Mechanism by which caffeine potentiates lethality of nitrogen mustard

(cell cycle/DNA damage/DNA repair/mitotic delay/nuclear fragmentation)

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ABSTRACT Caffeine is synergistic with many DNA-damaging agents in increasing lethality to mammalian cells. The mechanism is not well understood. Our results show that caffeine potentiates the lethality of the nitrogen mustard 2-chloro-N-(2-chloroethyl)-N-methylethanamine (HN2) by inducing damaged cells to undergo mitosis before properly repairing lesions in their DNA. Treatment with low doses of HN2 (0.5 μM for 1 hr) caused little lethality in baby hamster kidney cells (90% survival). These cells were arrested in G0 shortly after treatment with HN2 as shown by flow microfluorimetry and autoradiography. After an arrest of 6 hr, HN2-treated cells began to move into mitosis and from then on behaved like normal cells. Repair synthesis was shown to continue during the G0 arrest by using synchronized cells pulse labeled with [3H]thymidine after HN2 treatment and autoradiography. Caffeine (2 mM) increased the lethality of HN2 by 5- to 10-fold. It prevented the G0 arrest. Caffeine did not prevent these HN2-treated cells from entering or completing S phase but rather allowed them to divide without finishing the repair processes and as a consequence caused nuclear fragmentation after mitosis. Caffeine-induced nuclear fragmentation and enhanced lethality were proportional, as shown with dose–response curves and time dependence. In addition, both lethality and nuclear fragmentation were abolished by low doses of cycloheximide, an inhibitor of protein synthesis.

Caffeine is synergistic with many DNA-damaging agents in causing lethality in mammalian cells (1, 2). Among these agents are x-rays, UV light, and a wide variety of alkylating agents (3–5). Although the molecular mechanism by which caffeine post-treatment decreases cell survival is not known, it is generally believed to be caused by caffeine's inhibition of postreplication repair (6–8). Caffeine seems to maintain the molecular weight of newly synthesized DNA in damaged cells at a low level and prevent its subsequent conversion to high molecular weight DNA (9–11). The simple interpretation of the effect of caffeine is that it prevents completion of replication in regions where single-stranded DNA is exposed and consequently increases cell killing (11, 12). However, there are conflicting reports (13–15), and, as Cleaver pointed out (16), this interpretation might be oversimplified. Thus, alternative explanations for the effect of caffeine must be sought.

To understand the mechanism of caffeine-induced lethality, our laboratory has been studying effects of caffeine on baby hamster kidney cells treated with a variety of alkylating agents, including the nitrogen mustard 2-chloro-N-(2-chloroethyl)-N-methylethanamine (HN2). Treatment with low doses of HN2 (0.5 μM) for 1 hr caused little toxicity (90% survival). When these HN2-treated cells were exposed to caffeine (2 mM) for 12 hr after HN2 had been removed, survival was greatly reduced (15–20% survival). Treatment with caffeine alone produced no lethality. We present results consistent with the hypothesis that caffeine potentiates the lethal effect of HN2 by inducing damaged cells to undergo mitosis before they can repair the lesions in their DNA.

MATERIALS AND METHODS

[3H]Thymidine was purchased from New England Nuclear. HN2 was obtained from Merck. Serum and media were from Flow Laboratories. All other reagents were from Sigma.

Cell Cultures and Synchronization. Baby hamster kidney cells were grown in Dulbecco's modification of Eagle's medium supplemented with 5% calf serum, 5% horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified, 10% CO2 atmosphere. The generation time under these conditions was 12 hr. New cultures were started every month from frozen aliquots. Each new batch of cells was determined to be free of mycoplasma by using the method of Schneider et al. (17). Cells were synchronized at the G0/S boundary by first plating cells in complete media from confluent cultures and then treating with 0.5 mM hydroxyurea 7 hr after plating. Nine hours later hydroxyurea was removed and cells were rinsed twice and fresh medium without hydroxyurea was added back. Synchrony was monitored by flow microfluorimetry and [3H]thymidine incorporation.

Drug Treatment. Medium was changed immediately before drug treatment. HN2 was added to the cultures from frozen aliquots of a stock solution of 100 μM HN2 dissolved in sterile phosphate-buffered saline. All HN2 treatments were 0.5 μM for 1 hr unless indicated otherwise. At the end of treatment, medium containing HN2 was removed and cultures were rinsed once before fresh medium was added back. Concentration of caffeine in post-treatment was 2 mM throughout this study.

Flow Microfluorimetry. Cells were prepared for flow microfluorimetry as described by Yen and Pardee (18). Cells grown in 60-mm culture plates were washed three times with a hypotonic staining solution of propidium iodide (50 μg/ml in 0.1% sodium citrate). Cells were stained for 15 min with propidium iodide at 4°C and then dislodged, and the nuclei were suspended in the staining solution. Flow microfluorimetry was performed with a Biophysics Systems Cytofluorograf model 4500A.

Autoradiography. Autoradiography was performed by using a modification of Hamlin's procedure (19). Cells were grown in 35-mm culture plates. To determine the fraction of cells synthe-

Abbreviations: HN2, 2-chloro-N-(2-chloroethyl)-N-methylethanamine (a nitrogen mustard); DAPI, 4,6-diamidino-2-phenylindole.

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were calculated from treatment.

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sizing DNA at any one time, cells were pulsed for 30 min with [3H]thymidine (5 μCi/ml; 1 Ci = 3.7 x 10^10 becquerels). To determine the fraction of cells synthesizing DNA over a period of time, continuous labeling was done with [3H]thymidine (0.2 μCi/ml). At the end of labeling, cells were first rinsed two times with ice-cold phosphate-buffered saline and then fixed in methanol/acet acid, 2:1 (vol/vol), for 15 min at room temperature. After air drying, emulsion (Kodak NTB-2) was put on the plates, which were developed after 5 days with Kodak D19 developer. The nuclei were lightly stained with Giemsa stain

and the percent of labeled nuclei was determined by microscopic counting. A minimum of 300 cells was counted for each determination.

Fluorescent Staining. Cells were plated on round 15-mm glass coverslips placed in 24-well plates. Coverslips were washed in phosphate-buffered saline and fixed for 15 min sequentially in 50%, 75%, and 95% (vol/vol) ethanol and then air dried. They were then stained in 4,6-diamidino-2-phenylindole (DAPI) at a concentration of 0.5 μg/ml at room temperature for 10 min. The coverslips were then washed briefly in distilled water once and mounted onto microscopic slides with glycerol gel. Slides were observed under a Zeiss fluorescence microscope.

RESULTS

Our previous results showed that caffeine, at nontoxic concentrations (2 mM), potentiated the lethality in cells pretreated with low doses of HN2. This caffeine sensitivity of HN2-treated cells was time dependent; maximal lethal effect of caffeine was seen within 12 hr after HN2 treatment and cells became progressively insensitive to the lethal effect after 12 hr. Furthermore, this caffeine effect was shown with synchronized cells to be cell cycle-specific; HN2-treated cells were most sensitive in the first G2 phase after HN2 treatment (unpublished data).

The mechanism of caffeine-induced lethality was further investigated by using flow microfluorimetry to study the changes in cell cycle distribution that resulted from treatment with 0.5 μM HN2. Fig. 1A shows typical histograms of DNA content at various times after treatment with HN2. The fractions of the population in the various phases of the cell cycle were calculated from areas under the peaks. The fraction of cells in G2 + M progressively increased after HN2 treatment, beginning at 4 hr after HN2 was removed (Fig. 2B). More than 65% of the cells

![Fig. 1. Typical DNA histograms of cells at various times after HN2 treatment without (A) or with (B) caffeine post-treatment. DNA content corresponds to G1 phase (left peak), S phase (trough), and G2 + M phase (right peak).](image1)

![Fig. 2. Fraction of cells in G1 (A) and G2 + M (B) phases after HN2 treatment with (■) or without (□) caffeine post-treatment. These values were calculated from areas under the peaks in Fig. 1. Horizontal lines (----) are for the untreated controls with or without caffeine.](image2)

![Fig. 3. Fraction of cells in G2 after release from hydroxyurea. Cells were given HN2 treatment 1 hr before release from hydroxyurea. Caffeine was added to the appropriate plates immediately after release. ○, Untreated control cells; ●, untreated control cells with caffeine; □, HN2-treated cells; ■, HN2-treated cells with caffeine.](image3)
were in G_2 + M phase by 10 hr. At this time the mitotic index was less than 1%, indicating that the increase in the G_2 + M fraction was due to G_2 phase cells. By 12 hr cells began to leave G_2 to enter mitosis (as shown by flow microfluorimetry and cell number) and from then on behaved like normal cells. Throughout this period the fraction of cells in S phase remained constant. In the presence of caffeine (2 mM) this accumulation of cells in G_2 did not occur (Figs. 1B and 2).

By using cells synchronized at the G_1/S boundary by treatment with hydroxyurea, it was confirmed that these HN2-treated cells were delayed in G_2 phase for approximately 6 hr (Fig. 3). Post-treatment with caffeine allowed HN2-treated cells to traverse S and G_2 phases at a normal rate.

Concomitant to the reduction of G_2 delay, caffeine also caused a great increase in the number of nuclei with abnormally small DNA contents, less than the content of normal G_1 cells (Fig. 1B). This implies that much nuclear fragmentation is caused by post-treatment with caffeine. The flow microfluorimetry data were confirmed by direct observation. When HN2-treated cells were stained with a fluorescent dye, DAPI, after caffeine post-treatment, extensive fragmentation of nuclei was observed (Fig. 4). When metaphase chromosomes of cells treated with both HN2 and caffeine were spread, they were found to be completely pulverized (Fig. 5).

Caffeine might reduce the accumulation of G_2 cells either by preventing cells from passing through S phase or by overcoming the G_2 block, thereby allowing cells to go through mitosis without delay. The first possibility was eliminated by measuring DNA synthesis during 9 hr after HN2 treatment of exponentially growing cells in the presence and absence of caffeine. There was no significant difference in the amount of [3H]thymidine incorporated (1.4 × 10^6 and 1.6 × 10^6 cpm, respectively). This was further confirmed by the percent of labeled nuclei measured by continuous labeling and autoradiography. When exponentially growing cells were treated with HN2 and then continuously labeled with [3H]thymidine for 8 hr in the presence of caffeine, a large fraction of the labeled nuclei were fragmented, whereas none of the unlabeled nuclei were fragmented (Fig. 6A). This implies that DNA synthesis is essential for the fragmenting effect of HN2 followed by caffeine. When the experiment was repeated, but using 30-min pulse labeling just before fixation instead of continuous labeling, the situation was completely reversed. Only unlabeled nuclei were fragmented, observed beginning 4 hr after HN2 treatment, whereas the labeled nuclei were never fragmented (Fig. 6B). Together with the observation made with continuous labeling, this implies that this nuclei-fragmenting effect occurs some time after DNA synthesis is completed.

Rates of DNA synthesis in cells synchronized by hydroxyurea and treated with HN2 and caffeine were studied by pulse labeling and autoradiography. HN2-treated cells finished DNA replication synthesis (heavily labeled) without any delay, 4 hr after release from hydroxyurea, in the presence and absence of caffeine (Fig. 7). Control cells passed through S phase at the identical rate. After the percent of heavily labeled nuclei

![Fig. 5](image)

**FIG. 5.** Typical chromosome spreads of HN2-treated cells with (A) or without (B) caffeine post-treatment. Caffeine was added immediately after HN2 was removed and Colcemid (0.2 μg/ml) was added 5 hr later. Mitotic cells were harvested 3 hr later and metaphase chromosomes were spread and stained with Giemsa stain. (×525.)

![Fig. 6](image)

**FIG. 6.** Fragmented nuclei after continuous labeling (A) and pulse labeling (B) of HN2-treated cells with [3H]thymidine in the presence of caffeine. In each case, completely dark nuclei are labeled, while lighter nuclei are unlabeled but stained with Giemsa stain. (×300.)
posed as DNA-damaging induced moderately inhibit observed and time nuclear possible. that repair the time in (at labeled HN2 treated cells dropped, nuclei appeared in more than a third of the HN2-treated cells that were lightly labeled in contrast to the heavily labeled nuclei of cells in S phase (Fig. 7). This percentage of lightly labeled nuclei remained at high levels up to 9 hr after HN2 treatment, just before HN2-treated cells were ready to divide (at M'). Strikingly, caffeine abolished the lightly labeled nuclei in HN2-treated cells. Instead mitosis occurred at the normal time (M), and fragmented nuclei were observed during the same period in these cells only (Fig. 7B). These results show that repair synthesis was taking place during the G2 delay and that caffeine allowed mitosis to occur before much repair was possible.

A strong correlation was found between effects of caffeine on nuclear fragmentation and lethality. The dose–response curves and time dependence of caffeine-induced nuclear fragmentation and lethality were very similar (Fig. 8). When caffeine was added 12 hr after HN2 had been removed, both effects were greatly diminished. In addition, S. K. Das in this laboratory has previously observed that the enhanced lethality caused by caffeine can be abolished by cycloheximide at concentrations that moderately inhibit protein synthesis. Nuclear fragmentation induced by caffeine was also inhibited by cycloheximide (Table 1).

**DISCUSSION**

Numerous reports show that caffeine potentiates the lethality of DNA-damaging agents in mammalian systems (for references see ref. 20). Inhibition of postreplication repair has been proposed as a mechanism, on the basis of results showing that cells after exposure to UV or x-irradiation or after treatment with alkylating agents synthesized DNA that was of lower molecular weight than in the untreated controls (21, 22). Several hours later these low molecular weight DNAs were converted to normal, high molecular weight DNA. Thus it was postulated that there were gaps in the newly synthesized DNA, created when DNA polymerase encountered a blocking lesion (23), and that these gaps were later filled by a postreplication repair mechanism. In the presence of caffeine, the conversion from low to high molecular weight DNA was inhibited (9–11, 24). This was taken to be evidence that caffeine inhibits postreplication repair and that this inhibition eventually leads to cell death (11, 12).

Our results show that there is another effect that can account for caffeine's ability to inhibit repair and to potentiate lethality of DNA-damaging agents. There have been a number of reports of mitotic delay caused by x-irradiation (25, 26), ionizing radiation (27), neocarzinostatin (28), and 6-thioguanine (29). Rao and Rao (30) showed that the mitotic delay was due to damage to the chromosomes and not a metabolic block. With HN2, using cells synchronized at the G2/S boundary by hydroxyurea, we have shown that there was little delay in S phase. Instead, cells were delayed in G2 for 6 hr. Unscheduled DNA synthesis was detected during this G2 delay. When this kind of DNA synthesis was completed, cells proceeded into mitosis. This is consistent with...

![Graph](image_url1)

**Fig. 7.** Percentage of heavily labeled (•), lightly labeled (○), and fragmented (△) nuclei in hydroxyurea-synchronized cells after HN2 treatment; without caffeine (A) and with caffeine (B). Cells were treated with HN2 for 1 hr before they were released from arrest. At various times after release, cells were pulse labeled with [3H]thymidine (5 μCi/ml) for 30 min and then fixed for autoradiography. Heavily labeled nuclei are defined as those that are completely black, whereas lightly labeled nuclei are those that have scattered dark grains. Mitosis occurred at M for control cells and HN2-treated cells in the presence of caffeine. In the absence of caffeine, HN2-treated cells divided at M'.

**Fig. 8.** Dose–response curves of lethality (A) and nuclear fragmentation (B) of HN2 with no caffeine post-treatment (○), 2 mM caffeine post-treatment 0–12 hr (●), and 2 mM caffeine post-treatment 12–24 hr (◆). Lethality was measured by colony formation. Two hundred cells were plated after HN2 treatment and caffeine was added at the indicated intervals. Colonies were counted after 7 days. Percent survival is defined as the number of colonies of the treated cells relative to that of the untreated controls. Plating efficiency of untreated controls was 60–70%. Percent of intact nuclei was determined by microscopic counting of DAPI-stained as well as Giemsa-stained cells. More than 300 cells were counted for each determination.

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<tr>
<th>Table 1. Effect of cycloheximide on enhanced lethality and nuclear fragmentation induced by caffeine</th>
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<td><strong>Addition</strong></td>
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Caffeine (2 mM) and cycloheximide (0.2 μg/ml) were present for 12 hr after HN2 was removed. This concentration of cycloheximide inhibited protein synthesis by 75%. Other experimental details are in legend of Fig. 8.
with Tobey’s hypothesis that a surveillance mechanism operates in G₂ to ensure that damaged DNA is repaired before mitosis (31). However, in the presence of caffeine, HN2-treated cells were unable to become arrested in G₂ but entered mitosis at the usual time, and nuclei subsequently were fragmented in the divided cells. Karyotyping shows that chromosomes of such cells were completely pulverized, indicating numerous breaks. This is consistent with previous reports that post-treatment with caffeine causes numerous breaks in the DNA of cells treated with alkylating agents (32). However, our results indicate that the primary effect of caffeine is to induce HN2-treated cells to undergo mitosis, thereby not giving enough time for damaged cells to repair their DNA. This is very similar to what has been observed with ataxia telangiectasia cells after x-irradiation as reported by Painter and Young (33). They also suggested that caffeine, instead of impairing the ability of cells to repair damage, might be inducing cells to bypass damage-induced delays that allow them time to repair damage.

Note that Murnane et al. (15) recently reported that caffeine had no effect on removal of DNA damage caused by HN2. Moreover, their study failed to show any effect of caffeine on postreplication repair in conditions producing synergistic lethal effects. Instead they showed an influence by caffeine on initiation of DNA synthesis in damaged replicons, and they proposed that this effect was primarily responsible for the synergistic lethality of these drugs. However, we did not observe any significant decrease in the rate of DNA synthesis in HN2-treated cells, mainly because we used low concentrations of HN2 (0.5 μM), which killed only 10–15% of the cells. This low level of damage was not sufficient to cause significant inhibition in DNA synthesis but was enough to cause mitotic delay. On the other hand, Murnane et al. in their studies used much higher concentrations of HN2 (1.7 μM), which killed about 85% of the treated cells without caffeine post-treatment.

From our results, caffeine did not seem to act directly to potentiate lethality of HN2 but rather acted by means of some newly synthesized protein(s). HN2-treated cells were protected from the lethal effect of caffeine by low doses of cycloheximide (0.2 μg/ml). We have verified that this protection was not simply due to arresting of cells in G₁ or S, as cycloheximide can do (34). After HN2-treated cells had entered G₂ phase, they could still be protected from caffeine by cycloheximide (data not shown). This indicates that protein synthesis is required for the lethal as well as the nuclear fragmenting action of caffeine. This protein(s) might be required for normal mitosis and could be induced by caffeine.

Recently we have extended this work to study the effect of caffeine on cells treated with other alkylating agents and antimetabolites. These include methyl methanesulfonate, N-methyl-N'-nitro-N-nitrosoguanidine, 6-thioguanine, cis-diaminedichloroplatinum, and β-cystine arabinonucleoside. Cells treated with any one of these agents all showed G₂ arrest. This G₂ arrest appeared, for different agents, at different times after treatment that coincided with the time when the treated cells were maximally sensitive to the lethality of caffeine. In all cases, caffeine prevented the G₂ arrest and caused nuclear fragmentation.

Thus it is clear that nuclear fragmentation induced by caffeine is not limited to cells treated with crosslinking agents but also occurs in cells treated with mono-alkylating agents and possibly many agents that cause DNA damage.

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