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Intervention of Sulfur Mustard Toxicity by Downregulation of Cell Proliferation and Metabolic Rates†

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Key words: sulfur mustard; BAPTA AM; NHEK; cell proliferation; cell metabolism; DNA synthesis; RNA synthesis; protein synthesis; toxicity; protection.

Metabolically active and proliferating basal cells in the skin are most sensitive to the potent skin blistering chemical warfare compound HD (bis-(2-chloroethyl) sulfide). We previously described a Ca^{2+}-dependent mechanism of HD (0.3–1 mM) toxicity that was inhibited by the cell-permeant Ca^{2+} chelator BAPTA AM (1,2-bis(*O*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester). We describe some cellular effects of BAPTA AM that suggest a mechanism for its protective action. Monolayer log-phase normal human epidermal keratinocytes were incubated (37°C) first in keratinocyte growth medium (KGM) containing BAPTA AM (10–40 μM) for 30 min and then in KGM alone overnight prior to evaluation. The BAPTA AM inhibited cell growth in a concentration-dependent manner with some cellular degeneration above 30 μM (light microscopy). At 20–30 μM, BAPTA AM also inhibited cellular metabolic processes, as evidenced by a lower incorporation of [^{3}H]-thymidine (DNA synthesis, 54 ± 5%), [^{3}H]-uridine (RNA synthesis, 29 ± 6%) and [^{14}C]-valine (protein synthesis, 12 ± 2%) as well as a lower protein content per culture (30 ± 3%) compared with corresponding untreated controls. However, 20–30 μM BAPTA AM did not cause any demonstrable cytopathology based on morphological (electron microscopy) as well as biochemical (lactate dehydrogenase release, an indicator of cell viability loss) criteria, indicating a lack of acute toxicity. These results suggest that a mechanism of protection by BAPTA AM against HD may be via decreasing some metabolic, and therefore proliferative, rates. Published in 2000 by John Wiley & Sons, Ltd.

INTRODUCTION

Cellular toxicity due to DNA-damaging agents such as radiation and radiomimetic chemicals, e.g. sulfur mustard (HD), is known to be directly proportional to the rate of cell division. The most sensitive segment in the human skin for the toxic effects of the blistering compound HD is the basal epidermal layer containing the highly proliferative keratinocytes. We previously reported that HD-induced cytotoxicity in cultured normal human epidermal keratinocytes (NHEK) could be ameliorated by the chelation of intracellular free Ca^{2+} ions by BAPTA AM (1,2-bis (*O*-aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester).[1] The objective of the present study was to investigate whether the protection against HD by BAPTA is due to its effects on cell proliferation and associated metabolic events.

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EXPERIMENTAL

Materials

The NHEK and keratinocyte growth medium (KGM) were purchased from Clonetics Corporation (San Diego, CA). The BAPTA AM was purchased from Molecular Probes (Eugene, OR). Anhydrous dimethyl sulfoxide (DMSO) was purchased from Aldrich (Milwaukee, WI). [^{3}H]-Thymidine, [^{3}H]-uridine and [^{14}C]-valine were obtained from NEN (Boston, MA). Sulfur mustard (98% pure) was obtained from the US Army Edgewood Research, Development and Engineering Center, Aberdeen Proving Ground, MD. All other chemicals were of analytical reagent grade. Falcon tissue culture plasticware was purchased from Becton Dickinson (Lincoln Park, NJ).

Cell culture

The basic methodology of culture initiation from frozen stock and plating was as described by Rhoads *et al.*[2] The ambient conditions of incubation were a humidified atmosphere of 5% CO_2–95% air and a temperature of 37°C. Keratinocytes were subcultured until passage 3 using ca. 80% confluent cultures. For experiments,

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cells were grown in 150-cm^2 flasks or 24-well or 6-well plates starting at 5000–10000 cells cm^{-2}. These cells became confluent in 5–7 days. Cells were used either in log phase or when they were ca. 80–90% confluent, depending on the experiment.

Treatment of cells with HD

Cells were treated with diluted stock HD in KGM for a specified concentration and time. Sulfur mustard is very sparingly soluble in water and hydrolyses rapidly in aqueous medium ($t_{1/2}$ ~ 10–20 min, depending on the medium composition and temperature). Therefore, a special procedure was used for exposing cells to HD in KGM using a unique formulation developed by Broomfield and Gross[3] as described by Ray *et al.*[4]

Treatment of cells with BAPTA AM

Growth medium (KGM) from monolayer cultures was aspirated and then replaced by KGM containing a specified BAPTA AM concentration (0–40 μM). The cultures then were incubated inside a cell culture incubator for 30 min. At the end of this incubation period, growth medium was replaced by KGM for incubation overnight. To study the effects of BAPTA AM on HD-induced toxicity following BAPTA AM treatment for 30 min, BAPTA-AM-containing medium was replaced by 1 mM HD in KGM for 120 min and then the cells were incubated in KGM overnight.

Microscopy

For all microscopic studies, untreated control and BAPTA-AM- and/or HD-treated cells were incubated in KGM overnight and then evaluated by microscopic observations. Light microscopic observations were done using an inverted phase contrast microscope at 160 × magnification. All observations were recorded by polaroid photography. For scanning electron microscopy, the following procedure was used. Untreated control and experimental cells were detached from culture plates by mild trypsinization using 0.05% ultrapure trypsin in isosmotic buffered salt solution (pH 7.4) and then pelleted by centrifugation. These cells were then rinsed in 0.1 M sodium cacodylate buffer (pH 7.4, 190 mOsm) by resuspension and centrifugation. The resulting cell pellets were fixed for 4 h at 20°C in a combined fixative of 1.6% formaldehyde and 2.5% glutaraldehyde in sodium cacodylate buffer. Following primary fixation, pellets were washed three times in cacodylate buffer, post-fixed for 1 h in buffered 1% osmium tetroxide, rinsed in buffer three times and then finally rinsed in deionized water. Next the cells were dehydrated in graded ethanols, critical-point dried and sputter coated with gold–palladium to 12.5 nm thickness for examination by scanning electron microscopy.

Cell viability assay

Cell viability was determined by measuring lactate dehydrogenase (LDH) release, an indicator of cytotoxicity,[5] in the growth medium of control and experimental cells. The media were centrifuged to remove dislodged cells, if any. Released LDH was assayed according to the method of Gay *et al.*,[5] which is based on an increase in absorbance at 340 nm caused by the formation of NADH due to the LDH-catalyzed reaction between L-lactate and NAD.

Radiolabeled precursor incorporation

To study the effects of BAPTA AM on incorporation of the radiolabeled precursors [^{3}H]thymidine (DNA synthesis), [^{3}H]uridine (RNA synthesis) and [^{14}C]valine (protein synthesis), untreated control and BAPTA-AM (20 μM)-treated cells were incubated overnight (24 h) in KGM containing 0.2 μCi ml^{-1} of the respective radiolabeled compounds. Following this incubation, unincorporated radioactivity was removed by washing three times using KGM. Cells were dissolved in 0.2 N NaOH–1% Triton X-100. Radiolabeled nucleic acids and protein in an aliquot, neutralized by 0.2 N HCl–TRIS. HCl buffer (pH 7.4), were precipitated in ice-cold 5% trichloroacetic acid in the presence of 50 μg per assay carrier DNA. Incorporation of radioactivity

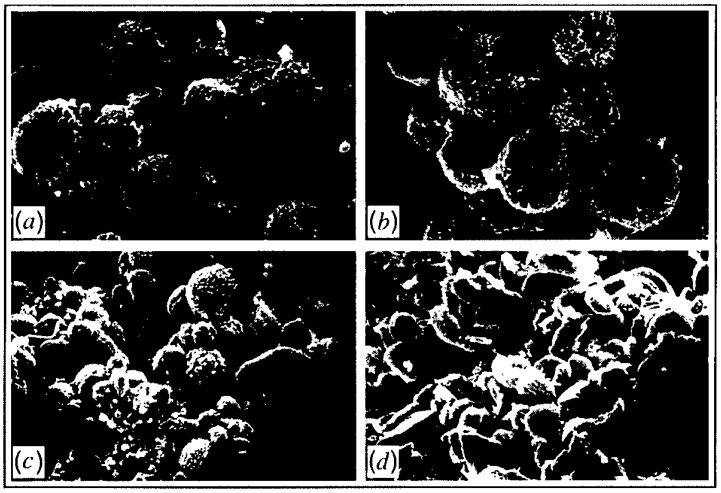

Figure 1. Effects of BAPTA AM on cell morphology and HD toxicity. Scanning electron micrographs of NHEK: (A) untreated control (2500 ×); (B) pretreated with 20 μM BAPTA AM (2500 ×); (C) exposed to 1 mM HD (1000 ×); (D) pretreated with BAPTA AM and then exposed to HD (1000 ×).

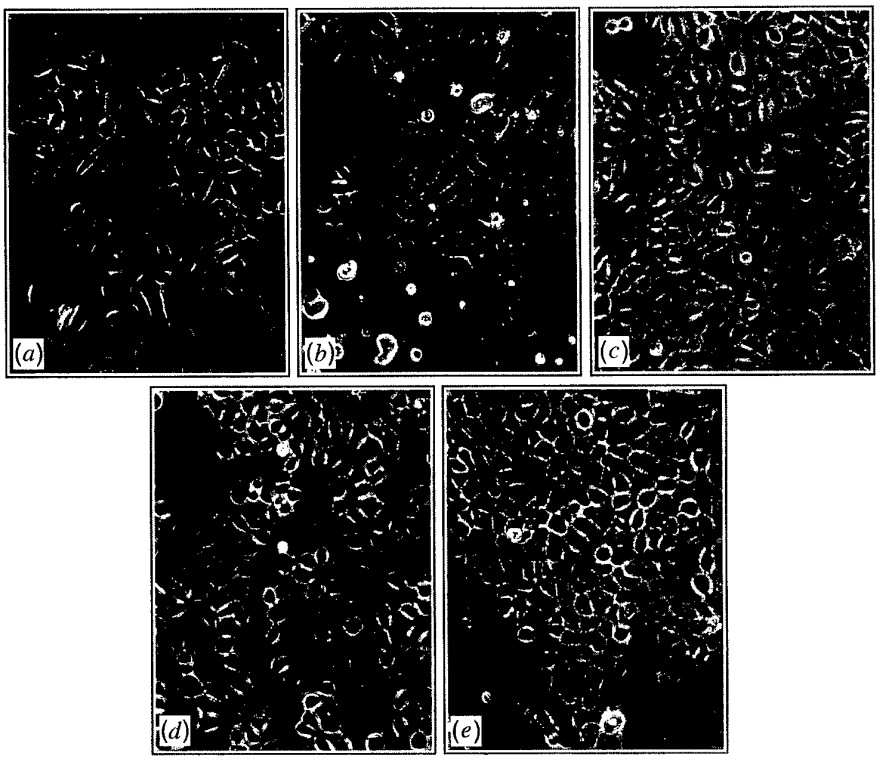

Figure 2. Concentration-dependent effects of BAPTA AM on cell growth and morphology. Light micrographs (magnification 160 ×) of NHEK cultures: (A) untreated control; (B) 10 μM BAPTA AM; (C) 20 μM BAPTA AM; (D) 30 μM BAPTA AM; (E) 40 μM BAPTA AM.

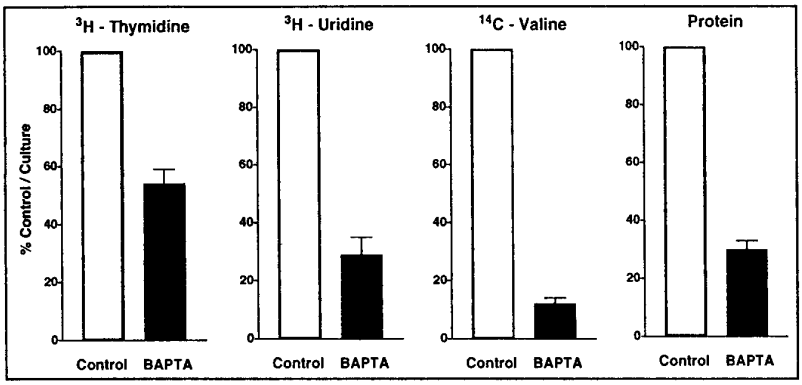

Figure 3. Effects of BAPTA AM on cellular incorporation of radiolabeled precursors and on protein content. Values are means ± SEM of three separate experiments.

was measured by filtration on Whatman GF/C 24 mm diameter glass-microfiber filters and scintillation counting using extremely quench-resistant Packard (Meriden, CT) Ultima Goldscintillation cocktail. To discount any possible quench error, duplicate background samples containing only non-radioactive carrier DNA were run under identical conditions and the average count was subtracted from experimental samples.

Protein assay

Total protein was assayed using a commercially available kit (Bio-Rad Laboratories, Herculis, CA) based on the Bradford method.[6] The protein assay was automated for use on the Cobas FARA II following the manufacturer's assay parameters.

RESULTS

The effects of BAPTA AM (20 μM) pretreatment on HD-induced changes in NHEK cultures are shown in Fig. 1 using scanning electron microscopy (SEM). In SEM, both untreated control (Fig. 1A) and BAPTA-AM-pretreated (Fig. 1B) cells had unremarkable surface features with a typical display of microvilli; HD caused a characteristic toxic display of blebbing and plasma membrane perturbations (Fig. 1C), which were pre-

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Table 1. Effect of BAPTA on HD-induced changes in cell viability and protein content[a]

Experimental condition	LDH release (U mg^{-1} protein)[b]	Total protein content (mg per culture)
Untreated control	0.02 ± 0.00	2.44 ± 0.15
1 mM HD	0.36 ± 0.04	1.18 ± 0.06
20 μM BAPTA AM	0.06 ± 0.01	1.60 ± 0.03
20 μM BAPTA AM, then 1 mM HD[c]	0.03 ± 0.00	1.45 ± 0.12

[a]Determinations were made 24 h following HD exposure. All values are means ± SD of three separate assays.
[b]One unit of LDH is defined as the amount of enzyme that converts 1 μmole of lactate to 1 μmole of pyruvate with the concomitant reduction of 1 μmole of NAD to 1 μmole of NADH per minute.
[c]The BAPTA AM (20 μM) pretreatment 30 min prior to HD (1.0 mM) exposure prevented LDH release and prevented the decrease in protein due to HD in NHEK.

vented by BAPTA AM pretreatment (Fig. 1D). The effects of different BAPTA AM concentrations (0–40 μM) on cell growth characteristics and morphology were studied by light microscopy (Fig. 2). Observations 24 h after BAPTA AM treatment showed inhibition of cell proliferation at all BAPTA AM concentrations and some morphological alterations due to BAPTA AM above 30 μM. Typical morphological effects were condensation of nuclei, flattening of cell bodies and the appearance of cytoplasmic vacuoles (Fig. 2B–E). All experiments in this study were therefore conducted using 20–30 μM BAPTA AM.

The effects of BAPTA AM pretreatment on cell metabolism and cell growth, assessed in terms of total cell protein, are shown in Fig. 3. The BAPTA AM (20–30 μM) decreased cellular incorporation of [^{3}H]thymidine (DNA synthesis), [^{3}H]uridine (RNA synthesis) and [^{14}C]valine (protein synthesis), as well as protein content, compared with the corresponding untreated controls.

The effects of BAPTA AM pretreatment on HD-induced changes in cell viability (LDH release) and cell growth (protein content in cultures) are shown in Table 1. Exposure of cells to 1 mM HD for 1 h increased the release of LDH in the incubation medium 20-fold during the subsequent 23 h compared with untreated control. In contrast, when the cells were pretreated with BAPTA AM and then exposed to HD, there was no increase in LDH release due to HD. There was about a 50% reduction in the total protein content of cultures treated with HD. Treatment of cultures with 20 μM BAPTA AM also reduced the total protein content by ca. 35%. However, BAPTA AM pretreatment of cultures completely prevented the decrease in protein content due to HD.

DISCUSSION

The results shown in Fig. 1 and Table 1 indicate that BAPTA AM pretreatment of cultured NHEK protects against both biochemical and morphological toxicities due to HD. The results shown in Figs 2 and 3 demonstrate that 20–30 μM BAPTA AM treatment inhibits cell proliferation and metabolism without any overt toxicity of its own. We published that one of the mechanisms of HD toxicity may be via an increase in intracellular free Ca^{2+} concentration leading to phospholipase A_2 (PLA_2) activation, arachidonic acid release from cell membrane and membrane fluidity decrease, and that the Ca^{2+} chelator BAPTA AM counteracts HD toxicity possibly by preventing these high Ca^{2+}-induced toxic mechanisms.[1] In the NHEK culture model, the protection by BAPTA AM against HD effects on cell morphology and viability was observed in cells either pretreated or post-treated (up to 4 h, but not 6 h, after HD exposure; data not shown), suggesting both pretreatment and therapeutic use of BAPTA AM. In this article, we propose another mechanism of protection by BAPTA AM via downregulation of cell metabolism and proliferation. This mechanism, however, does not exclude the other mechanism, i.e. via diminution of PLA_2 by BAPTA, that we proposed earlier.[1] The NHEK cultures pretreated with BAPTA AM become resistant to HD damage because the toxicity due to DNA-damaging agents is known to be directly proportional to the rate of cell division. One possibility remains that cells exposed to BAPTA AM and HD may not recover to function normally. Additional studies (data not shown) in our laboratory have shown that BAPTA-induced events that protect against HD appear to be irreversible. Also, BAPTA seems to protect against HD by preventing the initiation of events leading to vesication rather than by enhancing the recovery of HD injury. Besides the mechanisms suggested in this article, BAPTA also protects against HD via other mechanisms, e.g. by blocking the terminal differentiation response and by attenuating the apoptotic response due to HD.[7] We reported that BAPTA AM does not affect the activation of DNA ligase (a DNA repair enzyme) in HD-exposed NHEK, but delays its inactivation.[8] Whether BAPTA AM has any effect on the mechanisms of HD-damaged DNA repair remains to be investigated. In conclusion, a Ca^{2+} chelator like BAPTA AM may be considered as an HD antidote, assuming that BAPTA AM will prevent blister formation in the HD-exposed skin area, which will subsequently be repopulated by healthy cells infiltrating from the neighboring unexposed area. Whether BAPTA AM prevents or ameliorates HD injury in animal skin is being investigated in our laboratory.

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