Dithiocarbamates Induce Apoptosis in Thymocytes by Raising the Intracellular Level of Redox-active Copper*

(Received for publication, June 22, 1995)

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Dithiocarbamates are metal-chelating compounds that can exert either pro-oxidant or antioxidant effects in different situations. They have recently been found to potently inhibit apoptotic cell death, an activity attributed to their antioxidant action. However, when thymocytes were exposed to pyrrolidine dithiocarbamate, an oxidation of the glutathione pool occurred within 90 min. Longer incubation resulted in cell shrinkage, chromatin fragmentation, glutathione depletion, and eventual cell lysis, which is typical of apoptosis in these cells. These changes were inhibited by inclusion of non-permeable metal chelators in the incubation medium, suggesting that pyrrolidine dithiocarbamate exerts its toxic effect by transporting a redox-active copper into the cell. This was directly confirmed when sustained 8-fold elevations of intracellular copper were detected after addition of pyrrolidine dithiocarbamate. In agreement with this, supplementation of the incubation medium with submicromolar concentrations of copper significantly potentiated pyrrolidine dithiocarbamate toxicity. We conclude that pyrrolidine dithiocarbamate exerts a powerful pro-oxidant effect on thymocytes due to its ability to transport external redox-active copper into cells. The resulting increase in glutathione disulfide may also explain the temporary anti-apoptotic activity of this compound described in other systems.

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Dithiocarbamates (DCs)¹ are known to exert pro-oxidant and antioxidant effects in both cell-free and biological systems. Their biological effects include widespread use as agricultural insecticides, herbicides, and fungicides with an estimated annual global consumption of 25,000–35,000 metric tons (1). They have been used clinically in the treatment of various pathogenic fungi and bacteria (2) and recently even in the experimental therapy of AIDS (3). Their diverse functions also include use in aversion therapy against chronic alcoholism (disulfiram/Antabuse™) and as an antidote against nickel and copper poisoning (4). Many of the biological effects of DCs are based on their metal-chelating properties, and they have also been used for many years in analytical methods for determination of heavy metals, especially in organic samples due to their relatively lipophilic nature (2). In addition to binding metals, the free thiol groups of DCs can also react with sulfhydryl groups on other molecules. DCs have thus been reported to inhibit enzymes by covalent interaction with free protein thiol (2, 5) as well as to oxidize glutathione through a glutathione peroxidase-like activity (6, 7). DCs may also interfere with cellular detoxication mechanisms as they are reported to suppress hepatic microsomal drug metabolism (8) and to inhibit glutathione S-transferases (9). In addition, the diethyl-dithiocarbamate (DDC) derivative has been found to inhibit copper/zinc superoxide dismutase activity by withdrawal of essential metal from the enzyme (10, 11) and to deplete intracellular glutathione in a non-superoxide dismutase-dependent manner (12, 13).

A problem in evaluating the biochemical action of many DCs is that they are unstable, decomposing via two different pathways to form biologically active metabolites. Under acidic conditions, the decomposition of DCs to their corresponding amine and CS₂ is favored (14). CS₂ mediates protein cross-linking and is proposed to be an important molecule behind DDC-induced toxicity (15). Second, DCs can be metabolized by cytochrome P-450 to a S-methyl sulfoxide derivative, which in turn is a potent inhibitor of enzymes such as aldehyde dehydrogenase. Disulfiram is believed to exert its effects after this type of P-450-dependent metabolism (16, 17). The stability of DCs can be increased by modifying the aliphatic substitutions on the nitrogen atom. An example is pyrrolidine dithiocarbamate (PDTC), a much more stable analog of DDC (14).

DCs have recently found use in cell and molecular biology as antioxidants. Cell-free antioxidant effects include inhibition of lipid peroxidation in both liposomes and liver microsomes (18–20), while it has been widely assumed that similar effects occur when DCs are applied to intact cells. For example, PDTC potently inhibits oxidative activation of the transcription factor NFκB (21–23), while others report that the same compound inhibits apoptosis in thymocytes (24), leukemic cells (25), and in L929 fibroblasts (26). Reactive oxygen species have been proposed to be common mediators of apoptosis in many different cell systems (27), while antioxidants are often observed to exert an inhibitory effect on this type of cell death (24, 28). The widespread use of DCs in medicine and industry and its cytotoxic action in many cells, combined with reports of both pro-oxidant and antioxidant effects, led us to investigate whether a general antioxidant activity was indeed responsible for its ability to interfere with the apoptotic process. We now report that PDTC exerts a toxic pro-oxidant effect in thymocytes, inducing apoptotic cell shrinkage and chromatin fragmentation prior to cell lysis. This toxicity is dependent on the ability of the compound to transport external copper into the cells and thereby generate an intracellular oxidative stress.

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¹ This project was supported by grants from the Swedish Medical Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: DC, dithiocarbamate; DDC, diethyldithiocarbamate; PDTC, pyrrolidine dithiocarbamate; BPS, bathophenanthroline disulfonic acid; NFκB, nuclear transcription factor κB; HPLC, high pressure liquid chromatography; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; kb, kilobase(s); GSSG, glutathione disulfide.
Copper-dependent Induction of Apoptosis by Dithiocarbamates

**TABLE I**

Electrothermal atomization programs for copper and iron determination

| Set up | Temperature °C | Copper | Iron | Ramp time s | Copper | Iron | Hold time s |
|--------|---------------|--------|------|-------------|--------|------|-------------|
| Drying | 100           | 100    | 100  | 5           | 5      | 5    | 25          |
| Drying | 200           | 200    |      | 5           | 5      |      | 10          |
| Charring | 1200        | 1400   |      | 6           | 6      |      | 19          |
| Cleaning | 2450        | 2550   |      | 1           | 1      |      | 2           |
| Cooling | 2600         | 2700   |      | 1           | 1      |      | 3           |
| Cleaning | 20           | 20     |      | 1           | 1      |      | 1           |
| Cooling | 20           | 20     |      | 1           | 1      |      | 1           |

*The magnet and recorder started 23 s (copper) or 18 s (iron) after the start of this step and was kept on throughout the second cooling step.

**EXPERIMENTAL PROCEDURES**

**Materials**—PDTC, DDC, 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline disulfonic acid (bathocuproine disulfonic acid or BCP), and 1,7-phenanthrene were obtained from Aldrich. Molecular biology grade of the sodium salt and free acid of Hepes (buffer in the sample preparation for GFAAS), etoposide, 6-diphenyl-1,10-phenanthroline disulfonic acid (bathophenanthroline), diphenylamine, dansyl chloride, N-lauroyl sarcosine, and Pulse marker™ (0.1–200 kb, 225-1000 kb) were bought from Sigma. 

**Preparation of thymocytes for culture** was made as described (29). For those experiments, cells were incubated at a density of \( 10^6 \) cells/ml in a CO2 ventilated box, and the thymic glands were excised. Sprague-Dawley rats from B&K Universal (Sollentuna, Sweden) were sacrificed in a CO2 ventilated box, and the thymic gland swere excised.

**Precautions** were made to avoid contamination by copper and iron according to Zhang et al. (31) but using a modified atomization program (Table I). Stabilized temperature platform furnace technique was utilized; however, for iron an internal gas flow (99.9% argon) was 0 ml/min for copper and 100 ml/min for iron during this and the following step.

**RESULTS**

PDTC has been described as a potent inhibitor of thymocyte apoptosis (24, 25), and in confirmation of this we have found that it inhibits DNA fragmentation in thymocytes exposed to etoposide, glucocorticoids, or thapsigargin (IC50 approximately 10–20 \( \mu M \) PDTC in each case; data not shown). As DCS are reported to protect against oxygen toxicity in vivo (35, 36) and to possess antioxidant properties in vitro (18–20), and antioxidants in general are known to delay apoptosis in many model systems, it has been assumed that the metal-chelating and/or radical-scavenging properties of PDTC mediate its anti-apoptotic activity.

We have previously shown that a decrease of both intracellular GSH and reduced protein thiol is an early event in the apoptotic process of thymocytes that can be prevented by incubation of the cells with various antioxidants (28). However, when the GSH levels of thymocytes induced to undergo apoptosis in the presence of PDTC were measured, the dithiocarbamate was observed to inhibit DNA fragmentation without reversing GSH depletion (Fig. 1A). PDTC treatment alone was actually seen to oxidize the intracellular glutathione pool; GSSG content was elevated 1.5, 3, and 6 h after treatment, while GSH levels were progressively reduced by 6 h and thereafter (Fig. 1B). These results demonstrate that low concentrations of PDTC exert a pro-apoptotic effect on intracellular GSH/GSSG redox in thymocytes, and therefore a general antioxidant effect cannot explain the anti-apoptotic activity of this compound.

The observation that PDTC exerts a pro-apoptotic activity on the glutathione pool of thymocytes suggested that it could be toxic during longer incubations. Indeed, cell membrane permeability (measured with trypan blue) was markedly reduced when thymocytes were incubated overnight with different doses of PDTC (Fig. 2A). Even 4 \( \mu M \) PDTC was significantly toxic to the cells, and at 40 \( \mu M \) PDTC the cell viability fell below 50%. Lysis of the cells was accompanied by an increase in low molecular weight DNA fragmentation (Fig. 2B), although DNA damage was already evident at 1 \( \mu M \) PDTC before any loss in cell viability could be detected. To further investigate the mech-
anism of this cell death, a time-course study of the effect of
PDTC on chromatin degradation and cell volume was under-
taken (Fig. 3, A and B). 20 μM PDTC was found to give a
time-dependent induction of DNA fragmentation, first evident
after 6 h and increasing thereafter, while significant reduc-
tions in cell volume occurred with similar kinetics. When pulsed field
gel electrophoresis on DNA samples from the same experiment
was performed, 50-kb DNA fragments were present by 6 h (Fig.
3C). Formation of 200- and 50-kb DNA fragments was concen-
tration dependent, increasing from 1 to 40 μM PDTC (8-h in-
cubation, Fig. 3D). As cell membrane permeability was not
affected by PDTC at this time (Fig. 2 A), these data indicate
that the DC induces an apoptotic response (characterized by
chromatin fragmentation and cell shrinkage) prior to any sec-
ondary lysis of the cells. In parallel experiments, DDC (40 μM)
also gave a significant enhancement of thymocyte DNA frag-
mentation at 6 h (data not shown), indicating that these effects
are specific for the shared dithiocarbamate structure.

By virtue of its strong metal-chelating properties and high
membrane permeability (2), DDC has been reported to trans-
port metal ions across cell membranes (37). The ability of a
series of phenanthroline metal chelators to interfere with
PDTC-induced apoptosis of thymocytes was therefore tested.

Both the membrane-permeable copper-specific chelator neocu-
proine and its non-permeable analog BCPS were observed to
inhibit the DNA fragmentation that occurred after thymocytes
were exposed to PDTC (Table II). BCPS protection was dose
dependent (data not shown) and was gradually overcome when
PDTC concentrations were raised above 10 μM (Fig. 4). BPS
was also active as an inhibitor of PDTC toxicity (Table II).
As this non-permeable chelator is thought to be an iron-specific
reagent, these experiments cannot identify the metal ion in-
volved, while the lack of effect observed with the nonchelating
1,7-phenanthroline demonstrates that it is the metal-chelating
properties of these compounds that interfere with PDTC toxic-
ity (Table II). Both BCPS and BPS were completely inactive as
inhibitors of etoposide-induced DNA fragmentation, while as
reported previously, neocuproine exerted a weak protective
effect (Table II and Ref. 24).

The above results suggest that a PDTC-dependent transport
of external redox-active metal(s) into thymocytes was respon-
sible for its toxicity. GFAS was therefore employed to identify
the metal involved (Table III). The intracellular copper content
of thymocytes was raised 8-fold after a 1.5-h incubation with 10
μM PDTC. The copper content of PDTC-treated thymocytes
remained high at all later time points but was almost com-
Fig. 1. PDTC inhibition of etoposide-induced DNA fragmentation correlates with an oxidation and depletion of intracellular GSH.
Thymocytes were incubated at 37 °C, and intracellular levels of GSH were determined at various time points. A, cells were untreated (○) or
cultured with 25 μM etoposide (●) or 25 μM etoposide plus 20 μM PDTC (◆). DNA fragmentation was determined in the same samples using
diphenylamine. All results are mean ± S.D. (n = 3). B, average GSH (●) and GSSG (◆) levels (standardized against the levels in untreated cells)
of cells treated with 20 μM PDTC are shown after different times of incubation.

Fig. 2. PDTC induces a concentration-dependent cell lysis and DNA fragmentation. Thymocytes were incubated at 37 °C for 8 h (●) or
24 h (◆) with different doses of PDTC; then, cell viability was assayed by the trypan blue exclusion method (2–300 cells counted per sample) (A),
and the amount of fragmented DNA was determined using the diphenylamine assay (mean ± S.D., n = 3) (B).
completely abrogated if the cells were co-incubated with 50 μM BCPS. In this experiment, untreated cells showed a transient 2-fold increase of copper at 1.5 h, but levels at time points thereafter returned to the baseline. No increase in intracellular iron was detected after any of the treatments (Table III). To verify that this increase of intracellular copper mediated the oxidation observed during PDTC exposure, the intracellular levels of GSH and GSSG were determined in a similar experiment. GSSG levels were significantly elevated 1.5 h after adding PDTC, while co-incubation of the cells with the external copper chelator BCPS blocked this oxidation of the glutathione pool (Fig. 5).

These experiments indicate that PDTC transports redox-active external copper across the thymocyte cell membrane, thereby generating an oxidative stress within the cell that in turn induces apoptosis. Copper was not added to the media in

Fig. 3. Time- and dose-dependent induction of thymocyte apoptosis by PDTC. Thymocytes were incubated at 37 °C for different times as indicated, and then the amount of fragmented DNA was determined (mean ± S.D., n = 3) (A) or the mean cell volume was estimated using a Coulter counter (approximately 5000 cells counted per sample) (B). Cells were untreated (●) or cultured with 20 μM PDTC (●). C, samples were also processed for field-inversion gel electrophoresis analysis (see "Experimental Procedures"). Gels were stained with 0.5 μg/ml ethidium bromide for 1 h and visualized with a UV lamp. DNA equivalent to approximately 0.5 × 10⁶ cells was loaded in each lane, and the sizes were estimated with Pulse-marker™. D, samples taken from the experiment described in Fig. 2 were analyzed by field-inversion gel electrophoresis as described above. Cells were treated with PDTC (shown in lanes 1–6 in the order of 0, 1, 4, 10, 20, 40 μM PDTC) for 8 h at 37 °C.

**Table II**

Phenanthroline metal chelators inhibit PDTC-induced DNA fragmentation

| Conditions          | DNA fragmentation |
|---------------------|-------------------|
|                     | 24-h control      | 10 μM PDTC 24 h | 8-h control | 25 μM etoposide 8 h | %     |
| Untreated           | 25.4 ± 1.3        | 52.0 ± 0.7      | 19.8 ± 0.7 | 76.3 ± 1.4         |       |
| 1,7-Phenanthroline  | 25.6 ± 2.9        | 51.0 ± 0.8      | 21.2 ± 0.7 | 71.6 ± 1.8         |       |
| Neocuproine         | 36.0 ± 0.5        | 31.4 ± 0.5      | 18.3 ± 1.3 | 53.9 ± 1.7         |       |
| BPS                 | 24.9 ± 1.3        | 27.8 ± 1.8      | 23.2 ± 2.6 | 73.6 ± 1.4         |       |
| BCPS                | 26.7 ± 2.8        | 27.4 ± 1.4      | 21.2 ± 0.7 | 71.6 ± 1.8         |       |
any of these experiments, and therefore trace amounts of the metal (probably in the fetal calf serum supplement) are apparently sufficient for this process to occur. This suggested that supplementation of the media with external copper would potentiate PDTC toxicity. Consistent with this, in the presence of suboptimal 0.1 or 1 μM PDTC, addition of CuSO₄ to the culture media substantially increased DNA fragmentation and cell lysis. When the CuSO₄ concentration was raised above 0.4 μM, addition of 1 μM PDTC promoted necrotic rather than apoptotic cell death, reflected in reduced DNA fragmentation, and increased cell lysis (Fig. 6). Supplementation of the media with up to 1.6 μM CuSO₄ alone was not toxic to thymocytes in overnight incubation (Fig. 6).

**DISCUSSION**

Our results demonstrate that transport of extracellular copper from the media into cells is required for PDTC to induce apoptosis in thymocytes. The calf sera supplement is the most probable source of external copper in our experiments, as sera normally contain from 0.3–0.6 ppm of copper in mice (38) to 1 ppm of copper in humans (39). Elevated serum copper levels correlate with the progress of some lymphocyte-associated diseases such as AIDS (39), lymphomas (40), and also in age-related immune deficiency (38). We show here that increased copper levels in thymocytes generates an oxidative stress as measured by an increase in intracellular GSGS. The oxidative stress in turn is cytotoxic, inducing apoptosis at lower PDTC concentrations and necrotic lysis when the stress is enhanced (Fig. 2). Other oxidants including menadione and diamide have been reported to cause a similar dose-dependent cytotoxicity, where low amounts of oxidant induce apoptosis, whereas higher concentrations give rise to necrosis (41, 42). Copper is known to redox cycle and thereby generate reactive oxygen species such as hydroxyl radical (OH⁻) (43). Interestingly, the chelating agent 1,10-phenanthroline has been shown to enhance copper-dependent production of OH⁻ (44). As PDTC has a log β₂ of 10.9 for Cu²⁺ (45) and has not been reported to bind Cu⁺, it is likely the former that PDTC transports into cells. This Cu²⁺-PDTC complex may be reduced by endogenous reductants (GSH or ascorbic acid) and then redox cycle in the presence of oxygen. The resulting formation of reactive oxygen species would oxidize glutathione, probably in a very efficient way as DDC is reported to have a glutathione peroxidase-like activity (6, 7). Ultimately, the GSH pool of the cells would be depleted, consistent with observations in other systems (12, 13).

Since DDC has been reported to inhibit copper/zinc superoxide dismutase (10, 11), this provides another potential mechanism by which DCs can mediate an oxidative stress. However, this is unlikely to be the case in this study as millimolar concentrations of DDC are required to inhibit superoxide dismutase (13). The fact that we detected an increase in intracellular level of copper after treatment with PDTC (Table III) also strongly suggests that inhibition of superoxide dismutase is not relevant because such inhibition would be reduced, not potentiated, by addition of copper. The observation of Mohindru et al. (46) that extracellular copper potentiates a DDC-induced ar-
rest of cell proliferation further supports our findings that a direct copper-dependent oxidative stress is responsible for the cytotoxic effects of PDTC.

Bioactivation of DDC to a S-methyl sulfoxide derivative is known to be catalyzed by cytochrome P-450 (16, 17). In an attempt to determine whether the catabolism of PDTC was required to mediate its toxic effect, thymocytes were co-incubated with PDTC and metyrapone (a nonspecific cytochrome P-450 inhibitor). However, no effect on PDTC toxicity was seen (data not shown), indicating that cytochrome P-450-dependent bioactivation is not involved in the toxic effect of PDTC in our system. DDC can spontaneously decompose to diethylenamine and CS₂, whereas CS₂ has well documented toxicity (47). This type of reaction does not seem to be crucial in our system since the transport of copper is necessary for PDTC to mediate its toxic action, although the decomposition of PDTC inside cells may facilitate the intracellular release of chelated copper and thereby enhance the potential for oxidative damage to occur.

As mentioned above, PDTC has a completely different biological effect in a shorter time scale as it (a) prevents both thymocyte and leukemic cell apoptosis induced by different agents (24, 25) and (b) inhibits the activation of NF-kB and enhances the DNA binding of AP-1. Since these transcription factors are redox-regulated, and many apoptotic cells experience an oxidative stress, both of these effects have been assumed to be due to a general metal-chelating and/or radical-scavenging property of PDTC (21–24, 26). However, as PDTC is active at micromolar concentrations in the presence of millimolar intracellular GSH, it is unlikely that it exerts a significant effect by directly scavenging radicals. We find that it actually oxidizes GSH to GSSG within 1.5 h of treatment, i.e. in the same time period when it exerts an effect on transcription factor activity and apoptosis. Correlation between an increase of GSSG and inhibition of NF-kB activation by PDTC has been observed before (48), which makes it tempting to speculate that a GSSG increase mediates many of the inhibitory/regulatory effects of this compound. Preliminary experiments show that we can block the inhibitory action of PDTC on etoposide apoptosis by adding the external copper chelator BCPs, indirectly supporting the hypothesis that GSSG is involved in its antiapoptotic activity. GSSG is known to be an important mediator of protein function, for example in the regulation of NF-kB activation and DNA binding (49). It is therefore possible that the pro-oxidant effect of PDTC is responsible both for the initial prevention and subsequent induction of apoptosis. Similar phenomena involving oxidative stress have been observed before; for example, increasing the concentration of a redox-cycling quinone shifts the cellular response from proliferation to apoptosis (41). We are currently investigating the possibility that PDTC regulatory effects on apoptosis involves GSSG modulation of crucial enzymes/proteins in the machinery of apoptosis.

Acknowledgments—Kerstin Ström is acknowledged for assistance with the setup of the HPLC system, Bo Nilsson for help with the GFAAS measurements, and Elisabeth Wakeman for secretarial assistance.

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