbate seems to be preferentially taken up by cancer cells, due to their overexpression of GLUT receptors[34]; and third, cancer cells show an almost universal glycolytic phenotype, required to survive and to invade cells/tissues of their neighborhood.[3,35,36]

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