Biochemical Pharmacology, Vol. 57, pp. 19–26, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(98)00286-X

Prevention of Oxidant-Induced Cell Death in Caco-2 Colon Carcinoma Cells after Inhibition of Poly(ADP-ribose) Polymerase and Ca^{2+} Chelation: Involvement of a Common Mechanism

Jurgen M. Karczewski, Janny G. P. Peters and Jan Noordhoek*
Department of Toxicology, University of Nijmegen, NL-6500 HB Nijmegen, The Netherlands

ABSTRACT. The human colon carcinoma cell line Caco-2 was exposed to the oxidative stress-inducing agents menadione (MEN), 2,3-dimethoxy-1,4-naphthoquinone, and hydrogen peroxide. All three agents caused DNA damage which was assessed by alkaline unwinding. Further, all three agents induced intensive NAD^+ depletion, followed by a decrease in intracellular ATP and viability. Inhibition of poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30) by 3-aminobenzamide prevented the depletion of NAD^+. These cells had a higher viability and ATP content. The most pronounced effect was observed with 25 μM of MEN, while at higher levels a partial preservation of NAD^+ was observed with no effect on ATP or viability. The chelation of intracellular calcium by bis-(o-aminophenoxy)-ethane-*N*,*N*,N^1,N^1-tetraacidic acid/tetraacetoxymethyl) ester also prevented the dramatic loss of NAD^+, demonstrating that Ca^{2+} is an activating factor in PARP-mediated cell killing. BIOCHEM PHARMACOL **57**;1:19–26, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. menadione; hydrogen peroxide; DNA damage; calcium; poly(ADP-ribose) polymerase; 3-aminobenzamide

Activated oxygen species are well-documented mediators of cell injury under a variety of pathological and physiological conditions [1]. Oxygen radicals are involved in some types of gastrointestinal injury as in Crohn's disease [2], ulcerative colitis [3], and ischemia reperfusion [4, 5]. Oxidative injury was reported to develop according to a fairly general pattern occurring in different cells and involved basically: free-thiol oxidation and appearance of protein disulfides, depletion of the ATP pool, elevation of free cytosolic calcium, disassembly of cytoskeleton, increased plasma membrane peroxidation and permeability, release of cytosolic components, and induction of DNA strand breaks. MEN†, a well-studied source of radicals in hepatocytes, has a wide range of effects on cells. Exposure of hepatocytes to MEN perturbs the cytoskeleton of the cell, which can be recognized as membrane blebbing [6]. The bleb formation is associated with altered thiol and Ca^{2+} homeostasis [6–11]. The semiquinone of MEN and the reactive oxygen species generated by MEN were found to interact with DNA of hepatocytes [12–14] Chinese hamster ovary cells [15], and leukemic K562 cells [16].

DNA-damaging agents, including alkylating agents, ionizing radiation [17], and reactive oxygen species such as H_2O_2 [18–20], activate PARP. Poly(ADP-ribosylation) is a post translational modification of nuclear protein and enzymes, induced by DNA damage. ADP-ribosylation of histones causes a release of DNA from the nucleosomal structure. This process is known as histone shuttling and facilitates the access of repair enzymes to the damaged DNA [21]. The rapid depletion of NAD^+ following excessive DNA damage may disturb cellular ATP production and eventuate in necrotic cell death [22]. This suicide response to irreparable intracellular stress assures that unwanted mutations do not arise, but excessive activation of the same mechanism may also be responsible for aggravation of inflammatory diseases. Due to partial collapse of the antioxidant system and the subsequent cytokine-mediated hyperreactivity of mononuclear and polymorphonuclear leukocytes, patients suffering from inflammatory and autoimmune rheumatic diseases produce up to 30-fold levels of reactive oxygen species [23]. Recently, inhibition of PARP activity was found to partly suppress potassium peroxochromate-induced arthritis in mice and inhibited the phagocytic generation of reactive oxygen species [24].

We used a spontaneously differentiating colon carcinoma cell line Caco-2 as a model for studying the role of PARP in oxidative stress in the gastrointestinal epithelium. This cell line retains many of the morphological features and

* Corresponding author: Dr. Jurgen Karczewski, Department of Toxicology, University of Nijmegen, P.O. Box 9101, NL-6500 HB Nijmegen, The Netherlands. Tel. 31 24 361366; FAX 24 3541802; E-mail: J.Karczewski@toxi.kun.nl

† *Abbreviations:* 3-ABA, 3-aminobenzamide; BAPTA-AM, bis-(o-aminophenoxy)-ethane-*N*,*N*,N^1,N^1-tetraacetic acid/tetra (acetoxymethyl)-ester; DIM, 2,3-dimethoxy-1,4-naphthoquinone; H_2O_2, hydrogen peroxide; MEN, menadione; KH, Krebs–Henseleit; PARP, poly(ADP-ribose)polymerase; SSB, single-strand breaks; and DMEM, Dulbecco's modified Eagle's medium.

Received 6 November 1997; accepted 4 May 1998.

enzyme level characteristics of normal human enterocytes [25]. The present study describes the effect of induced oxidative stress on the DNA of Caco-2 cells and the subsequent depletion of NAD^+ and ATP. We show the involvement of PARP in the cytotoxicity of quinones and H_2O_2 in this cell type. Further, the role of calcium was studied with respect to depletion of the cellular NAD^+ pool.

MATERIALS AND METHODS

Chemicals

MEN was purchased from Aldrich. DIM was prepared according to Gant *et al.* [26]. The identity was checked with mass spectrometry. Monobromobimane was from Calbiochem. Other chemicals and enzymes were obtained from Sigma. All chemicals were of analytical grade. All cell culture materials were purchased from ICN, except for flasks and tissue culture plates that were from Greiner and gentamicine.

Cell Culture and Incubation

Caco-2 cells were maintained at 37° in DMEM, containing 10% (v/v) fetal bovine serum, 10 mM HEPES, 1% nonessential amino acids, 5 mM L-glutamine, and 5 μg/mL of gentamicine in an atmosphere containing 5% CO_2. Cells (1×10^5) were seeded in 24-well culture plates and became confluent after 4 days. Cell cultures were supplied with medium every second day and used on day 7 of culture.

Incubations with MEN were performed in 24-well tissue plates in an atmosphere as described above. Cells were washed with Krebs–Henseleit buffer (KH, formulation in mM: 1.2 $MgSO_4.7H_2O$, 2.5 $CaCl_2$, $2H_2O$, 4.7 KCl, 94 NaCl, 11.6 D-glucose, 25 $NaHCO_3$, 1.2 KH_2PO_4 and 5 L-glutamine, pH 7.4) and supplied with 0.5 mL of KH. MEN and DIM were dissolved in dimethylsulfoxide and control cells were treated with equal volumes of the solvent (0.3% of final volume).

Neutral Red Uptake Assay

Viability was determined according to Borenfreund and Puerner [27]. Briefly, after incubation the monolayer was washed with KH. Fresh complete DMEM with 50 μg/mL of neutral red was added to the wells. After 30 min of incubation at 37°, cells were washed rapidly with 40% formaldehyde-10% $CaCl_2$, to remove extraneously adhering, unincorporated dye. Neutral red was extracted with 500 μL of 1% acetic acid-50% ethanol and 150 μL were transferred to a 96-well titerplate. The plate was measured on a Thermomax microplate reader (Molecular Devices) equipped with a 550 nm filter. The readings were expressed as percentages of the nonexposed cells.

Cellular ATP and NAD^+ Content

Cells were treated with 300 μL 5% of (v/v) perchloric acid and neutralized with 700 μL of 0.8 M potassium phosphate buffer. Insoluble material was removed by centrifugation (5 min, 13,000 *g*) and stored at −80° until use. ATP and NAD^+ were separated using a Spectra-Physics HPLC system consisting of an SP8800 ternary pump, an SP8875 autosampler, an SP4600 integrator, and a Merck 100 RP-18 column (LichroCART 125-4 Lichrospher). Twenty μL of supernatant was injected on the column at 40° and separated with a flow of 1 mL/min. The elution buffer was a 0.1 M potassium phosphate buffer, pH 6.0 that was filtered through a 0.45 mm filter before use. ATP and NAD^+ were detected by a Kratos Spectroflow 773 UV detector at 260 nm. ATP and NAD^+ contents were expressed as percentages of the control cells.

Cellular GSH Content

Reduced glutathione was determined by HPLC after derivatization with monobromobimane as described by Cotgreave and Moldeus [28]. After incubation, cells were washed with KH and 100 μL of aqua pure was added to the well. GSH was derivatized by adding 100 μL of 2 monobromobimane dissolved in 50 mM of *N*-ethylmorpholine and incubating this mixture for 5 min in the dark. Protein was denatured by inclusion of 25 μL of 40% trichloric acid and removed by centrifugation (5 min, 13,000 *g*). Twenty μL of GSH derivatives was separated by HPLC using a Merck 100 RP-18 column at 40° and a flow rate of 1 mL/min. Buffer A was 128 mL of methanol and 2.5 mL of acetic acid diluted to 1 L with aqua pure, adjusted to pH 3.9 with 5 N of NaOH. Buffer B was 900 mL of methanol and 2.5 mL of acetic acid, diluted to 1 L with aqua pure. A linear gradient from 0% B at 0 min, 50% B at 8 min to 0% B at 17 min was used to elute the GSH derivatives that were detected using a Shimadzu RF-530 fluorescence detector at λ_{ex} = 385 nm and λ_{em} = 480 nm. Glutathione content was expressed as percentage of the control cells.

Determination of DNA SSB

The formation of SSB in DNA was measured by alkaline unwinding and determination of ethidium bromide fluorescence on an LS50 spectrofluorometer (Perkin Elmer) with excitation at 520 nm and emission at 590 nm according to the method of Birnboim and Jevcak [29]. After incubation with DNA-damaging agents, cells were washed once with KH. Cells were detached from the culture plates with 200 μL of trypsin/EDTA for 12 min at 37° after which 200 μL of culture medium was added to inactivate the trypsin. Cells were separated from the solution and resuspended in 100 μL of 250 mM *meso*-inositol containing 10 mM of

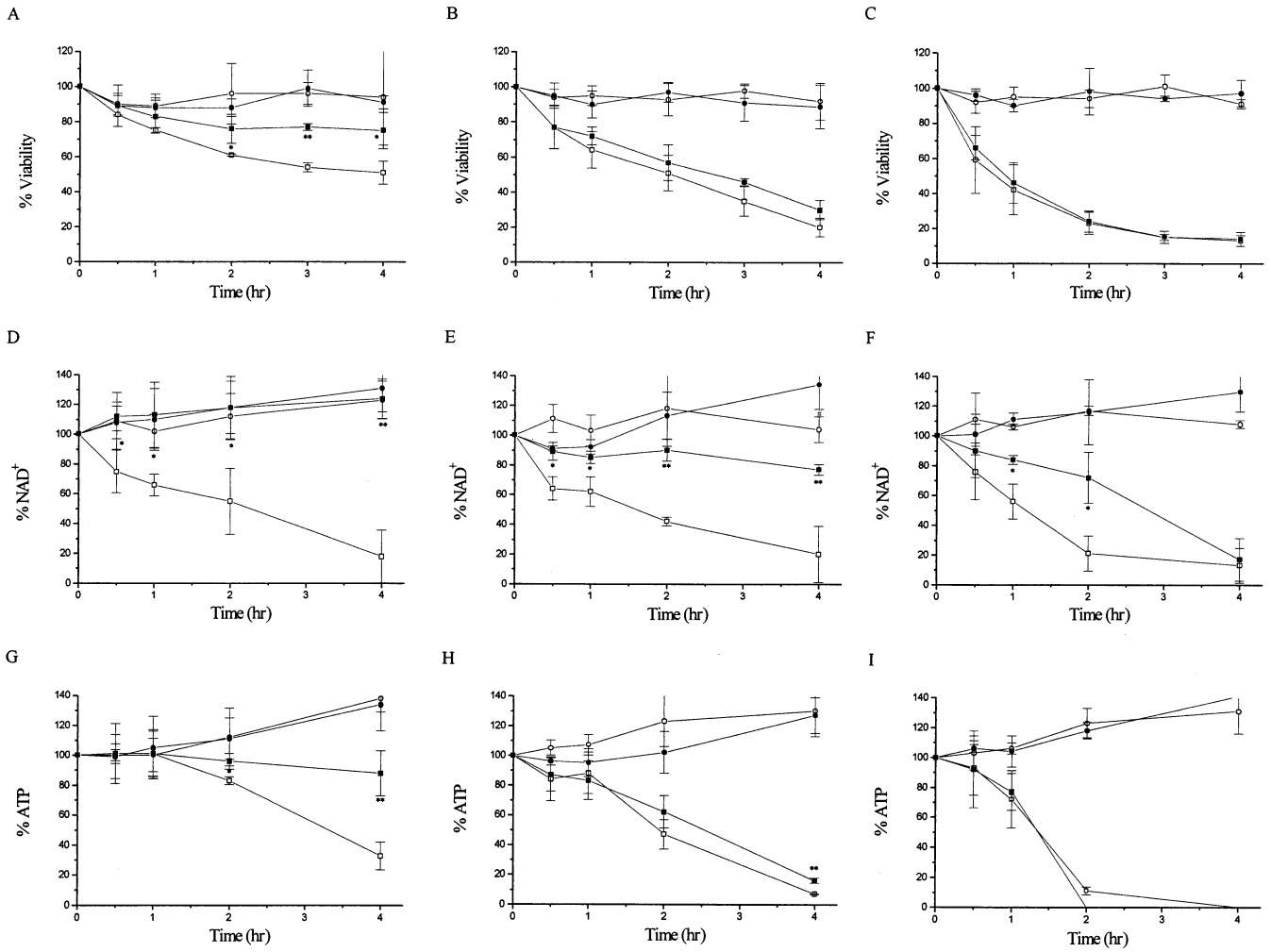

FIG. 1. Effect of 3-ABA on MEN-treated Caco-2 cells. Caco-2 cells were untreated (○) or exposed to 10 mM of 3-ABA (●), MEN (□), or to a combination of MEN and 3-ABA (■). The concentration of MEN was 25 μM in graphs A, D and G; 50 μM in graphs B, E and H; and 100 μM in C, F and I. Viability (1-A, B, C), NAD^+ (1-D, E, F), and ATP (1-G, H, I) were measured as described in Materials and Methods. The [ATP] and [NAD^+] in control cells were 38.7 ± 8.1 and 7.8 ± 1.0 nmol/mg protein, respectively. Shown are the means ± SD of three independent experiments (*: $P \leq 0.05$, **: $P \leq 0.01$ compared to MEN-exposed cells).

$Na_3PO_4.12H_2O$ and 1 mM of $MgCl_2.6H_2O$, pH7.2, and 100 μL of 9 M urea containing 10 mM of NaOH, 2.5 mM of EDTA.$2H_2O$, 0.1% SDS was added. Cell were lysed during an incubation period of 30 min on ice. The alkaline lysates were incubated for 10 min on ice followed by 10 min at 15°. DNA unwinding was stopped by adding 200 μL of 14 mM β-mercaptoethanol/1 M glucose. The lysates were sonicated briefly and 750 μL of ethidium bromide 13.4 μg/mL 13.3 mM NaOH was added. DNA unwinding was calculated using the formula: $(F - F_{min})/(F_{max} - F_{min})*100$, where F is the fluorescence of the sample and F_{min} is the background fluorescence of a sample that was damaged by being passed 3 times through a 12 × 0.4 mm injection needle. F_{max} is the fluorescence of samples kept at pH 11.0, which is below the pH needed to induce unwinding of single-stranded DNA. DNA SSB were expressed as percentages of the control values in order to normalize the variation in DNA unwinding of the control cells at the start of the experiment.

Statistics

Results are expressed as means ± SD of three to six independent experiments. Statistical significance between two groups was determined by means of an unpaired Student's *t*-test. Statistical differences between groups were determined by means of a one-way analysis of variance (ANOVA), followed by Dunnett's multiple-comparison test. A probability of $P \leq 0.05$ was considered significant.

RESULTS

As expected, MEN induced concentration-dependent toxicity and depletion of ATP in the human colon carcinoma cell line Caco-2 (Fig. 1). The depletion of NAD^+, however, occurred at the same rate with all concentrations of MEN (ANOVA, $P \leq 0.01$), indicating a process that was already maximally activated with an exposure of 25 μM of MEN. MEN was able to induce DNA damage as depicted in

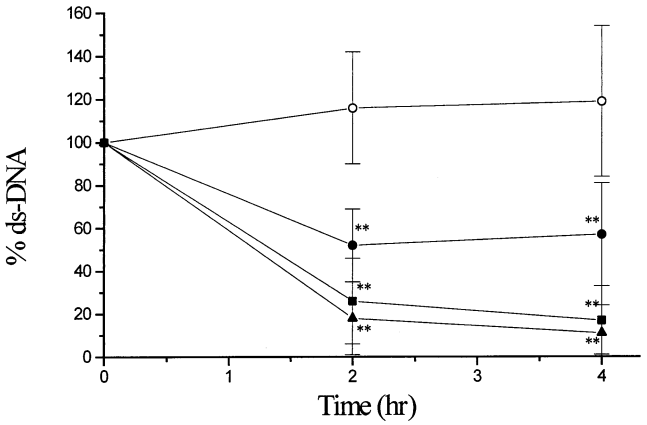

FIG. 2. Effect of MEN, DIM, and H_2O_2 on DNA of Caco-2 cells. Caco-2 cells were untreated (○) or exposed to 25 μM of MEN (●), 300 μM of DIM (■), or 10 mM of H_2O_2 (▲). DNA strand breaks were measured as described in Materials and Methods. Shown are the means ± SD of four independent experiments (: $P \leq 0.01$ compared to untreated cells).**

Fig. 2. H_2O_2 was used as a control since this agent is capable of inducing DNA damage in several cell lines. DIM, in contrast to MEN, is not able to arylate and is therefore a pure redoxcycler. DIM was used to assess whether oxygen species solely derived from redoxcycling can induce DNA damage in this cell line. Both H_2O_2 and DIM caused considerable amounts of single-strand breaks and MEN also generated SSB.

DNA damage is a trigger for the activation of PARP in all types of mammalian cells. To estimate the contribution of PARP in the toxicity of MEN in intestinal cells, the effect of the relatively specific inhibitor 3-ABA was studied. Caco-2 cells were exposed to 25, 50, and 100 μM of MEN in combination with 3-ABA and the effect on viability, cellular NAD^+, and ATP was assessed (Fig. 1). Incubation of the cells with 3-ABA alone resulted in a slight increase in NAD^+, but the inhibitor had no effect on viability or ATP content. 3-ABA clearly prevented the drop in NAD^+ in cells that were treated with 25 μM of MEN and the protecting effect was also present with a higher concentration of MEN. The ATP depletion observed with 25 μM was partly reversed by 3-ABA, although at higher concentrations of MEN the inhibitor was not able to prevent the depletion of ATP.

Exposure of Caco-2 cells caused a rapid depletion of GSH with all concentrations of MEN. The addition of the PARP inhibitor during the damaging treatment did not protect the cellular GSH pool (Fig. 3).

An increase in free intracellular calcium is considered to be a major factor in the cytotoxicity of quinones [30]. We used the intracellular calcium chelator BAPTA-AM to assess the role of free cytosolic calcium in PARP-mediated cell killing. Figure 4 shows that BAPTA-AM partly prevented the decrease in viability and completely prevented the depletion of NAD^+. Although BAPTA-AM slightly decreased the cellular ATP content of control cells, the use of this chelator resulted in substantially higher levels of ATP in MEN-exposed cells.

In order to exclude the possibility that the action of PARP is restricted to MEN-induced toxicity, cells were exposed for 4 hr to 300 μM DIM or 10 mM of H_2O_2. Both agents were also capable of damaging cellular DNA (Fig. 2). The concentrations of DIM and H_2O_2 were chosen to induce a similar degree of toxicity as 25 μM MEN. Table 1 shows that both DIM and H_2O_2 decreased the viability to 60%, which is comparable to the observation made with the experiment using 25 μM MEN. The depletion of NAD^+ by both agents was also similar to the observed levels in the MEN-treated cells. H_2O_2 was more effective than DIM in disturbing the levels of ATP. Again, 3-ABA rescued the DIM-exposed cells from an intensive NAD^+ depletion, while the inhibitor only partly preserved the NAD^+ content of the H_2O_2-treated cells. A similar pattern was observed for the ATP levels. With respect to the viability, 3-ABA offered a similar protection against DIM or H_2O_2. BAPTA-AM also partly prevented the decrease of viability in H_2O_2-treated cells, but the chelator did not show this effect on DIM-treated cells. Chelation of calcium also prevented the loss of NAD^+ with both mediators of oxidative stress, but the effect was more pronounced in DIM-treated cells. The ATP levels of BAPTA-AM- and DIM-treated cells was much lower than in DIM-exposed cells. Addition of BAPTA-AM to H_2O_2-treated cells caused a slight but significant increase in ATP compared to treatment with H_2O_2 alone.

DISCUSSION

Several toxic properties of quinones have been attributed to their ability to damage DNA via generation of free radicals and activated oxygen species. Several investigations have

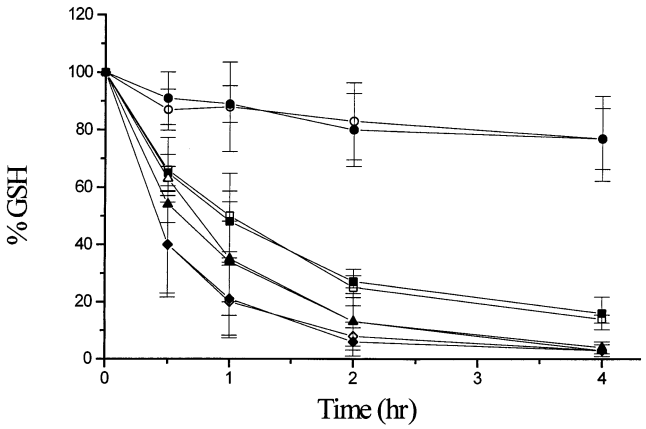

FIG. 3. Effect of 3-ABA on the GSH content of MEN-treated Caco-2 cells. Caco-2 cells were untreated (○) or exposed to 25 μM (□), 50 μM (△), and 100 μM MEN (◇). The solid symbols represent the combination of MEN and 10 mM of 3-ABA. GSH was extracted and measured as described in Materials and Methods. The GSH content of the control cells was 41.7 ± 3.1 nmol/mg protein. Shown are the means ± SD of three independent experiments.

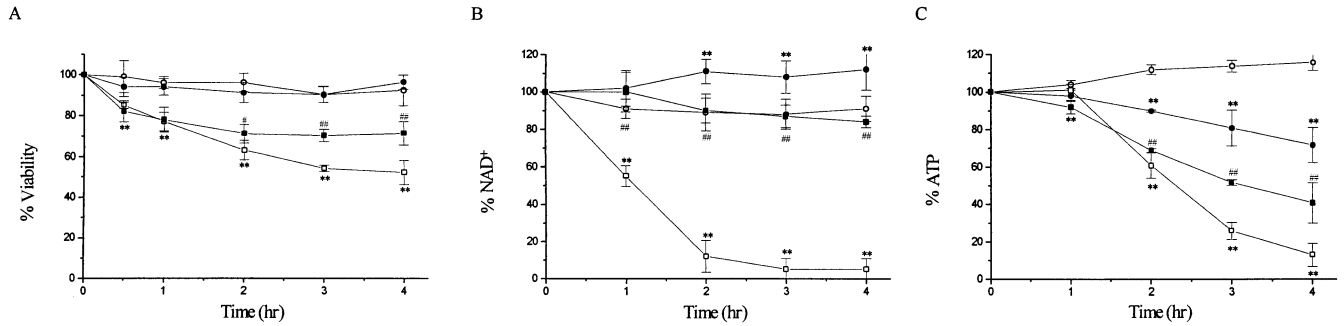

FIG. 4. Effect of 100 μM of BAPTA-AM on toxicity of 25 μM of MEN. The effect of the intracellular calcium chelator BAPTA-AM on MEN-induced toxicity was analyzed with the neutral red assay (A), intracellular NAD^+ (B), and ATP content (C). Caco-2 cells were untreated (○) or exposed to 25 μM of MEN (□). BAPTA-AM was added alone (●) or simultaneously with MEN (■). Shown are the means ± SD of four independent experiments (*: $P \leq 0.05$, **: $P \leq 0.01$ compared to untreated cells; #: $P \leq 0.05$, ##: $P \leq 0.01$: compared to MEN-exposed cells).

indicated that oxygen species such as H_2O_2 or the hydroxyl radical (generated from H_2O_2 via Fenton-type reactions) may be the most damaging.

Exposure to MEN, DIM, and H_2O_2 induced single-strand breaks in the DNA of Caco-2 cells. In most mammalian cell types, strand breaks are recognized by poly(ADP-ribose) polymerase [17, 31]. The involvement of PARP in our experiments was supported by two observations. First, MEN caused a concentration-independent depletion of the cellular NAD^+ content of Caco-2 cells. This indicates that the degradation of NAD^+ was at its maximum rate with a dose of 25 μM of MEN. Exposure to DIM and H_2O_2 resulted in similar levels of NAD^+. Obviously, the depletion of NAD^+ was independent of the source of oxidative stress or the concentration of MEN in this study. Further, 3-ABA, a potent inhibitor of PARP, was able to prevent this depletion. The preserving effect was more pronounced at the lower levels of MEN exposure. These cells also contained more ATP and had a higher viability. The preservation of the viability by ABA is attributable to inhibition of necrosis rather than apoptosis. In apoptosis, PARP is inactivated by proteases of the ICE family [32] and this should reflect in a partial or temporal preservation of the NAD^+ pool [33]. However, we observed an unchanged rate of NAD^+ depletion, which indicates that apoptosis may play only a modest role in our experiments. Eguchi *et al.* [34] showed that cellular ATP levels determine whether cells undergo apoptosis or necrosis. In these experiments, treatment of Jurkat cells with calcium ionophore A23187 induced apoptosis under ATP-supplying conditions but induced necrotic cell death under ATP-depleting conditions. It is possible that MEN decreased the ATP content of Caco-2 cells to such a degree that necrotic cell death prevailed over apoptosis. Thus, the decrease in neutral uptake during exposure to menadione is probably related to necrotic cell death. With higher concentrations of MEN, the depletion of NAD was still affected by 3-ABA, but the decrease in ATP and viability was not prevented. It is likely that MEN caused extensive damage to cell components other than DNA. The active site of several enzymes contains one or more thiols which are directly related to enzymatic activity. MEN has been shown to directly inhibit glutathione reductase [35] and glyceraldhyde 3-phosphate dehydrogenase [36]. It is conceivable that inactivation of these and other enzymes may induce cell killing at a much faster rate than overactivation of PARP alone. In spite of treatment with 3-ABA, some depletion of NAD^+ occurred with the treatment with 50 and 100 μM MEN. This loss of NAD^+ may result from leakage due to an increasing loss of membrane integrity rather than PARP activity. The use of

TABLE 1. Effect of 3-ABA and BAPTA-AM on the oxidative stress-induced toxicity

Experiment	% viability	% NAD^+	% ATP
Control	100.0 ± 3.41	100.0 ± 6.09	100.0 ± 0.98
3-ABA	96.3 ± 3.62	100.4 ± 3.69	101.9 ± 1.98
BAPTA-AM	78.0 ± 5.27**	98.4 ± 4.89	56.1 ± 6.21**
DIM	60.6 ± 2.39**	23.8 ± 5.25**	51.4 ± 3.87
DIM + 3-ABA	83.3 ± 5.30##	107.1 ± 4.16##	70.8 ± 2.04##
DIM + BAPTA-AM	60.6 ± 3.77	83.2 ± 8.81##	25.9 ± 2.92##
H_2O_2	59.6 ± 2.69**	16.9 ± 7.49**	23.1 ± 4.75**
H_2O_2 + 3-ABA	83.3 ± 3.62##	69.2 ± 5.18##	51.9 ± 2.06##
H_2O_2 + BAPTA-AM	83.0 ± 2.35##	57.9 ± 7.01##	30.9 ± 4.17##

Caco-2 cells were incubated with DIM (300 μM) or H_2O_2 (10 mM) for 4 hr. 3-ABA (10 mM) and BAPTA-AM (100 μM) were added simultaneously with DIM or H_2O_2. Shown are % viability, % NAD, and % ATP as means ± SD of six independent experiments.

**: $P \leq 0.01$ compared to untreated cells.

##: $P \leq 0.01$ compared to DIM- or H_2O_2-treated cells.

higher concentrations of quinones may also explain why 3-ABA did not protect isolated hepatocytes from 50–200 μM MEN [37] or 400 μM DIM-treated hepatoma cells [30]. With higher concentrations of MEN, the role of PARP in cytotoxicity may become questionable, since this enzyme is inhibited by MEN (IC_{50} = 420 μM; [38]).

MEN intensively depleted the cellular reduced GSH. The protection offered by 3-ABA was not mediated by preserving the cellular GSH content, since no effect on GSH was observed with the use of 3-ABA. In some cell types such as human peripheral lymphocytes, it was found that 3-ABA was able to preserve the GSH pool during treatment with H_2O_2 by retaining the levels of $NADP^+$ [39]. Glutathione reductase is responsible for the reduction in oxidized glutathione and utilizes NADPH as a cofactor. The reduction of $NADP^+$ to NADPH is mediated by the hexose monophosphate shunt, which displays increased activity during oxidative stress. The experiments of Baker and Baker [40] describe a 600% rise in hexose monophosphate shunt activity in 50 mM of MEN-exposed Caco-2 cells. In quinone-exposed hepatocytes, a decrease in NAD^+ was followed by an increase in $NADP^+$ [37]. Thus, the preservation of NAD^+ may benefit the interconversion of NAD^+ to $NADP^+$ by NAD^+ kinase. In our experiment, we did not observe an effect of MEN or 3-ABA on the $NADP^+$ levels (data not shown). It is likely that NAD^+ kinase is not present or of any significance in intestinal tissue.

A close relationship has been found between the sustained increase in cytosolic Ca^{2+} and the toxicity of MEN and other agents that induce oxidative stress [41]. The level of free calcium in the nucleus is connected to that of the cytosol. Nicotera *et al.* [42] described an ATP-dependent mechanism by which nuclear Ca^{2+} levels respond to a rise in cytosolic calcium. A consequence of the increased free Ca^{2+} in the nucleus is the activation of Ca^{2+}-dependent endonucleases [43]. More evidence for an MEN-stimulated DNA fragmentation follows from experiments with isolated hepatocytes and moderate concentrations of MEN (100 μM). Chromatin condensation and progressive DNA fragmentation were observed, which correlated with the rise in cytosolic calcium [44]. The results from our experiments showed that chelation of Ca^{2+} with BAPTA-AM preserved the cellular NAD^+ pool and viability of MEN-treated Caco-2 cells. Similar results were obtained with H_2O_2- and DIM-treated cells, which means that oxidative stress interferes with Ca^{2+} homeostasis and leads to a depletion of the NAD^+ pool. This depletion can still be assigned to the activation of PARP. Poly(ADP-ribosylation) causes a release of DNA from the nucleosomal structure. This process is known as histone shuttling and facilitates the access of repair enzymes to the damaged DNA [21]. Relaxation of DNA by PARP may also increase the accessibility for endonucleases that are activated by the rise in cytosolic calcium. Thus, the combination of PARP-induced DNA relaxation followed by endonucleolytic activity results in feedback activation of more PARP. Evidence for this mechanism follows from experiments with isolated liver nuclei in which DNA fragmentation by Ca^{2+} dependent endonucleases was elicited by the presence of NAD^+, ATP, and a submicromolar concentration of Ca^{2+}. This DNA fragmentation was inhibited by 3-ABA [45]. Thus, the chelation of Ca^{2+} prevents the activation of endonucleases and indirectly, PARP, thereby preserving the NAD^+ pool. There are some reports that claim that the ADP-ribosylation process inactivates Ca^{2+}-dependent endonucleolytic activity [46]. However, there are implications that other calcium-dependent mechanisms may damage DNA. Dybukt *et al.* [30] showed that exposure of Ca^{2+}-dependent endonuclease free murine hepatoma cells to 400 μM DIM protected the cells from NAD^+ depletion by 3-ABA and BAPTA-AM, although the loss of viability was not prevented by 3-ABA.

The results presented in this paper clearly show the involvement of PARP and Ca^{2+} in oxidative stress-mediated death in cells derived from the human gastrointestinal tract. MEN, DIM, and H_2O_2 induced DNA damage and caused an intensive NAD^+ depletion, followed by a decrease in intracellular ATP and viability. Both inhibition of PARP by 3-ABA and chelation of Ca^{2+} prevented the depletion of NAD^+. These cells had a higher viability and ATP content. The role of PARP in cell killing was more evident in weakly exposed cells. This level of exposure is probably a better model for the level of oxidative stress present in gastrointestinal cells that are suffering from inflammation. It would be interesting to know whether PARP is active in Crohn's disease, ulcerative colitis, and ischemia reperfusion. Nicotinamide is a biogenic inhibitor of PARP and may offer an interesting approach for controling this type of necrotic cell death.

This work was supported by a grant from the Dutch Organization for Scientific Research (NWO), The Hague, The Netherlands (Grant no. 902-22-112).

References

1. Bast A and Goris RJ, Oxidative stress. Biochemistry and human disease. *Pharm Weekbl Sci* **11:** 199–206, 1989.
2. Iantomasi T, Marraccini P, Favilli F, Vincenzini MT, Ferretti P and Tonelli F, Glutathione metabolism in Crohn's disease. *Biochem Med Metabol Biol* **53:** 87–91, 1994.
3. Babbs CF, Oxygen radicals in ulcerative colitis. *Free Radical Biol Med* **13:** 169–181, 1992.
4. Nilsson UA, Olsson LI, Thor H, Moldeus P and Bylund-Fellenius AC, Detection of oxygen radicals during reperfusion of intestinal cells *in vitro*. *Free Radical Biol Med* **6:** 251–259, 1989.
5. Osborne DL, Aw TY, Cepinskas G and Kvietys PR, Development of ischemia/reperfusion tolerance in the rat small intestine. An epithelium-independent event. *J Clin Invest* **94:** 1910–1918, 1994.
6. Thor H, Smith MT, Hartzell P, Bellomo G, Jewell SA and Orrenius S, The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. *J Biol Chem* **257:** 12419–12425, 1982.
7. DiMonte D, Ross D, Bellomo G, Eklöw L and Orrenius S,

Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes. *Arch Biochem Biophys* **235:** 334–342, 1984.
8. DiMonte D, Bellomo G, Thor H, Nicotera P and Orrenius S, Menadione-induced cytotoxicity is associated with protein thiol oxidation and alterations in Ca^{2+} homeostasis. *Arch Biochem Biophys* **235:** 343–350, 1984.
9. Mirabelli F, Salis A, Perotti M, Taddei F, Bellomo G and Orrenius S, Alterations of surface morphology caused by the metabolism of menadione in mammalian cells are associated with the oxidation of critical sulfhydryl groups in the cytoskeletal proteins. *Biochem Pharmacol* **37:** 3423–3427, 1988.
10. Mirabelli F, Salis A, Marinoni V, Finardi G, Bellomo G, Thor H and Orrenius S, Menadione-induced bleb formation in hepatocytes is associated with the oxidation of thiol groups in actin. *Arch Biochem Biophys* **264:** 261–269, 1988.
11. Nicotera P, Moore M, Mirabelli F, Bellomo G and Orrenius S, Inhibition of hepatocyte plasma membrane Ca^{2+}-ATPase activity by menadione metabolism and its restoration by thiols. *FEBS Lett* **181:** 149–153, 1985.
12. Cantoni O, Fiorani M, Cattabeni F and Bellomo G, DNA breakage caused by hydrogen peroxide produced by the metabolism of 2-methyl-1, 4-naphthoquinone (menadione) does not contribute to the toxicity of the cytotoxic action of the quinone. *Biochem Pharmacol* **42:** S220–S222, 1991.
13. Fischer-Nielsen A, Corcoran GB, Poulsen HE, Kamendulis LM and Loft S, Menadione-induced DNA fragmentation without 8-oxo-2′deoxyguanosine formation in isolated rat hepatocytes. *Biochem Pharmacol* **49:** 1469–1474, 1995.
14. Morrison H, Jernström B, Nordenskjöld H, Thor H and Orrenius S, Induction of DNA damage by menadione (2-methyl-1,4-naphthoquinone) in primary cultures of rat hepatocytes. *Biochem Pharmacol* **33:** 1763–1769, 1984.
15. Martins EA and Meneghini R, DNA damage and lethal effects of hydrogen peroxide and menadione in Chinese hamster cells: Distinct mechanisms are involved. *Free Radical Biol Med* **8:** 433–440, 1990.
16. Morgan WA, Harley JA and Cohen GM, Quinone-induced DNA single strand breaks in rat hepatocytes and human chronic myelogenous leukaemic K562 cells. *Biochem Pharmacol* **44:** 215–221, 1992.
17. Lautier D, Lagueux J, Thibodeau J, Menard L and Poirier GG, Molecular and biochemical features of poly(ADP-ribose) metabolism. *Mol Cell Biochem* **122:** 171–193, 1993.
18. Schraufstatter IU, Hinshaw DB, Hyslop PA, Spragg RA and Cochrane CG, Glutathione cycle activity and pyridine nucleotide levels in oxidant-induced injury of cells. *J Clin Invest* **76:** 1131–1139, 1985.
19. Schraufstatter IU, Hinshaw DB, Hyslop PA, Spragg RA and Cochrane CG, Oxidant injury of cells. *J Clin Invest* **77:** 1312–1320, 1986.
20. Schraufstatter IU, Hyslop PA, Hinshaw DB, Spragg RA, Sklar LA and Cochrane CG, Hydrogen peroxide-induced injury of cells and its prevention by inhibitors of poly(ADP-ribose) polymerase. *Proc Natl Acad Sci USA* **83:** 4908–4912, 1986.
21. Realini CA and Althaus FR, Histone shuttling by poly(ADP-ribosylation). *J Biol Chem* **267:** 18858–18865, 1992.
22. Sims JL, Berger SJ and Berger NA, Poly(ADP-ribose)polymerase inhibitors preserve nicotinamide adenine dinucleotide and adenosine 5′-triphosphate pools in DNA-damaged cells: Mechanism of stimulation of unscheduled DNA synthesis. *Biochemistry* **22:** 5188–5194, 1983.
23. Miesel R, Kröger H, Zuber M, Gratz R and Neth J, Elevated levels of oxygen- and nitrogen-centered radicals in patients with rheumatic diseases. *Z Rheumatol* **53:** 6–?, 1994.
24. Miesel R, Kurpisz M and Kröger H, Modulation of inflammatory arthritis by inhibition of poly (ADP-ribose) polymerase. *Inflammation* **19:** 379–387, 1995.
25. Pinto M, Robine-Leon S, Appay MD, Kedinger M, Triadou N, Dussaulx E, Lacroix B, Simon-Assman P, Haffen K, Fogh J and Zweibaum A, Enterocyt-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol Cell* **47:** 323–330, 1983.
26. Gant TW, Ramakrishna Rao DN, Mason RP and Cohen GM, Redox cycling and sulphydryl arylation: Their relative importance in the mechanism of quinone cytotoxicity to isolated hepatocytes. *Chem-Biol Interact* **65:** 157–173, 1988.
27. Borenfreund E and Puerner JA, Toxicity determined *in vitro* by morphological alterations and neutral red absorption. *Toxicol Lett* **24:** 119–124, 1985.
28. Cotgreave IA and Moldeus P, Methodologies for the application of monobromobimane to the simultaneous analysis of soluble and protein thiol component of biological systems. *J Biochem Bioph Meth* **13:** 231–249, 1986.
29. Birnboim HC and Jevcak JJ, Fluorometric method for rapid detection of DNA strand breaks in human white blood cells produced by low doses of radiation. *Cancer Res* **41:** 1889–1892, 1981.
30. Dypbukt JM, Thor H and Nicotera P, Intracellular Ca^{2+} chelators prevent DNA damage and protect hepatoma 1C1C7 cells from quinone-induced cell killing. *Free Radical Res* **8:** 347–354, 1990.
31. De Murcia G and Menissier de Murcia J, Poly(ADP-ribose) polymerase: A molecular nick-sensor. *Trends Biochem Sci* **19:** 172–176, 1994.
32. Patel, T, Gores GJ and Kaufmann SH, The role of proteases during apoptosis. *FASEB J* **10:** 587–597, 1996.
33. Nosseri C, Coppola S and Ghibelli L, Possible involvement of poly(ADP-ribosyl) polymerase in triggering stress-induced apoptosis. *Exp Cell Res* **212:** 367–373, 1994.
34. Eguchi Y, Shimizu S and Tsujimoto Y, Intracellular ATP levels determine cell death fate by apoptosis or necrosis. *Cancer Res* **57:** 1835–1840, 1997.
35. Bellomo G, Mirabelli F, DiMonte D, Richelmi P, Thor H, Orrenius C and Orrenius S, Formation and reduction of glutathione-protein mixed disulfides during oxidative stress. A study with isolated hepatocytes and menadione (2-methyl-1,4-naphthoquinone). *Biochem Pharmacol* **36:** 1313–1320, 1987.
36. Redegeld FAM, Moison RMW, Barentsen HM, Koster AS and Noordhoek J, Interaction with cellular ATP generating pathways mediates menadione-induced cytotoxicity in isolated rat hepatocytes. *Arch Biochem Biophys* **280:** 130–136, 1990.
37. Stubberfield CR and Cohen GM, Interconversion of NAD(H) to NADP(H). A cellular response to quinone-induced oxidative stress in isolated hepatocytes. *Biochem Pharmacol* **38:** 2631–2637, 1989.
38. Banasik M, Komura H and Ueda K, Inhibition of poly(ADP-ribose) synthetase by fatty acids, vitamins and vitamin-like substances. *FEBS Lett* **263:** 222–224, 1990.
39. Marini M, Frabetti F, Brunelli MA and Raggi MA, Inhibition of poly (ADP-ribose) polymerization preserves the glutathione pool and reverses cytotoxicity in hydrogen peroxide-treated lymphocytes. *Biochem Pharmacol* **46:** 2139–2144, 1993.
40. Baker S and Baker RD, Caco-2 cell metabolism of oxygen-derived radicals. *Digest Dis Sci* **38:** 2273–2280, 1993.
41. Nicotera P, McConkey D, Svensson SA, Bellomo G and Orrenius S, Correlation between cytosolic Ca^{2+} concentration and cytotoxicity in hepatocytes exposed to oxidative stress. *Toxicology* **52:** 55–63, 1988.
42. Nicotera P and Rossi AD, Nuclear Ca^{2+}: Physiological regulation and role in apoptosis. *Mol Cell Biochem* **135:** 89–98, 1994.

43. Arends MJ, Morris RG and Wyllie AH, Apoptosis, the role of endonucleases. *Am J Pathol* **136:** 593–608, 1990.
44. McConkey DJ, Hartzell P, Nicotera P, Wyllie AH and Orrenius S, Stimulation of endogenous endonuclease activity in hepatocytes exposed to oxidative stress. *Toxicol Lett* **42:** 123–130, 1988.
45. Jones DP, McConkey DJ, Nicotera P and Orrenius S, Calcium-activated DNA fragmentation in rat liver nuclei. *J Biol Chem* **264:** 6398–6403, 1989.
46. Yoshihara K, Tanigawa Y, Burzio L and Kiode SS, Evidence for adenosine diphosphate ribosylation of Ca^{2+}, Mg^{2+}-dependent endonuclease. *Proc Natl Acad Sci USA* **72:** 289–293, 1975.