Diphenyleneiodonium (DPI) is a broad-spectrum flavoprotein inhibitor commonly used to inhibit oxidant production by the NADPH oxidase of phagocytic and nonphagocytic cells. A previous study has shown that DPI can sensitize T24 bladder carcinoma cells to Fas-mediated apoptosis. We observed DPI to deplete intracellular reduced glutathione (GSH) in T24 cells and a range of other primary and transformed cell types. The effect was immediate, with 50% loss of intracellular GSH within 2 h of treatment with DPI. The glutathione was quantitatively recovered in the extracellular medium, indicating that efflux was occurring. The loss of GSH was blocked with bromosulfophthalein, an inhibitor of the canalicular GSH transporters. We conclude that DPI induces a dramatic efflux of cellular GSH from T24 cells via a specific transport channel. This provides a potential mechanism for its proapoptotic effect, and it also has important implications for the regulation of glutathione homeostasis in cells.

Diphenyleneiodonium (DPI) is a flavoprotein inhibitor whose targets include the phagocyte NADPH oxidase, nitric oxide synthase, xanthine oxidase, cytochrome P450 reductase, and NADH:ubiquinone oxidoreductase (1−5). Electron transport through the flavin moieties of these complexes causes reduction of DPI to its radical form, followed by irreversible phenylation of either the flavin or adjacent amino acid and heme groups (6, 7). Despite its nonspecific mode of action, DPI has been used extensively in recent years to block NADPH oxidase activity in nonphagocytic cells, where the resultant oxidase can sensitize T24 bladder carcinoma cells to Fas-mediated apoptosis. We measured GSH and GSSG levels before and after treatment with DPI and anti-Fas antibody to determine if the switch in sensitivity to Fas is associated with changes in the glutathione redox couple in T24 cells. To our surprise, we found that DPI triggered rapid GSH efflux, with >50% loss over 2 h. This provides a potential mechanism for its proapoptotic effect, and it also has important implications for the regulation of glutathione homeostasis in cells.

EXPERIMENTAL PROCEDURES

Reagents—Human anti-Fas IgM (clone CH-11) was purchased from Upstate Biotechnology (Lake Placid, NY). The glutathione transport inhibitor dibromosulfophthalein (DBrS) was purchased from Société d’Études et de Recherches Biologiques (Paris, France), and the caspase substrate Ac-Asp-Glu-Val-Asp (DEVD-AMC) was purchased from Peptide Institute, Inc. (Osaka, Japan). L-Methionine, dansyl chloride, DPI, and bromosulfophthalein (BSP) were from Sigma Chemical Co. Cell culture materials were from Invitrogen New Zealand Ltd.

Cell Culture—The T24 bladder carcinoma and Jurkat T lymphocyte cell lines were obtained from American Type Culture Collection (Manassas, VA). T24 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 µM glutamine. Jurkat T cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 55 µM 2-mercaptoethanol, 100 units/ml penicillin, and 100 µg/ml streptomycin. Fas expression on Jurkat T cells was confirmed by flow cytometry using anti-Fas IgM (clone CH-11) followed by FITC-conjugated goat anti-mouse IgM. Cells were harvested by centrifugation and washed twice with PBS before use in apoptosis assays.

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kat cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human umbilical vein endothelial cells were isolated from umbilical cords by collagenase digestion (33) and cultured in Medium 199 supplemented with 20% fetal bovine serum, 100 μg/ml heparin, 30 μg/ml endothelial cell growth factor, 25 units/ml penicillin, and 25 μg/ml streptomycin on fibronectin-cultured plates (34). Fibroblasts were isolated from human infant foreskins and cultured in minimum essential medium containing 15% fetal bovine serum, 2 mM glutamine, 25 units/ml penicillin, and 25 μg/ml streptomycin as described previously (35). All cell types were maintained at 37°C in a humidified atmosphere with 5% CO₂.

RESULTS

DPI Sensitizes T24 Cells to Fas-mediated Apoptosis—T24 cells are one of the many cell lines known to be resistant to Fas-induced apoptosis, despite surface expression of the receptor. As shown previously (9), pretreatment of T24 cells with the flavoprotein inhibitor DPI sensitized these cells to anti-Fas antibody (Fig. 1). Whereas the antibody or DPI alone had little effect on the cells, characteristic apoptotic changes were detectable within 6 h of a combination treatment. Cells were detachment from the plate, and extensive membrane blebbing was evident. Caspase-3-like activity was increased 6-fold above control levels, indicative of apoptosis (Fig. 1). By 24 h, >60% of these cells were unable to exclude trypan blue (Fig. 1). DPI was equally effective at causing cell death when added concurrently or 2 h after the addition of anti-Fas antibody and also when it was present for 1 h and then washed away before antibody addition (Fig. 1).

Effect of DPI on Intracellular GSH Levels—To determine whether DPI affected the redox balance of the cells, we measured intracellular GSH and GSSG levels using the dansyl chloride HPLC assay. To our surprise, we observed a rapid depletion of intracellular GSH in the DPI-treated T24 cells (Fig. 2). The loss was apparent within 30 min of adding 10 μM DPI. After 2 h of treatment, GSH levels had dropped to 47 ± 10% (S.D.; n = 9) of the control value, and after 4 h only 29 ± 6% (S.D.; n = 5) of the GSH remained in the cells (Fig. 3). Concurrent with the loss in cellular GSH, GSSG levels also dropped after incubation with DPI (Fig. 2, inset). Two h of treatment decreased T24 GSSG levels to 42 ± 12% (S.D.; n = 11) of control, and 4 h of treatment with DPI decreased T24 GSSG levels to 27 ± 12% (S.D.; n = 5). Whereas the direct GSSG/GSH ratio remained constant, GSH levels are squared when calculating the glutathione redox couple; therefore, we would predict a more oxidizing intracellular environment. Determination of the absolute reduction potential is dependent on the cell volume, but by substituting the percentage losses in GSH and GSSG into the Nernst equation (38), we calculated that DPI would cause a loss in reduction potential of 9 mV at 2 h and 16 mV at 4 h.²

The anti-Fas antibody itself had a minimal effect on the GSH levels (Fig. 3). When DPI was present for 1 h and then washed away, and the cells were incubated for an additional hour in fresh media, GSH levels were almost identical to those in cells that had been exposed to DPI for the entire 2-h period (Fig. 3), indicating that GSH continued to decrease after DPI removal. This correlates with the ability of DPI pretreatment and removal to sensitize the cells to apoptosis (Fig. 1). Treatment with increasing concentrations of DPI caused a dose-dependent loss in cellular GSH, giving an IC₅₀ of ~3 μM (Fig. 4). This compares with the 0.5 μM DPI observed for inhibition of super-
were transferred to fresh media for 1 h and then incubated with DPI (10 µM) and anti-Fas antibody (0.25 µg/ml) for up to 4 h as indicated (control, ○, DPI, ■, Fas, □). DPI and Fas added concurrently, ○ and 1 h with DPI, followed by 1 h in fresh media (△). GSH levels are expressed in nanomoles/well, with each 15-mm well containing ~140,000 cells and 40 µg of protein, and are duplicate determinations from one of three representative experiments.

 oxide production by the neutrophil NADPH oxidase (39).

To investigate whether the DPI-induced GSH loss is specific to T24 cells, we measured GSH levels in Jurkat T lymphocytes and in primary cultures of endothelial cells and fibroblasts after exposure to DPI. All cell types suffered a progressive loss in intracellular GSH after DPI treatment, with untreated cells either maintaining or increasing their GSH levels over the 4-h period (Fig. 5). Thus, DPI induces a rapid depletion of the glutathione pool in both primary and transformed cells, suggesting a common target in these cell types.

Mechanism of GSH Loss in DPI-treated Cells—We collected the extracellular medium of DPI-treated T24 cells to determine whether glutathione had been exported from the cells. A marked increase in the area of a new peak with a retention time of ~7 min was observed (Fig. 6A). Due to a large excess of cystine in the medium, any GSH present extracellularly is likely to react via thiol-disulfide exchange to form a GSH-cysteine mixed disulfide (40). To confirm that the unknown peak was the mixed disulfide, the products of the reaction between GSH and cystine were also analyzed by HPLC. The major peak of this reaction eluted at the same time as the unknown peak, and when the extracellular medium was incubated with DTT before the derivatization procedure, the disulfide peak disappeared, and GSH was detected (data not shown). Quantification of extracellular levels after reduction with DTT showed that almost all of the GSH lost from the

FIG. 3. Time course of GSH loss with DPI treatment. T24 cells were transferred to fresh media for 1 h and then incubated with DPI (0–20 µM) for 2 h. GSH levels are expressed as nanomoles/well and are duplicate determinations.

FIG. 4. Concentration curve of GSH loss with DPI treatment. T24 cells were transferred to fresh media for 1 h and then incubated with DPI (0–20 µM) for 2 h. GSH levels are expressed as nanomoles/well and are duplicate determinations.

FIG. 5. DPI decreases intracellular GSH in a variety of cell types. Human umbilical vein endothelial cells (●, ○), fibroblasts (■, □), and Jurkat T lymphocytes (▲, △) were incubated in fresh media for 1 h, and then 10 µM DPI was added or the cells were left untreated for up to 4 h. Cells were derivatized with dansyl chloride and separated by HPLC. Untreated cells, filled symbols; cells exposed to DPI, open symbols. Results are expressed as a percentage of control and are the mean ± range of a representative experiment performed in duplicate. Human umbilical vein endothelial cells and fibroblasts contained ~3.38 ± 0.01 and 1.39 ± 0.06 nmol GSH/well, respectively, with 1 × 10⁶ Jurkat T cells containing 2.89 ± 0.04 nmol GSH/well. This equates to 28, 16, and 3 fmol/cell, respectively.

It is possible that GSSG could be exported from the cells and react with any trace cysteine present to give the mixed disulfide. Addition of GSSG to DMEM produced some of the mixed disulfide, but a significant amount of the GSSG remained after reduction and compared their effects with that of DPI. Buthionine sulfoximine, an inhibitor of γ-glutamylcysteine synthetase that catalyzes the rate-limiting step of GSH synthesis, and acivicin, an inhibitor of the enzyme γ-glutamyl transpeptidase involved in recycling extracellular GSH, both decreased T24 GSH levels by ~30% over a 4-h period (Fig. 7). This was considerably slower than DPI, whereas adding both together lowered GSH to a level approaching that of DPI. However, neither caused the accumulation of extracellular GSH. Incubation of T24 cells in cystine-free medium, which will impair both new synthesis and the recycling pathways, induced a rapid drop in intracellular GSH similar to that of DPI. However, there was again no corresponding increase in GSH in the cell supernatant, indicating a different mode of action than DPI (Fig. 7). Unlike DPI, incubation with buthionine sulfoximine, acivicin, and cystine-free medium all resulted in a loss in the total GSH levels (Fig. 7). We conclude that DPI decreases intracellular GSH
levels by enhancing the rate of GSH efflux rather than by inhibiting precursor uptake or biosynthesis.

GSH transport has been studied almost exclusively in hepatocytes, with GSH carrier systems described in both the sinusoidal and the canalicular membranes, mediating release of GSH into the blood and the bile, respectively (41–44). There is some evidence that these same transporters exist in the plasma membrane of other mammalian cells (45, 46). When T24 cells were incubated with DPI and L-methionine, an inhibitor of the low affinity sinusoidal GSH transporter (47, 48), the extent of intracellular GSH loss was the same as that with DPI alone, suggesting that this carrier was not responsible for the observed efflux (Fig. 8). BSP prevents GSH efflux from the canalicular membrane of hepatocytes, inhibiting both the high and low affinity transport components (42, 49). Addition of BSP alone to T24 cells resulted in some loss of intracellular GSH, probably because BSP is a substrate for the glutathione S-transferases (50). When cells were treated with BSP together with DPI, a concentration-dependent reduction in the DPI-induced GSH loss was observed (Fig. 8). Notably, 1 mM BSP prevented the DPI-induced GSH loss completely, maintaining intracellular GSH at the same levels as BSP alone. An analogue, DBSP, also dose-dependently reduced the DPI-mediated GSH loss, with complete inhibition occurring at 4 mM. However, it also lowered T24 GSH levels (Fig. 8). These results strongly suggest that a BSP- and DBSP-sensitive carrier, probably one of the canalicular-like transporters, is mediating the DPI-induced efflux of GSH out of T24 cells. Because BSP and DBSP lower intracellular GSH levels themselves, and they also enhance Fas-mediated apoptosis (data not shown), we were unable to use them to test whether DPI still sensitizes T24 cells to apoptosis when GSH efflux is prevented.

FIG. 6. Glutathione species present in the extracellular medium of T24 cells. A, T24 cells were transferred to fresh DMEM for 1 h, and then 10 μM DPI was added (1), or the cells were left untreated (2). After 3 h, the cell supernatant was derivatized with dansyl chloride and separated by HPLC. Fresh DMEM is shown in 3. B, GSSG was added to DMEM and incubated for 3 h at 37 °C (1). Fresh DMEM is shown in 2. C, T24 cells were transferred to fresh DMEM for 1 h and washed in phosphate-buffered saline, and then cystine-free medium was added, and the cells were treated with 10 μM DPI (1) or left untreated (2) for 3 h. GSH was added to cystine-free medium and incubated for 3 h at 37 °C (3).

DISCUSSION

We have discovered that the flavoprotein inhibitor DPI causes a rapid loss of intracellular GSH from cultured cells, with the missing GSH recovered outside of the viable cell. This phenomenon was not cell type-specific because it was observed in all the primary and transformed cell types tested. The turnover of cellular glutathione is well characterized, and GSH efflux is an integral component of this pathway (Fig. 9) (32). Our results indicate that the rate of GSH efflux is susceptible to modulation. Indeed, efflux can be increased to such an extent that it outpaces the capacity of the cell for resynthesis and leads to severe depletion of intracellular GSH within a few hours.

The increased GSH efflux was prevented by inhibitors of the
canalicular GSH transporters, indicating a specific efflux mechanism. GSH transporters involved in extrusion of the tripeptide are poorly characterized and remain difficult to clone, and there is a general reliance on inhibitor studies to distinguish their activity (43, 44). The concentration at which BSP inhibited efflux favors the low affinity canalicular transport component as being the transporter responsible for DPI-induced GSH loss. Higher concentrations are required to inhibit it compared with the high affinity form (42), although it is not even definitive whether the two are different proteins.

We do not know how DPI is triggering GSH efflux. A DPI-sensitive flavoprotein may directly regulate the activity of a GSH transporter or associated regulatory proteins. Alternatively, a cell might respond to general metabolic changes upon treatment with DPI, such as an accumulation of NAD(P)H, by increasing the rate of GSH efflux. We are able to exclude two alternate mechanisms. Firstly, increased amounts of DPI did not further increase the rate of GSH loss from the cells, indicating that a direct conjugation reaction was not responsible for GSH loss. Also, DPI could have acted by inhibiting glutathione reductase, leading to the formation of GSSG and its export. We could not detect evidence of significant GSSG formation inside or outside of the cells, and the glutathione reductase inhibitor bis-chloroethyl-nitrosourea had no effect on GSH levels (data not shown).

The ability of DPI to lower intracellular GSH levels provides a feasible but as yet unproven mechanism for its ability to sensitize T24 cells to Fas-mediated apoptosis. A depletion of intracellular GSH has been described in a number of different apoptotic systems (51–53), with several studies showing that GSH loss in cells undergoing apoptosis is the result of accelerated efflux rather than depletion by oxidation (54, 55). In Jurkat cells treated with anti-Fas antibody, GSH efflux occurred via a specific membrane channel, the low affinity canalicular-like transporter, and the efflux was a downstream event dependent on caspase activation (54). In contrast, others have shown that U937 cells and HepG2 cells induced to undergo apoptosis with puromycin efflux intracellular GSH, that the extrusion precedes other apoptotic changes, and that maintenance of GSH was able to delay apoptosis (55). The results are not necessarily in direct conflict. It may be that efflux of GSH is a common process in apoptotic cells but that in some cell types, the loss of GSH is required early to allow successful induction of the apoptotic program. It is also of interest that the antiapoptotic protein Bel-2 is linked to changes in GSH metabolism in cells, in particular, a shift in the cellular redox state toward a more reduced environment (56), increased intracellular GSH, altered compartmentalization, and even modulation of GSH efflux pathways (57–60).

In our model, the time course of GSH efflux is consistent with sensitization, as is the ability to remove the DPI before activation of the Fas pathway and still see GSH efflux and sensitization. At this point, however, GSH efflux is associated with rather than responsible for the sensitization of T24 cells to Fas-mediated apoptosis. A crucial test of this hypothesis is to prevent GSH efflux and see if DPI still sensitizes the cells to apoptosis. Unfortunately, the transport inhibitors also depleted intracellular GSH, and they enhanced Fas-mediated apoptosis themselves. It will be important to identify the target of DPI and elucidate the pathways involved in controlling GSH efflux, so that more selective compounds and/or molecular approaches can be used to determine the role of DPI in sensitizing the tumor cells to apoptosis.

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