Effects of diethyldithiocarbamate and endogenous polyamine content on cellular responses to hydrogen peroxide cytotoxicity

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In exponential-phase Chinese-hamster cells, 0.1 mM-diethyldithiocarbamate (DDC) afforded greater than 1 log survival protection to cultures treated before and during exposure to 1 mM-H₂O₂. Both DDC and H₂O₂ treatment stimulated the activity of ornithine decarboxylase (ODC), the first enzyme in polyamine synthesis, within 4 h of exposure. DDC, and to a lesser degree H₂O₂, also stimulated the activity of spermidine N¹-acetyltransferase (SAT), the rate-limiting enzyme in polyamine catabolism. The increase in SAT activity, after exposure to DDC or another stress (heat shock), was inhibited in cells depleted of putrescine and spermidine by α -difluoromethylornithine (DFMO), the enzyme-activated suicide inhibitor of ODC. Pretreatment with DFMO or heat shock also induced resistance to H₂O₂ cytotoxicity. Since SAT activity is low in resting cells, yet stimulation of enzyme activity depends on endogenous spermidine pools, these results suggest that the expression of SAT activity occurs by a mechanism involving a stress-dependent displacement of spermidine into a new intracellular compartment. The stimulation of ODC and SAT activities does not appear to be a necessary component of the mechanism by which DDC protects cells from H₂O₂ cytotoxicity, although spermidine displacement may be a common facet of the cellular response to stress.

INTRODUCTION

The investigation of chemical modifiers in cancer therapy reflects the ultimate objective of improving the therapeutic index via enhanced selectivity and efficacy of present treatment modalities. In recent years, a renewed interest in diethyldithiocarbamate (DDC), an *N*-substituted dithiocarbamate, has revealed its capacity to modify responses to radiation, hyperthermia and certain chemotherapeutic agents. Paradoxically, both sensitizing and protective effects have been observed in a variety of experimental systems. Enhancement of radiation [1], hyperthermia [1,2] and bleomycin [3] toxicity during DDC exposure has been well documented in mammalian cell cultures. On the other hand, protective effects of DDC have been repeatedly observed during whole-body irradiation in mice [4–7] with potential selective radioprotection of bone marrow [5].

Our laboratory is interested in the mechanisms by which physical and chemical agents (stresses) induce cytotoxicity, and we have found that the naturally occurring polycations, the polyamines, appear to modulate cytotoxic responses to a number of these agents. For example, depletion of putrescine (1,4-diaminobutane) and spermidine by α -difluoromethylornithine (DFMO), the enzyme-activated suicide inhibitor of ornithine decarboxylase (ODC), enhances cell killing by hyperthermia [8] and a number of cytotoxic drugs [9]. Thus, in addition to their well-documented role in specific processes

essential to normal cell growth and differentiation [9], the polyamines appear to be involved in cellular responses to a variety of exogenous stresses. We have recently observed that DDC induces polyamine oxidation by a mechanism involving stimulation of SAT [10]. We therefore investigated whether DDC could modulate the response of cells to an exogenous oxidative stress in the form of H₂O₂, and whether the polyamines were involved in the mechanism of H₂O₂-induced cell death.

MATERIALS AND METHODS

Cell culture procedures

Chinese-hamster ovary (CHO) cells were maintained in exponential growth as monolayer cultures in McCoy's 5A (modified) medium supplemented with 10% (v/v) fetal bovine serum at 37 °C. Cultures were heat-shocked in a temperature-controlled (± 0.1 °C) water bath by immersion of sealed flasks. Cell survival was assayed by using colony formation as an end-point, as reported previously [8].

Chemicals and chemical treatments

DDC was purchased from Sigma Chemical Co., H₂O₂ (30%, v/v) was obtained from Mallinckrodt, and DFMO was kindly given by Merrell Dow Research Institute (Cincinnati, OH, U.S.A.). These chemicals were diluted with triple-distilled water and sterilized by passage

Abbreviations used: DDC, diethyldithiocarbamate; DFMO, α -difluoromethylornithine; ODC, ornithine decarboxylase (EC 4.1.1.17); SAT, spermidine N¹-acetyltransferase.

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through a 0.2 μm -pore-size filter before addition to the culture medium. Drugs were removed from cell cultures by draining and rinsing twice with warm medium. In all experiments, DDC was added to culture medium 30 min before addition of H_2O_2 . L-[1- ^{14}C]Ornithine (60 mCi/mmol) and [1- ^{14}C]acetyl-CoA (45.1 mCi/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.).

Enzyme assays

ODC activity was measured as previously described [11]. Briefly, cell pellets were disrupted by sonication into ODC assay buffer, consisting of 0.05 M-sodium/potassium phosphate (pH 7.2), 0.1 mM-EDTA, 1.0 mM-dithiothreitol, 20 μM -pyridoxal phosphate and 100 μM -phenylmethanesulphonyl fluoride, at a concentration of 10^7 cells/ml. Enzyme activity was then assayed by measurement of $^{14}\text{CO}_2$ liberation from this lysate after centrifugation (5000 g for 5 min) during a 30 min interval at 37 $^\circ\text{C}$. One unit of ODC activity is defined as the liberation of 1 pmol of $^{14}\text{CO}_2$ /min.

SAT activity was determined as reported by Pegg and co-workers [12,13]. Cell pellets were resuspended in 50 mM-Tris (pH 7.5)/2.5 mM-dithiothreitol/0.1 mM-EDTA at a concentration of 4×10^7 cells/ml. Each assay contained 3 mM-spermidine, 50 mM-Tris, pH 7.8, and 8 μM -[1- ^{14}C]acetyl-CoA in a total volume of 100 μl . Activity was measured by the incorporation of ^{14}C into N^1 -acetylspermidine and expressed as units per 10^6 cells; one unit is defined as the production of 1 pmol of N^1 -acetylspermidine/min. Although only enzyme-activity data are presented here, we have reported actual product (N^1 -acetylspermidine) formation resulting from stress exposure elsewhere [14]. Further, we have confirmed that the stress-stimulated SAT activities are immunoreactive with polyclonal anti-SAT antibody (generously given by Dr. A. E. Pegg; results not shown).

RESULTS

Effects of DDC on H_2O_2 cytotoxicity

Addition of H_2O_2 to cell cultures resulted in cell killing that was dependent on the cell density (Table 1), H_2O_2 concentration, and time of treatment (Fig. 1). DDC was not toxic to these hamster cells when cultures were

Table 1. Cell-density dependence of H_2O_2 -induced cytotoxicity and the effect of heat shock on this response

The heat-shock treatment sequence was 45 $^\circ\text{C}$ heat shock for 10 min, 24 h incubation at 37 $^\circ\text{C}$, replating at indicated cell densities, followed 3 h later by 1 h treatment with H_2O_2 .

Cell density (cells/cm ²)	Cell survival (%) after exposure to H_2O_2 (0.3 mM)		Increase after heat shock (fold) (B/A)
	No heat shock (A)	Heat shock (B)	
1.2 ± 10^4	0.001 ± 0.001	0.040 ± 0.013	40
2.8 ± 10^4	0.020 ± 0.008	0.140 ± 0.007	7
8.0 ± 10^4	0.300 ± 0.114	1.220 ± 0.320	4

Fig. 1. Effects of DDC on H_2O_2 cytotoxicity

(a) Colony survival was assayed after 2 h exposures to increasing concentrations of H_2O_2 (●), or DDC plus H_2O_2 (○). (b) Survival was evaluated after exposure to 0.1 mM-DDC (■), 1.0 mM- H_2O_2 (●), or the drugs combined (○) for various times as indicated. Each data point represents mean $\pm$ S.E.M. for triplicate plates.

treated with concentrations up to 0.1 mM for times up to 3 h. Addition of 0.1 mM-DDC to cultures 30 min before H_2O_2 (1 mM) treatment resulted in substantial protection, greater than 1 log, against H_2O_2 -induced cell killing.

DDC and H_2O_2 modulate the activity of polyamine-metabolizing enzymes

The effects of DDC and H_2O_2 on enzymes of polyamine metabolism were studied by enzyme-activity assays for ODC and SAT, the rate-limiting enzymes for polyamine synthesis and catabolism respectively. Exponential-phase monolayer cultures were treated with 10 μM -DDC, 10 μM - H_2O_2 or the combination of these two agents for times up to 48 h. These lower concentrations, compared with those used in the experiments described in Fig. 1, were used because DDC or H_2O_2 concentrations higher than 10 μM became extremely toxic during long exposures, and we wished to compare equivalent concentrations of DDC and H_2O_2 . Cells exposed continuously to DDC expressed a greater than 10-fold increase in ODC activity at 6 h and a 7-fold increase in SAT activity at 24 h (Fig. 2), over those activities measured in the untreated controls. Continuous exposure to this low concentration of H_2O_2 yielded a more modest elevation of ODC activity over the time course measured, and SAT activity was only stimulated 2-fold over this same time period. The combination of DDC and H_2O_2 suggested an approximately additive effect of these agents on enzyme activity. These results demonstrate that low concentrations of DDC and H_2O_2 can stimulate polyamine-metabolizing enzymes.

Polyamine-dependent stimulation of SAT

Since Pegg has suggested that stimulation of SAT may involve the displacement of spermidine from its normal intracellular location(s) to other cellular compartment(s) [9], we examined the requirement for endogenous polyamines for the observed stimulation of SAT by DDC. Cultures were treated with 5 mM-DFMO for 48 h before DDC exposure to displace normal spermidine pools by depletion. This treatment decreased putrescine and

Fig. 2. Stimulation of polyamine-metabolizing enzymes by DDC and H₂O₂

The activity of ODC (a) and SAT (b) was measured during continuous exposure to 10 μM-H₂O₂ (□), 10 μM-DDC (○) or the drugs combined (■); ●, untreated controls.

Fig. 3. Polyamine dependence of SAT stimulation

SAT activity was measured during continuous exposure to 20 μM-DDC in control cultures (○) and in cultures depleted of endogenous polyamines by pretreatment with 5 mM-DFMO for 48 h (●).

spermidine to less than 10% of their normal values [8] and virtually eliminated the time-dependent stimulation of SAT observed in DDC-treated cultures with unperturbed polyamine contents (Fig. 3). Similarly, stimulation of SAT by other stresses, such as heat shock, was inhibited in polyamine-depleted cells (results not shown). Thus DDC and other stresses stimulate SAT activity by a polyamine-dependent mechanism. It is as yet unclear whether the increase in ODC activity by these agents also requires normal concentrations of endogenous putrescine and spermidine.

Modification of H₂O₂ cytotoxicity by DFMO

Since DDC protected cells from the toxic effects of H₂O₂, and stimulated SAT only in cultures with normal polyamine contents, it seemed possible that the mechanism of DDC-protection involved polyamine catab-

Fig. 4. Modification of H₂O₂ cytotoxicity by DFMO

Colony survival was evaluated in response to 1.0 mM-H₂O₂ in control cultures (●), in cultures depleted of endogenous polyamines with 5 mM-DFMO for 48 h (○), and in polyamine-depleted cultures treated with 0.1 mM-DDC 30 min before H₂O₂ challenge (■).

olism. In order to test this hypothesis, we treated cells with DFMO for 48 h to deplete putrescine and spermidine, thereby preventing stimulation of SAT (Fig. 3) and subsequent polyamine catabolism. Cultures were then challenged with H₂O₂ in the presence or absence of DDC. Surprisingly, DFMO treatment itself afforded survival protection to H₂O₂-treated cultures (Fig. 4). DDC treatment of polyamine-depleted cultures had no effect on the survival of these cells after H₂O₂ challenge, and was similar to the survival of cultures not treated with DFMO (Fig. 1). Therefore polyamine depletion by DFMO can also protect cells from H₂O₂ damage, but the mechanism of DDC protection does not appear dependent on SAT stimulation.

Heat-shock-induced resistance to H₂O₂ damage

The results so far indicate that DDC can protect cells from H₂O₂-induced cell death and stimulate ODC and SAT. Further, the mechanism of SAT stimulation by DDC is polyamine-dependent, endogenous polyamines influence cellular responses to H₂O₂, and the stimulation of SAT is not necessary for the protection against H₂O₂ damage. An explanation of these results is that polyamine displacement, resulting from either DDC or DFMO treatment, modulates cellular oxidative-stress responses. To test this hypothesis, we asked whether another agent which affects intracellular polyamines would render cells less sensitive to H₂O₂-induced cell killing. Heat shock is such an agent, as it affects intracellular polyamine contents [15] and induces SAT [14]. Cultures were either heat shocked or not, incubated for 24 h (a time at which we have determined that SAT activity remains elevated), replated at a range of densities and then challenged with H₂O₂. As shown in Table 1, heat shock protected these cells from H₂O₂ damage at all cell densities, even though killing was cell-density dependent.

DISCUSSION

DDC has been observed to induce either sensitization or protection toward the cytotoxic effects of a number of physical and chemical stresses [1–7]. Sensitization has been attributed, in part, to DDC's ability to chelate copper and thus inhibit copper-dependent anti-oxidant enzymes, such as superoxide dismutase [1,16]. Protection has often been ascribed to the ability of DDC to scavenge free radicals [5], and to exhibit a glutathione-like activity based on its potent capacity to reduce cytochrome *c* [17]. In the present study, we first asked whether DDC would protect or sensitize Chinese-hamster cells to H_2O_2 . In the range 10–100 μM , DDC decreased the cytotoxic potential of this form of oxidative stress. Second, in relation to our interest in the role of polyamines in growth and stress responses, we asked whether DDC affected polyamine metabolism. We found that DDC and H_2O_2 stimulated ODC and SAT, the rate-limiting enzymes in polyamine synthesis and catabolism respectively. We have confirmed these results by directly measuring product formation under these conditions (results not shown). After finding that SAT stimulation by DDC depended on intracellular putrescine and spermidine contents, we asked whether the mechanism by which DDC protected cells from H_2O_2 damage necessitated activation of SAT. The results presented in Fig. 4 suggested that this was not the case, but further implicated the polyamines in cell survival responses to H_2O_2 .

To summarize, we find that physical (heat shock) and chemical (DDC, DFMO) stresses can induce resistance to H_2O_2 -induced cell death. Each of these stresses affects polyamine metabolism, but in unique ways. DDC and heat shock stimulate polyamine synthesis and catabolism, whereas DFMO depletes endogenous putrescine and spermidine. Whether all three of these agents are acting to protect cells from H_2O_2 damage by the same mechanism is unclear from the data here. One common effect of DDC and heat shock relates to their capacity for stimulation of SAT. Pegg [9] has suggested that spermidine displacement is necessary for enzyme activation by exogenous toxic agents. DDC, heat shock and DFMO all appear to cause displacement of spermidine from normal intracellular sites. DFMO depletes endogenous polyamines, inhibits SAT activation, and thus can be viewed as displacing normal polyamine pools via their depletion. Heat shock and DDC appear to have more subtle effects on polyamine localization, as demonstrated by the fact that polyamine pool sizes are not substantially depleted after treatment [15]. Since SAT expression is minimal in resting cells, and is only stimulated by DDC or heat shock in cells with normal contents of polyamines, it follows that these stresses may be activating SAT by changing the usual intracellular location of these amines. More definitive experiments to test further this stress-induced spermidine-displacement hypothesis are required.

Precisely how polyamine displacement might affect cellular responses to oxidative stresses is unclear. The exact mechanism by which H_2O_2 inactivates cells is

unknown. Peroxide is known to induce DNA damage and generate lipid peroxidation [18], and has recently been reported to promote protein fragmentation [19] and inhibit ATP production [20]. Future studies investigating specific aspects of these processes will hopefully increase our understanding of the mechanisms by which chemicals such as DDC, and natural intracellular factors such as the polyamines, modulate cellular responses to oxidative stresses.

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