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INTRACELLULAR Ca^{2+} CHELATORS PREVENT DNA DAMAGE AND PROTECT HEPATOMA 1C1C7 CELLS FROM QUINONE-INDUCED CELL KILLING

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Exposure of hepatoma 1c1c7 cells to 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) resulted in a sustained elevation of cytosolic Ca^{2+}, DNA single strand breaks and cell killing. DNA single strand break formation was prevented when cells were preloaded with either of the intracellular Ca^{2+} chelators, Quin 2 or BAPTA, to buffer the increase in cytosolic Ca^{2+} concentration induced by the quinone. DMNQ caused marked NAD^+ depletion which was prevented when cells were preincubated with 3-aminobenzamide, an inhibitor of nuclear poly-(ADP-ribose)-synthetase activity, or with either of the two Ca^{2+} chelators. However, 3-aminobenzamide did not protect the hepatoma cells from loss of viability. Our results indicate that quinone-induced DNA damage, NAD^+ depletion and cell killing are mediated by a sustained elevation of cytosolic Ca^{2+}.

KEY WORDS: Cytosolic Ca^{2+}, quinone, oxidative stress, DNA, poly-(ADP)-rybosylation, ATP.

ABBREVIATIONS: Quin 2, 2-{[2-*bis*(carboxymethyl)-amino-5-methylphenoxy]-methyl}-6-methoxy-8-*bis*-(carboxymethyl)-aminoquinoline; Fura 2, 1-[2-(5-carboxyaxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N′,N′-tetracetic acid; BAPTA, *bis*-(o-aminophenoxy)-ethane-N,N,N′,N′-tetracetic acid; Quin 2 AM, Fura 2 AM, and BAPTA AM are the tetraacetoxymethyl esters of Quin 2, Fura 2, and BAPTA, respectively.

INTRODUCTION

Metabolism of xenobiotics which generate free radicals and active oxygen species is often associated with formation of DNA strand breaks or base oxidation.[1] It has been suggested that radical-induced DNA strand breaks may be involved in the toxicity of hydrogen peroxide, radiomimetic chemicals such as bleomycin, and several quinones, including mitomycin C, adriamycin, daunomycin and menadione.[2] Formation of strand breaks associated with free radical generating systems also results in the activation of enzymes involved in DNA repair and nucleic acid synthesis. However, DNA repair does not necessarily result in cytoprotection. For example, activation of nuclear poly-(ADP-ribose)-synthetase, which is involved in the control of DNA excision repair following DNA damage, has been implicated as a potentially lethal mechanism during oxidative stress.[3] Since poly-(ADP-ribose)-synthetase catalyses the cleavage of NAD^+ to yield nicotinamide and extend the poly-(ADP)-ribose chains, it has been proposed that extensive DNA strand breakage may lead to severe loss of NAD^+, ATP depletion and cell death.[3] On the other hand, DNA damage caused by

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free radicals may also involve changes which are not easily repaired and may cause cell killing. Adriamycin-induced formation of DNA double strand breaks is believed to be mediated by topoisomerase II,[4] and other cytotoxic drugs and/or high Ca^{2+} levels have been shown to lock topoisomerase into a form which cleaves, but does not religate, DNA.[5] In addition, studies in this laboratory have recently shown that an endogenous endonuclease which is activated by a sustained elevation of cytosolic Ca^{2+} can cause DNA fragmentation in hepatocytes exposed to oxidative stress.[6] However, the patterns of DNA degradation caused by endonucleases differ depending on the cell type,[7] and some cells lack typical Ca^{2+}-dependent endonuclease activity.

In the present study the mechanism of DNA damage induced by the redox cycling quinone, DMNQ, was investigated using hepatoma (hepa) 1c1c7 cells. Hepa 1c1c7 cells, which have retained most of the properties of normal liver cells do not express Ca^{2+}-dependent endonuclease activity (J. Dypbukt and P. Nicotera unpublished observations). Thus, they appeared to be a suitable model for investigation of the possible involvement of Ca^{2+}-dependent mechanisms, other than endonuclease, in DNA damage caused by oxidative stress. The results of this study show that oxidative stress induced by DMNQ caused single strand breakage in hepa 1c1c7 cells, which was markedly decreased when cells were preloaded with intracellular Ca^{2+} chelators to buffer the quinone-induced changes in cytosolic Ca^{2+}. In addition, Ca^{2+} chelators prevented the loss of NAD^+ and cell viability without affecting the GSH and ATP depletion induced by quinone metabolism. This suggests that the depletion of NAD^+ was a consequence of the elevation of cytosolic Ca^{2+} and dissociates the NAD^+ loss from the depletion of ATP during oxidative stress. Taken together, our results indicate that DNA single strand breakage and loss of cell viability during oxidative stress are mediated by Ca^{2+}-activated processes.

MATERIALS AND METHODS

Murine hepatoma 1c1c7 cells (a gift from Dr. Paul Talalay, Johns Hopkins University School of Medicine, Baltimore, MD.) were grown in α-minimal essential medium without nucleosides (GIBCO, Chagrin Falls, Ohio) supplemented with 10% fetal calf serum as previously described.[8] Cells were exposed to DMNQ (a gift from Dr. G. Cohen, Department of Pharmacology, School of Pharmacy, University of London, U.K.) or other additions in a modified Krebs-Henseleit buffer at 37°C under an atmosphere of O_2:CO_2 (5:95%). Loading with fura 2 to measure cytosolic Ca^{2+}, or with quin 2 or BAPTA to buffer quinone-induced changes in cytosolic Ca^{2+}, was performed as reported previously.[8] Reduced and oxidized glutathione were measured as in ref 8. NAD^+ and ATP were assayed according to Jones *et al.*[9] DNA single strand breakage was measured by the alkaline elution technique described by Kohn.[10] Results were then calculated and expressed as the double logarithmic plot, normalized with respect to the elution of an internal standard (^{3}H-Thymidine-labelled L1210 cells exposed to 3 Gy radiation).

RESULTS

Exposure of hepa 1c1c7 cells to DMNQ resulted in GSH depletion and concomitant formation of GSSG. As observed previously, intracellular accumulation of GSSG

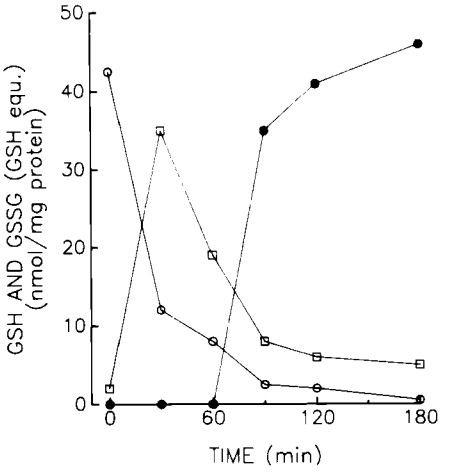

FIGURE 1 Glutathione oxidation in hepa 1c1c7 cells exposed to DMNQ. Cells were exposed to DMNQ 400 μM and GSH and GSSG were assayed as in ref 8. Symbols: (○) intracellular GSH; (□) intracellular GSSG; (●) extracellular GSSG. One typical experiment of 4.

was followed by its extrusion and recovery in the incubation medium (Figure 1). GSSG formation wholly accounted for the disappearance of GSH, indicating that DMNQ metabolism was purely oxidative. Intracellular levels of NAD^+ and ATP decreased concomitantly, and after 30 min of incubation cells were markedly depleted of these nucleotides (Figure 2a and 2b). Oxidation of cellular thiols and loss of ATP have both been shown to result in disruption of intracellular Ca^{2+} homeostasis, with an associated sustained increase of the cytosolic free Ca^{2+} concentration. In hepa 1c1c7 cells preloaded with fura 2 and exposed to DMNQ, the cytosolic Ca^{2+} concentration increased progressively, reaching maximal levels after loss of glutathione and ATP was extensive (Figure 3). Elevation of cytosolic Ca^{2+} concentration was sustained for the following period of 1 h (*i.e.* 2 h from the DMNQ addition). During the first 2 h of incubation cells did not leak LDH or detached from the dishes.

Oxidizing species are known to cause direct damage to DNA by producing alkali-labile strand breaks. In addition, recent studies have indicated that elevation of cytosolic Ca^{2+} can result in the activation of nuclear enzymes which cause DNA fragmentation.[6] Alkaline elution of DNA from hepa 1c1c7 cells exposed to DMNQ for 2 hours indicated that a large amount of DNA strand scissions were formed (Figure 4a), whereas neutral elution under identical conditions revealed that formation of double strand breaks did not occur (not shown). When hepatoma cells were preloaded with an intracellular Ca^{2+} chelator, either quin 2 or BAPTA, the number of single strand breaks was markedly reduced (Figure 4a). To test whether the protective effect of quin 2 and BAPTA was due to chelation of metals other than Ca^{2+} (*e.g.* Fe^{2+}) or scavenging of free radicals, naked DNA isolated from hepa 1c1c7 cells was incubated with H_2O_2 and $FeSO_4$ in absence or presence of intracellular Ca^{2+} chelators at concentrations identical to those present in the cytosol of loaded hepatoma cells. As shown in Figure 4b, strand breakage measured after exposure of naked DNA to the combination of H_2O_2 and $FeSO_4$, was not affected by 200 μM quin 2 free

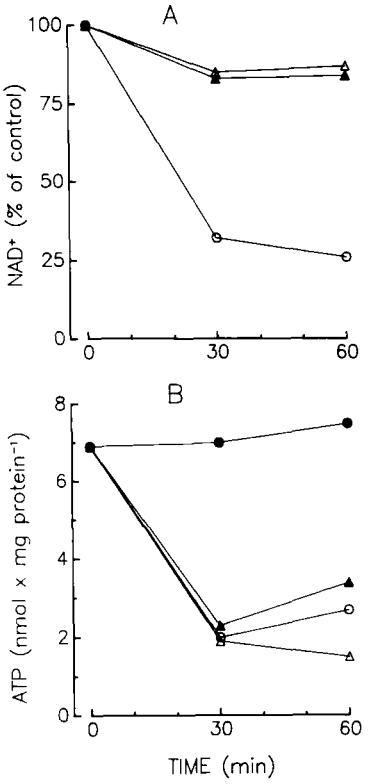

FIGURE 2 NAD^+ and ATP depletion in hepa 1c1c7 cells exposed to DMNQ. Panel A.: Cells were incubated in the presence of DMNQ 400 μM (○) or preincubated with 3-aminobenzamide, 6 mM for 40 min (△) or with BAPTA-AM 30 μM (▲) for 20 min, prior to the incubation with 400 μM DMNQ. Panel B. Experimental procedure was as in A. (●) Control, (○) 400 μM DMNQ; (△) 400 μM DMNQ plus 6 mM 3-aminobenzamide; 400 μM DMNQ plus 30 μM BAPTA-AM (▲). Mean of 4 experiments were standard deviation did not exceed 5%.

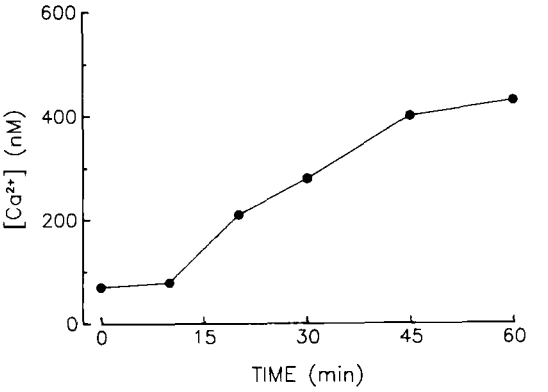

FIGURE 3 Elevation of cytosolic Ca^{2+} in DMNQ-treated hepa 1c1c7 cells. Cells were grown on quartz coverslips and loaded with fura 2-AM as described in Materials and Methods and then incubated as reported (8), in the presence of 400 μM DMNQ.

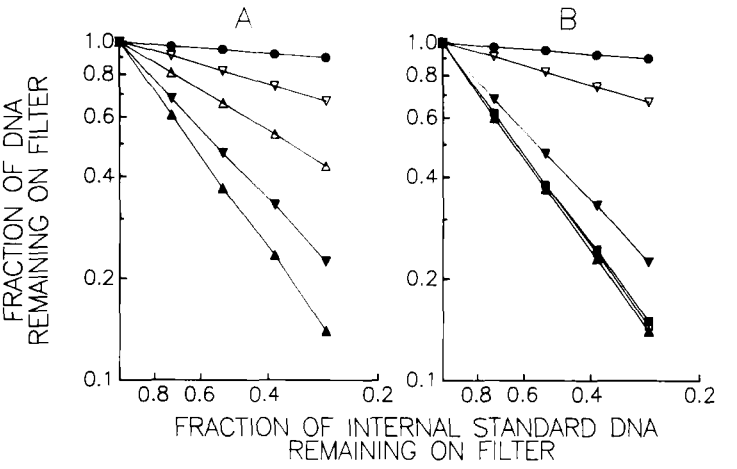

FIGURE 4 DNA strand breaks in hepa 1c1c7 cells exposed to DMNQ, and protective effect of intracellular Ca^{2+} chelators. Confluent hepa 1c1c7 cells seeded on costar 60 mm dishes were preincubated in the absence or the presence of intracellular Ca^{2+} chelators (Quin 2 AM or BAPTA AM) and were subsequently exposed for 2 h. to DMNQ in a rocking incubator at 37°C, under an atmosphere of CO_2:O_2 (95:5%). DNA strand breaks were measured according to Kohn et al. (ref. 10). Panel A: (●) control; (▼) 200 μM DMNQ or (▲) 400 μM; (▽) 200 μM DMNQ plus 20 μM quin 2 AM; (△) 400 μM DMNQ plus 10 μM BAPTA AM. Panel B: Hepa cells were lysed on alkaline elution filters and exposed to the combination of 1 mM H_2O_2 and 10 μM $FeSO_4$ in the absence (□) or in the presence of 200 μM quin 2 free acid or (■) 200 μM BAPTA free acid (▲). (●) Control; (▼) Hepa cells incubated with 200 μM DMNQ as described above for 2 h., or (▽) preincubated with 20 μM quin 2 AM and then exposed to 200 μM DMNQ for the same time. Mean of 4 separate experiments where standard deviation did not exceed 5%.

acid. The protective effect of 20 μM quin 2 AM, which yields (after intracellular hydrolysis) about 200 μM quin 2 is also illustrated in Figure 4b for comparison.

To test the effect of intracellular Ca^{2+} chelators on the DMNQ-induced NAD^+ depletion, NAD^+ content was measured in cells preloaded with BAPTA. NAD^+

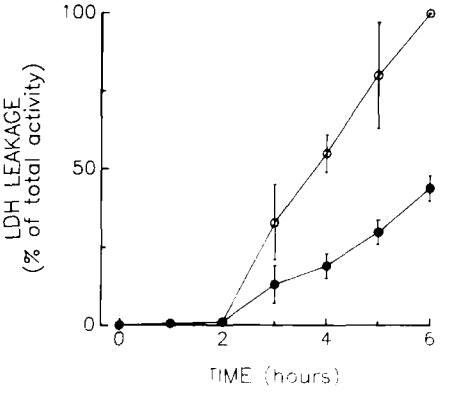

FIGURE 5 Loss of viability in hepa 1c1c7 cells exposed to 400 μM DMNQ. Open symbols DMNQ; closed symbols DMNQ plus 30 μM BAPTA AM. Results are the mean ± S.D. of 4 different experiments.

depletion was prevented by BAPTA, as shown in Figure 2b. Similar protection was afforded by preincubating hepa 1c1c7 cells with 3-aminobenzaminde, an inhibitor of poly-(ADP-ribose)-synthetase[3] (Figure 2b). Neither BAPTA nor 3-aminobenzamide prevented the loss of ATP, suggesting that ATP depletion was not a direct consequence of loss of NAD^+ in this system.

Both elevation of cytosolic Ca^{2+},[11] and NAD^+ depletion[3] have been proposed to cause lethal effects in cells exposed to oxidative stress. To test this hypothesis, hepa 1c1c7 cells were preloaded with the same concentrations of either of the two Ca^{2+} chelators or 3-aminobenzamide, which had been found to prevent DNA damage and NAD^+ loss. Figure 5 illustrates the protective effect of BAPTA on the loss of cell viability caused by DMNQ. Conversely, concentrations of 3-aminobenzamide which prevented NAD^+ loss were uneffective in protecting hepa cells (data not shown).

DISCUSSION

Several toxic properties of quinones have been attributed to the ability of free radicals and activated species generated during their intracellular metabolism, to cause DNA damage.[12] Many investigations have indicated that species such as H_2O_2 or the hydroxyl radical (generated from H_2O_2 intracellularly by Fenton-type reactions) may be the most damaging. Hydroxyl radicals are highly effective at producing DNA strand breaks, probably via addition to a DNA base which yields intermediate base-peroxy radicals.[12] These will in turn result in the oxidation of thymine and guanine to thymine 5,6-glycol and 8-hydroxyguanine, respectively.[13] However, the observation that DNA strand breaks produced by radiation (via hydroxyl radicals) are readily repaired, whereas strand breaks induced by H_2O_2 or superoxide anions are repaired less quickly, has suggested that damage by certain free radicals may not simply involve a direct attack on DNA.[14] Support for this hypothesis has come from studies showing that the repair kinetics of DNA single strand breaks generated by either radiation or H_2O_2 at 4°C were identical, whereas resealing of H_2O_2-induced DNA breaks at 37°C occurred at a much slower rate.[15] Evidence that DNA damage during oxidative stress may be inflicted by Ca^{2+}-dependent enzymes rather than direct radical attack has come from studies by Cantoni and co-workers[16] and work in our laboratory demonstrating that Ca^{2+} may mediate DNA damage by activating a Ca^{2+}-dependent endonuclease.[6,9]

The results presented here demonstrate that intracellular Ca^{2+} chelators can prevent DNA single strand breakage and NAD^+ depletion in hepa 1c1c7 cells exposed to DMNQ. Furthermore, quin 2 and BAPTA protected the cells from DMNQ-induced killing by means of their ability to supplement cell buffering capacity for Ca^{2+}, and minimize changes in cytosolic Ca^{2+} produced by DMNQ (cytosolic Ca^{2+} measured by fura 2 in the presence of BAPTA after 60 min of incubation with DMNQ was 108 $\pm$ 27 nM; mean $\pm$ S.D. from 3 different experiments; cgf. Figure 3). Neither BAPTA nor Quin 2 reacted with DMNQ, and DMNQ-induced GSH and ATP depletion was not modified by preloading the cells with either chelator.

Interestingly, Ca^{2+} chelators prevented NAD^+ depletion. NAD^+ loss during oxidative stress may result from a strand break-stimulated increase in poly-ADP-ribosylation of the nuclear enzymes involved in DNA repair.[3] The sparing of NAD^+ by the Ca^{2+} chelators may therefore be explained by their ability to prevent the formation of DNA single strand breaks. In addition, recent work in our laboratory has demon-

strated that poly-(ADP-ribose)-synthetase activity in isolated liver nuclei is stimulated by Ca^{2+}.[9] Thus, it is conceivable that buffering of Ca^{2+} may directly result in decreased poly-(ADP-ribose)-synthetase activity.

An uncontrolled NAD^+ cleavage by poly-(ADP-ribose)-synthetase has been suggested to ultimately cause ATP depletion and cell death.[3] However, this does not appear to be the case in our experimental system, where BAPTA or quin 2 can prevent NAD^+ depletion and protect from cell killing without affecting DMNQ-induced ATP depletion. Previous studies have indeed indicated that ATP depletion during oxidative stress can result from at least two mechanisms other than increased NAD^+ utilization: (i) early inhibition of glycolytic enzymes and (ii) mitochondrial dysfunction.[17,18] The observation that 3-aminobenzamide prevented DMNQ-induced NAD^+ loss, but did not protect cells from DMNQ toxicity, further rules out the possibility that cell death is a direct consequence of NAD^+ loss. The discrepancy between results of the present study and other findings[3] remains unclear and requires further investigation, although it may simply reflect a difference in the cell type employed.

The Ca^{2+}-dependent mechanism(s) involved in DNA single strand break formation remain to be identified. Ca^{2+} may play an ancillary role by supporting calmodulin dependent processes in the nucleus[19] and regulating chromatin conformation, or it may directly activate excision enzymes. Two Ca^{2+} dependent enzymes which can cause DNA double strand breakage are endonucleases and topoisomerase II. Hepatoma cells lack endonuclease activity (nuclei from hepatoma 1c1c7 cells incubated under conditions similar to those previously employed to study endonuclease activity in liver nuclei, did not exhibit DNA fragmentation typical of Ca^{2+}-dependent endonucleases), and neutral elution did not reveal double strand break formation even after prolonged exposure to DMNQ, implying that topoisomerase II was not involved. Thus, a novel mechanism seems to be responsible for Ca^{2+}-dependent single strand breakage. The hypothesis that Ca^{2+}-dependent conformational changes of the chromatin structure may facilitate DNA single strand formation during oxidative stress is at present under investigation.

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