JOURNAL OF APPLIED TOXICOLOGY
J. Appl. Toxicol. **20**, S125–S128 (2000)
DOI: 10.1002/jat.671

N-Acetylcysteine and Endothelial Cell Injury by Sulfur Mustard

Kevin B. Atkins,[1] Irfan J. Lodhi,[2] Lauren L. Hurley[2] and Daniel B. Hinshaw[1,2,*]
[1]University of Michigan Medical School, Ann Arbor, MI 48105, USA
[2]VA Medical Center, Ann Arbor, MI, USA

Key words: *N*-acetylcysteine; endothelial cell injury; glutathione; GSH; NFκB.

Understanding the underlying mechanisms of cell injury and death induced by the chemical warfare vesicant sulfur mustard (HD) will be extremely helpful in the development of effective countermeasures to this weapon of terror. We have found recently that HD induces both apoptosis and necrosis in endothelial cells (*Toxicol. Appl. Pharmacol.* 1996; 141: 568–583). Pretreatment of the endothelial cells for 20 h with the redox-active agent *N*-acetyl-L-cysteine (NAC) selectively prevented apoptotic death induced by HD. In this study, we tested the hypotheses that pretreatment with NAC acts through two different pathways to minimize endothelial injury by HD: NAC pretreatment acts via a glutathione (GSH)-dependent pathway; and NAC pretreatment acts to suppress HD-induced activation of the nuclear transcription factor NFκB. We used a fluorescence microscopic assay of apoptotic nuclear features to assess viability and electrophoretic mobility shift assays (EMSAs) to assess the activity of NFκB following exposure to HD. The cells were treated with 0–10 mM GSH for 1 h prior to and during exposure to 0 or 500 μM HD for 5–6 h. Cells were also treated with 50 mM NAC or 200 μM buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, alone or in combination overnight prior to exposure to 0 or 500 μM HD for 5–6 h. Externally applied GSH up to a concentration of 5 mM had no toxic effect on the cells. Mild toxicity was associated with 10 mM GSH alone. There was a dose-related enhancement of viability when 2.5 and 5 mM GSH were present during the HD exposure. Pretreatment with BSO alone had no discernible toxicity. However, pretreatment with this inhibitor of GSH synthesis potentiated the toxicity of HD. Pretreatment with 50 mM NAC, as previously reported, provided substantial protection. Combining pretreatment with both BSO and NAC eliminated the protective effect of NAC pretreatment alone on HD injury. These observations are highly suggestive that NAC enhances endothelial survival via GSH-dependent effects and confirms and extends the work of others with different models that externally supplied GSH alone may be a fairly effective countermeasure against HD injury of endothelium. We next examined the hypothesis that HD may activate the nuclear transcription factor NFκB by performing EMSAs with nuclear extracts of endothelial cells following exposure to 0, 250 or 500 μM HD. This demonstrated an up to 2.5-fold increase (scanning densitometry) in activation of NFκB binding to its consensus sequence induced by 500 μM HD after 5 h of HD exposure. Paradoxically, treatment of the endothelial cells alone with 50 mM NAC activated NFκB, although HD-induced activation of NFκB was partially suppressed by NAC at 5 h. Factor NFκB is an important transcription factor for a number of cytokine genes (e.g. tumor necrosis factor, TNF), which can be activated following stress in endothelial cells. Taken together, these observations suggest that the protective effects of NAC may be mediated by enhanced GSH synthesis. The increased GSH may act to scavenge HD and also prevent oxidative activation of NFκB. Under some conditions, NAC may act as an oxidizing agent and thus increase NFκB activity. The NFκB-dependent gene expression may be important in inducing endothelial cell death as well as in generating a local inflammatory reaction associated with the release of endothelial-derived cytokines. Copyright © 2000 John Wiley & Sons, Ltd.

INTRODUCTION

In recent years there has been a growing concern about the proliferation of chemical weapons and an increasing interest in the ability to combat them. One such agent, sulfur mustard (HD), is used as a vesicant. Sulfur mustard is both mutagenic and carcinogenic, most likely as a result of DNA damage due to alkylation.[1] Exposure to HD causes irritation, edema, vesication, ulceration and ultimately necrosis. These effects are dependent upon the dose and location of HD exposure.

Endothelial cells are one target of HD-mediated injury. Increased capillary leakage has been observed during HD injury.[2] We have also found that cultured vascular endothelial cell monolayers demonstrate increased permeability in response to HD.[3] This increased permeability may be the result of altered endothelial morphology. Vascular endothelial cells exposed to HD undergo microtubule depolymerization, disruption of long actin filament stress fibers, loss of

* Correspondence to: D. B. Hinshaw, University of Michigan Medical School, Ann Arbor, MI 48105, USA.

Received 21 October 1998
Revised 22 November 1999
Accepted 22 November 1999

adherence and ultimately apoptosis or necrosis.[4] *N*-Acetylcysteine (NAC) induces reorganization of endothelial stress fiber networks and prevents HD-induced cellular rounding, loss of adherence and apoptosis.[4]

N-Acetylcysteine is an antioxidant and redox-active agent reported to prevent apoptosis in lymphocytes, neurons and vascular endothelial cells.[5] In addition, this thiol-containing compound is a precursor for glutathione synthesis. Glutathione, a cysteine-containing tripeptide, plays an important role in the detoxification of xenobiotics and in the scavenging of reactive oxygen species and free radicals. It has been demonstrated that some of the major metabolic products of HD are glutathione conjugates[6] and that HD is excreted as a glutathione metabolite.[7] In rat lung tissue slices exposed to HD there was a depletion of intracellular glutathione.[8] Glutathione levels are reduced in neuroblastoma–glioma hybrids and human peripheral lymphocytes exposed to HD.[9,10]

Sulfur mustard exposure of the human keratinocyte cell line SVK-14 is accompanied by decreased viability.[11] Pretreatment with glutathione increased the survivability of SVK-14 cells exposed to HD. Pretreatment of cultured rat lung slices with cysteine esters, which raise cellular cysteine levels available for glutathione synthesis, is reported to be protective in HD injury.[12] Reciprocally, reduction of cellular glutathione prior to HD exposure sensitized cells to the cytotoxic effects of HD.[10] In these same experiments, pretreatment with NAC, which raised intracellular glutathione, was protective against HD-induced cytotoxicity. Therefore, it was of interest to determine in our model whether there is any relationship between the protective effects of NAC and glutathione levels. Furthermore, depletion of glutathione is associated with potentiation of tumor-necrosis-factor-mediated activation of the transcription factor NFκB in L6 myoblasts.[13] Because NAC has been demonstrated to inhibit the activation of NFκB, we investigated whether this transcription factor might be activated in response to HD injury.

METHODS AND MATERIALS

Cell culture

Bovine pulmonary artery endothelial cells (National Institute of Aging, Aging Cell Culture Repository, Camden, NJ) were grown in F12 (Ham) nutrient mixture supplemented with 10% fetal bovine serum and antibiotics (Gibco, Grand Island, NY) and maintained at 37°C under a 5% CO_2-humidified atmosphere. Cells were passaged using 0.05% trypsin and 0.02% EDTA (Gibco) and were at confluence for all experiments reported herein.

Cell viability

Confluent endothelial cells pretreated or not with glutathione-modifying agents were subsequently treated with or without 500 μM HD for 6 h. The medium was then removed and saved and the cells were harvested using trypsin/EDTA. The harvested cells and media were centrifuged and the resulting pellet resuspended. An aliquot was stained with a dye mixture (10 μM acridine orange and 10 μM ethidium bromide) and examined by fluorescence microscopy according to the criteria described previously.[4]

Glutathione assay

Endothelial cells treated with or without 500 μM HD in the presence or absence of pretreatment were harvested as described above. The cell pellet ($0.25–0.5 \times 10^6$ cells) was resuspended in 50 μl of 0.2% Triton-X100 containing 2.5% sulfosalicylic acid. The cells were vortexed and then centrifuged at 12000 rpm for 5 min. Then 25 μl of supernatant was transferred to a tube. Total cellular glutathione (GSH) was subsequently measured as described.[14]

Electrophoretic mobility shift assay

Confluent endothelial cells were treated with or without 50 mM NAC for at least 16 h. Cells were then treated with or without 250 μM HD or 500 μM HD for 2 or 5 h. Cells were harvested by scraping and the cells and media were pelleted. The pellet was processed for nuclear protein as described.[15] The nuclear extracts were normalized for protein content (Bio-Rad, Hercules, CA) and analyzed for binding to a radiolabeled consensus nuclear factor sequence (NFκB; 5′GGGGACTTTCCGCTGGGGACTTTCCAGGGGGACTTTCC 3′) as described.[16] Specific binding was determined utilizing a 100-fold excess unlabeled NFκB sequence.

Statistics

Data were analyzed by a two-tailed Student's *t*-test.

RESULTS

Effects of glutathione-altering conditions on endothelial cell viability following Sulfur mustard exposure

The agents buthionine sulfoximine (BSO) and NAC were used to alter the synthesis of GSH in endothelial cells prior to HD exposure. Pretreatment of the endothelial cells with 0.2 mM BSO (a specific inhibitor of γ-glutamyl cysteine synthetase) did not alter the viability of uninjured control endothelial cells (Table 1). However, GSH depletion with BSO pretreatment did potentiate the toxic effect of 500 μM HD on endothelial viability, which was largely demonstrated as an increase in the number of necrotic cells detected 6 h after HD exposure (Table 1). The increased viability of endothelial cells exposed to 500 μM HD that had been pretreated with 50 mM NAC was abrogated by concurrent pretreatment with BSO (Table 1).

When exogenous GSH (0–10 mM) was present during HD exposure, there was a dose-dependent increase in survival of endothelial cells exposed to 500 μM HD detected at 6 h, although with 10 mM GSH there was some associated toxicity detected, even in the control, which diminished some of the dose-related benefits seen with the lower concentrations of GSH (Table 2).

Table 1. Effects of buthionine sulfoximine (BSO) and *N*-acetylcysteine (NAC) on sulfur-mustard (HD) treated endothelial cells[a]

	Control			500 μM HD		
	% V	% A	% N	% V	% A	% N
Untreated	90.3 ± 3.1	3.6 ± 2.1	6.1 ± 2.8	50.4 ± 4.0	33.1 ± 5.0	16.3 ± 4.5
0.2 mM BSO	91.0 ± 3.0	2.7 ± 0.6	6.3 ± 3.1	35.7 ± 8.4	32.7 ± 10.3	31.7 ± 17.8
50 mM NAC	93.3 ± 3.6	2.5 ± 1.0	4.3 ± 2.6	69.3 ± 1.7	21.2 ± 4.3	9.5 ± 4.4
BSO + NAC	92.7 ± 4.0	2.0 ± 1.0	5.3 ± 3.2	51.7 ± 4.7	37.0 ± 5.7	11.3 ± 1.2

[a]Confluent endothelial cells were pretreated with or without 0.2 mM BSO for at least 6 h prior to treatment with or without 50 mM NAC for 16 h. The medium was changed, followed by treatment with or without 500 μM HD for 6 h. Cell viability was determined as described in Methods and Materials: V, viable; A, apoptotic; N, necrotic. Values reported as mean ± SD (n = 4).

Table 2. Effect of exogenous glutathione (GSH) on sulfur-mustard (HD)-induced injury in endothelial cells[a]

	Control			500 μM HD		
	% V	% A	% N	%V	%A	%N
GSH (mM)						
0	90.3 ± 3.1	3.6 ± 2.1	6.1 ± 2.8	50.4 ± 4.0	33.1 ± 5.0	16.3 ± 4.5
2.5	88.0 ± 0.0	5.0 ± 2.6	7.0 ± 2.6	72.7 ± 5.9	15.7 ± 5.5	11.7 ± 4.2
5.0	88.0 ± 5.5	4.8 ± 2.5	9.8 ± 5.6	75.4 ± 5.6	11.3 ± 1.3	11.3 ± 4.2
10.0	83.0 ± 4.0	4.0 ± 1.7	13.3 ± 3.0	66.7 ± 6.4	10.7 ± 3.2	23.0 ± 3.6

[a]Confluent endothelial cells were pretreated with 0–10 mM GSH for 1 h. The cells were then treated with or without 500 μM HD for 6 h without a medium change. Cell viability was determined as described in Methods and Materials: V, viable; A, apoptotic; N, necrotic. Values reported as mean + SD (n = 4).

Effects of glutathione-altering agents on total cellular glutathione levels in sulfur-mustard-injured endothelial cells

When intracellular GSH levels were measured, it was determined that NAC pretreatment alone was associated with an approximate 60% increase in GSH levels in uninjured endothelial cells (Fig. 1). Treatment with BSO alone or in combination with NAC in uninjured endothelial cells was associated with a substantial reduction of approximately 60% of total intracellular GSH levels. Total GSH levels following exposure to 500 μM HD were reduced significantly in endothelial cells (ca. 40%), but were substantially preserved in endothelial cells pretreated with 50 mM NAC prior to HD exposure (Fig. 1).

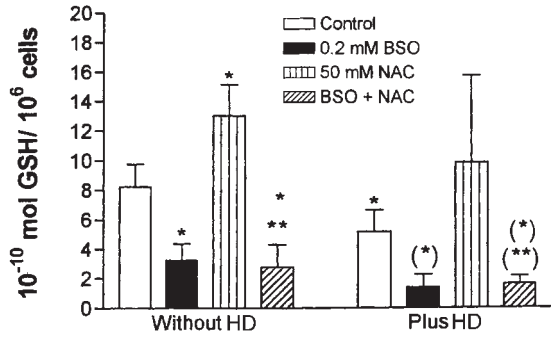

Figure 1. Effects of buthionine sulfoximine (BSO) and *N*-acetylcysteine (NAC) on glutathione (GSH) levels in endothelial cells. Cells were treated with or without 0.2 mM BSO for 6 h prior to additional treatment with or without 50 mM NAC for 16 h. The medium was changed and cells were treated with or without 500 μM HD for 6 h. Total cellular GSH was determined as described in Methods and Materials (n = 3): *$P \leq 0.02$ vs (−HD) control; **$P \leq 0.005$ vs (−HD) NAC; (*)$P \leq 0.02$ vs (+HD) control; (**)$P \leq 0.005$ vs (+HD) NAC.

Effect of Sulfur mustard on nuclear transcription factor NFκB activity in endothelial cells

Nuclear transcription factor NFκB activity was measured using electrophoretic mobility shift assays in experiments where endothelial cells were exposed to 250 or 500 μM HD and nuclear extracts were made at 2 or 5 h after exposure to the vesicant with or without prior treatment with 50 mM NAC. An increase in NFκB binding to its consensus sequence was detected in extracts taken from cells exposed to 500 μM HD as early as 2 h, with an increase seen at 5 h. The NAC treatment in combination with HD exposure was associated with variable effects on the NFκB activity induced by HD alone (Fig. 2). Not until 5 h after exposure to 500 μM HD was there any inhibitory effect (88% reduction in NFκB activity vs HD alone by scanning densitometry) of NAC treatment on NFκB activity (Fig. 2). Interestingly, NAC treatment alone was associated with a 52% increase in NFκB activity compared with the control (Fig. 2).

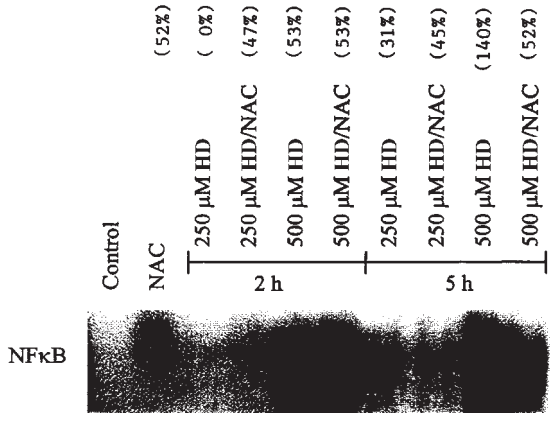

Figure 2. Electrophoretic mobility shift analysis of nuclear extracts of endothelial cells exposed to sulfur mustard (HD). Confluent endothelial cells were pretreated with or without 50 mM *N*-acetylcysteine (NAC) for at least 16 h. Cells were then treated with 0, 250 or 500 μM HD for 2 or 5 h without a medium change. Nuclear extracts were prepared and 5 μg of protein was incubated with a ^{32}P-labeled consensus NFκB oligonucleotide (30 000 cpm) for 20 min at room temperature. The binding reactions were then resolved by polyacrylamide gel electrophoresis and exposed to X-ray film at −70°C. Numbers in parentheses represent the percentage increase of each band compared with the control as determined by scanning densitometry.

DISCUSSION

In this study we have confirmed and extended the work of others demonstrating that exogenous GSH exerts marked protective effects in cellular injury secondary to HD exposure. The experiments with the GSH-altering agents, NAC and BSO, confirm that at least part of the beneficial effect of NAC on endothelial cell survival following HD injury is directly related to its stimulatory effect on GSH synthesis, because inhibition of the stimulatory effects of NAC on GSH synthesis by concurrent treatment with BSO abrogated any beneficial effects on endothelial cell viability and total GSH levels.

Our preliminary observations with electrophoretic mobility shift assays of nuclear extracts from endothelial cells following HD treatment suggest that HD can lead to activation of the nuclear transcription factor NFκB. Paradoxically, NAC alone activated NFκB but also demonstrated substantial inhibitory effects on NFκB activation in cells exposed to 500 μM HD later (5 h) in the time course of injury. Paradoxical effects of NAC in other biological systems in which oxidant generation was enhanced under some conditions by NAC are consistent with these observations.[17] Using a scopoletin assay,[18] we have found in a cell-free system that oxidation of scopoletin nearly doubles when 50 mM NAC is present (unpublished observations). Competing effects of NAC, such as oxidative activation of NFκB versus enhanced reducing capacity within the cell as a result of NAC-enhanced GSH synthesis, may account for these puzzling observations. Taken together, these observations suggest that agents that can stimulate GSH synthesis within potential target cells of HD injury may enhance cellular survival by increasing cellular levels of GSH, a potential scavenger of HD, as well as inhibiting activation of the nuclear transcription factor NFκB, which is an important regulator of the expression of a number of endothelial-cell-derived cytokines. These cytokines (e.g. tumor necrosis factor, TNF) play critical roles in amplifying inflammatory responses involving injured endothelium and may even play an important role in potentially acting as autocrine signals in the pathways governing programmed cell death. Redox-active compounds, which stimulate intracellular GSH synthesis and also limit activation of the nuclear transcription factor NFκB, may be particularly helpful as chemical countermeasures to the toxic effects of the vesicant HD.

Acknowledgements

This work was supported by the US Army Medical Research and Material Command under contract MIPR CVEM6785 and in part by the Department of Veterans Affairs. The views, opinions and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REFERENCES

1. Papirmeister B, Gross CL, Meier HL, Petrali JP, Johnson JB. *Fundam. Appl. Toxicol.* 1985; **5**: S134–S149.
2. Papirmeister B, Feister AJ, Robinson SI, Ford RD. *Medical Defense Against Mustard*. CRC Press: Boca Raton, FL, 1991.
3. Hinshaw DB, Lodhi IJ, Hurley LL, Atkins KB, Dabrowska MI. *Toxicol. Appl. Pharmacol.* 1999; **156**: 17–29.
4. Dabrowska MI, Becks LL, Lelli JL, Levee MG, Hinshaw DB. *Toxicol. Appl. Pharmacol.* 1996; **141**: 568–583.
5. Tsai J-C, Jain M, Hsieh C-M, Lee W-S, Yoshizuma M, Patterson C, Perrella MA, Cooke C, Wang H, Haber E, Schlegel R, Lee M-E. *J. Biol. Chem.* 1996; **271**: 3667–3670.
6. Black RM, Brewster K, Clarke Rj, Hambrook JL, Harrison JM, Howells DJ. *Xenobiotica* 1992; **4**: 405–418.
7. Davison C, Rozman RS, Smith PK. *Biochem. Pharmacol.* 1961; **7**: 64–74.
8. Langford AM, Hobbs MJ, Upshall DG, Blain PG, Williams FM. *Hum. Exp. Toxicol.* 1996; **15**: 619–624.
9. Ray R, Legere RhH, Majerus BJ, Petrali JP. *Toxicol. Appl. Pharmacol.* 1995; **131**: 44–52.
10. Gross CL, Innace JK, Hovatter RC, Meier HL, Smith WJ. *Cell Biol. Toxicol.* 1993; **9**: 259–267.
11. Smith CN, Lindsay CD, Upshall DG. *Hum. Exp. Toxicol.* 1997; **16**: 247–253.
12. Wilde PE, Upshall DG. *Hum. Exp. Toxicol.* 1994; **16**: 247–253.
13. Sen CK, Khanna S, Reznick AZ, Roy S, Packer L. *Biochem. Biophys. Res. Commun.* 1997; **237**: 645–649.
14. Griffith OW. *Anal. Biochem.* 1980; **106**: 207–212.
15. Scheiber E, Matthias P, Muller MM, Schaffner W. *Nucleic Acid Res* 1989; **17**: 6419.
16. Dignam JD, Lebovitz RM, Roeder RG. *Nucleic Acid Res.* 1983; **11**: 1475–1489.
17. Sprong CR, Winkelhuyzen-Janssen ML, Aarsman CJM, VanOirschot JFLM, Van der Bruggen T, Sweder van Asbeck B. *Am. J. Respir. Crit. Care Med.* 1998; **157**: 1283–1293.
18. DeLaHarpe J, Nathan CF. *J. Immunol. Methods* 1985; **78**: 323–336.

