Poly(ADP-ribose) polymerase (PADPRP) [E.C.2.4.2.30] has been proposed to play a key role in the NAD⁺ depletion following alkylation of DNA in sulfur mustard (HD) exposures. Papirmeister et al. (Fundam Appl Toxicol 5:S134, 1985) hypothesized that activation of PADPRP was central to the subsequent depletion of NAD⁺ and activation of proteolytic enzymes leading to vesication. NAD⁺ depletion following HD exposure has been previously documented and the results have been used to infer the effect of HD exposure on PADPRP. The present study was undertaken to demonstrate the direct effect of HD on PADPRP activity. HeLa cells culture were used as the model system.

At 10 μM HD PADPRP activity was increased above the levels of controls in the first hour. The activity peaked at 4 hrs and by 6 hrs had returned to control levels. The 24-hour level of PADPRP activity was again elevated above the controls.

The 100 μM HD exposures had maximal enzymatic response in HeLa cells within the first hour. The level had decreased 40% from the maximum by the second hour reaching a plateau at 30% of the maximum response after 4 hrs. Cells exposed to 100 μM HD showed enzyme levels at or below those seen with the 10 μM dose after 24 hours. The doses of HD used did not decrease viability as measured by trypan blue dye exclusion within 24 hr.
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INTRODUCTION

Sulfur mustard (2,2'-dichlorodiethyl sulfide or HD) is a bifunctional alkylating agent which reacts with a wide variety of biological molecules. It is a strong alkylating agent of purine bases in DNA (Kohn 1983). Early studies strongly implicate DNA as a principal cellular target in HD toxicity (Papirmeister et al. 1991). A hypothesis of HD pathogenesis (Papirmeister et al. 1985) suggests that the pathology is initiated by alkylation of DNA. Subsequent breaks in DNA cause activation of the chromosomal enzyme Poly(ADP-ribose) Polymerase (PADPRP) [E.C.2.4.2.30], which utilizes nicotinamide adenine dinucleotide (NAD+) as a substrate to ADP-ribosylate, a variety of nuclear proteins. Depletion of NAD+ results in the inhibition of glycolysis, and alteration of metabolic pathways that lead to pathological changes characterized by cell death and necrosis.

As predicted by this hypothesis, research has shown that HD exposure will reduce NAD+ levels in several models, including human skin on nude mice (Gross et al. 1985), lymphocytes (Meier et al. 1987), keratinocytes (Smith et al. 1990), HeLa (Smith et al. 1992), and the hairless guinea pig (Yourick et al., 1991). These depletion of NAD+ has varied in a time- and concentration-dependent manner following exposure to HD in all the various models tested.

HeLa cells have previously demonstrated stimulation of PADPRP activity following exposure to a monofunctional alkylating agent, methylnitrosourea (MNU) (Sudhakar et al., 1979). Since previous work has used NAD+ depletion is an indirect measure of PADPRP activity, this study was undertaken to demonstrate the direct effect of HD on PADPRP activity. During the course of this study we determined the time- and concentration-dependent effects of HD on PADPRP activity in HeLa cell cultures.

METHOD AND MATERIALS

Reagents

Sulfur mustard (HD, 2,2'-dichlorodiethyl sulfide) with purity of 98% was obtained from Chemical Research, Development and Engineering Center, Aberdeen Proving Ground, MD. Eagle's MEM with non-essential amino acids Earle's BSS (Sigma Cell Culture, St. Louis, MO.) was used for culturing and and washing the cells. Fetal calf serum (FCS) was obtained from HyClone Lab, Inc., Logan, Utah. The radiolabeled Adenine-2,8-3H-NAD was purchased from New England Nuclear, Boston, Ma. All other reagents were purchase from Sigma Chemical Co., St. Louis, MO.
Cell Culture

HeLa cultures are maintained in 150 cm² culture flask (Corning Glass Works, Corning, NY) in MEM with 10% FCS at 37°C and 5% CO₂. When the cultures are 80-90% confluent, the media was removed and replaced with media containing HD (0.1 to 100.0 μM) and the cells were incubated for 60 minutes in an approved fume hood at room temperature to remove any hazardous vapors. After 1 hour, the media was removed and replaced with fresh MEM without HD to incubate an additional 0-23 hours. The cells were harvested by removing the media and adding phosphate buffered saline containing trypsin and EDTA to the flask. The detached cells were pipetted into a conical centrifuge tube containing MEM with 10% FCS and centrifuged at 250xg for 10 minutes. The supernate was removed and the cells suspended in 10 ml of 50 mM Tris buffer (pH 8.0) and 25 mM MgCl₂. The cultures were maintained on ice until the PADPRP assay was initiated.

Poly(ADP-Ribose) polymerase assay.

The cell suspension was sonicated on ice with a microtip and a setting of 50 in a Branwill Biosonik III sonicator. The samples were pulsed for 6 seconds and cooled for 1 minute. This was repeated twice for maximal liberation of poly(ADP-ribose) polymerase (Sudhakar et al., 1979). The standard assay involved the addition of sample (50 μg wet weight of cells) to a reaction mixture (50 mM Tris (pH 8.0) and 25 mM MgCl₂) with a final volume of 125 μl. The reaction mixture was incubated for 20 seconds at 25°C and the reaction initiated by the addition of 15 μl of [Adenine-2,8-3H]-NAD at a final concentration of 100 μM NAD (with a specific activity of 60 mCi/mMol). This provided NAD in excess. The reaction is proceeded for 45 seconds at 25°C and was then stopped by the addition of 2 ml of 70% TCA. The sample was placed on ice for at least 30 minutes, and the resulting precipitate collected on 0.45 μm pore size filters (Whatman, Hillsboro, Or.), washed with 10 ml of 20% TCA and then 20 ml of 95% ethanol, and dried; radioactivity was determined by liquid scintillation spectrometry (Sudhakar et al., 1979).

RESULTS

HD increased PADPRP activity in HeLa cells in all concentrations tested (0.1 μM to 100 μM) at 24 hrs after HD exposure (Fig. 1). A dose dependency was seen over the HD concentration range of 0.1 μM to 10 μM. On the other hand, at 100 μM the amount of increase dropped off but was still higher than controls. There was no HD-induced loss of viability over the concentration range used at 24 hr (Data not shown).
Since previous studies have shown NAD+ depletion within the first four hours after HD exposure (Smith et al. 1990, Meier et al. 1987), time studies over the 1- to 6-hr period post HD were conducted. At 10 μM HD elevated PADPRP activities were first seen at 3 hour with peak enzyme activity at 4 hours in HeLa cells (Fig. 2). The PADPRP activity at this dose began to decline after 4 hours.

**Figure 1.** Concentration response of PADPRP activity following HD exposure of HeLa cells in culture. Enzyme assays were conducted 24 hours post exposure.

When the effects of 100 μM PADPRP activity were assessed over the initial time periods after HD exposure maximum enzyme activation occurred at 1 hour (Fig. 2). This activity decreased to 60% of maximum response by the second hour and to 30-40% of maximum response at 4 and 6 hrs.

**Figure 2.** Time dependent response of PADPRP activity following HD exposure of HeLa cells. Two concentrations of HD were used, 10 and 100 μM, and PADPRP activities are presented as percent maximal response.

Figure 2 also shows that a second elevation of PADPRP activity develops after 9 hours post-exposure and is evident at 24 hours at the lower concentration.
DISCUSSION

Previous studies could not definitively establish that NAD+ depletion is due to PADPRP activation. Much of the previous work has involved the use of inhibitors of this enzyme to prevent NAD+ loss. This work has shown that exposure of HeLa cells to HD will activate PADPRP an enzyme that uses NAD+ as a substrate (Papirmeister et al. 1985). We have demonstrated time- and dose-dependent response of PADPRP to HD which correlates with NAD+ response to HD. The 100 μM exposure causes a rapid activation of PADPRP enzyme which occurs within the first hour. Measurement of NAD+ levels in a comparable cell culture systems (HeLa and human epidermal keratinocytes, HEK) have shown that NAD+ levels begin decreasing in 1-2 hours after exposure to HD. The observation that PADPRP activity plateaus after 4 hours is enhanced by studies in human keratinocytes that NAD+ depletion occurs over the period of 1-4 hours after HD exposure then plateaus through the 4- to 8-hour time period (Smith et al., 1990).

The observation that increased PADPRP activity is again seen at 24 hours post-HD would provide a basis for recent data suggesting that NAD+ levels undergo a second decline during the 8- to 24-hr time period (Martens, M.L. and Smith, W.J., unpublished observations). The nature of this second elevation in PADPRP activity and NAD+ decrease is unknown but is consistent with a central role of PADPRP activation in the metabolism disruptions seen following HD. Further characterization of this PADPRP activity may lead to a method for assessing cellular damage after HD exposure as well as to potential therapeutic interventions for the HD lesion.
REFERENCES


