EFFECTS OF GLUTATHIONE-OXIDIZING AGENTS ON MICROTUBULE ASSEMBLY AND MICROTUBULE-DEPENDENT SURFACE PROPERTIES OF HUMAN NEUTROPHILS

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ABSTRACT

In human peripheral blood polymorphonuclear leukocytes and lymphocytes, GSH-oxidizing agents promote the movement of surface-bound concanavalin A (Con A) into caps and inhibit the assembly of microtubules (MT) that is normally induced by Con A binding. Con A capping and inhibition of MT assembly occur when GSH levels in cell suspensions are decreased by 30-70%, and return of GSH to control levels is accompanied by the appearance of cytoplasmic MT and by inhibition of the capping response with Con A. Oxidation of GSH markedly stimulates the hexose monophosphate shunt, and regeneration of GSH occurs rapidly.

The data indicate that MT cannot be assembled or maintained in the face of decreased GSH levels. Thus, GSH homeostasis becomes critical during physiological events such as phagocytosis which simultaneously induce the assembly of MT and the production of agents like H_2O_2 that can oxidize GSH.

Although a great deal of information is presently available about the conditions required for microtubule (MT) assembly in vitro, there is still no clear understanding of how assembly is regulated in vivo. Nath and Rebhun (21) have recently shown that oxidation of GSH in fertilized sea urchin eggs inhibits mitotic spindle formation and causes dissolution of preformed spindles. We proposed that intracellular GSH may also influence the organization and function of cytoplasmic MT. To test this hypothesis, we examined the effects of GSH oxidation on the assembly of MT in human peripheral blood polymorphonuclear leukocytes (PMN). Two relatively specific oxidizing agents were employed: diamide (diazene dicarboxylic and bis-*N*,*N*-dimethylamide) (15, 16) and BHP (tertiary butylhydroperoxide) (30).

PMN were chosen for study first because MT assembly can be reproducibly induced by brief exposure of a variety of ligands including the plant lectin concanavalin A (Con A) (8, 10, 25), and second because the presence or absence of MT in Con A-treated PMN can be inferred from the distribution of surface-bound lectin. Thus, Con A shows a homogeneous surface distribution on cells that are competent to assemble MT but moves into a single aggregate – a cap – on the surface of cells treated with agents that bind with tubulin subunits and inhibit their polymerization (colchicine and the new carbamate anti-MT agent, R17934) (22-27).

MATERIALS AND METHODS

Materials

Diamide was obtained from the Sigma Chemical Co., St. Louis, Mo. and BHP from ICN Pharmaceuticals, Inc., Cleveland, Ohio. Con A (3 × crystalline) was

obtained from Miles-Yeda, Rehovot, Israel. Diamide is the most selective of a group of GSH-oxidizing agents developed by Kosower and co-workers (15, 16), 1 mol of this compound oxidizes 2 mol of GSH in a two-step chemical reaction which produces GSSG plus inert reduced diamide. In erythrocytes, Kosower et al. showed that GSH can be regenerated at 37°C after exhaustion or removal of diamide in the presence of glucose and an intact hexose monophosphate shunt (HMS). BHP is an organic hydroperoxide that serves as a substrate for GSH peroxidase and is not decomposed by catalase. Srivistava and co-workers (30) have shown that BHP is as effective as diamide in oxidizing erythrocyte GSH. As for diamide, the reaction with GSH is stoichiometric, and spontaneous regeneration of GSH follows exhaustion of the BHP. Srivistava et al. (30) reported that diamide leads to the formation of a small proportion of mixed disulfides of GSH with protein sulfhydryls, whereas BHP and other organic hydroperoxides appear to specifically convert GSH to GSSG.

Cells

Leukocytes from peripheral blood of normal human donors were isolated from buffy coat as previously described (24, 26). Cell suspensions contained approximately 80% PMN and 20% mononuclear cells.

Fluorescence Labeling of Cells

Cells (10^6 leukocytes in 0.5 ml of phosphate-buffered saline [PBS]) were labeled at 37°C for 5 min with fluorescein isothiocyanate-conjugated con A (FITC-Con A), 10 μg/ml, then fixed, washed, and examined as before (24, 26). PMN were identified by phase-contrast microscopy before scoring the surface distribution of FITC-Con A. The distribution of fluorescence was observed on at least 100 cells in any experimental group. Data are expressed as percent of random, capped, or patched cells. As described previously, randomly labeled cells show a uniform distribution of FITC-Con A, and capped cells show FITC-Con A concentrated in a single polar aggregate on the membrane. Patched cells show small fluorescent aggregates that are intracellular and cannot be removed with the competing sugar α-methyl-D-mannose presumably due to pinocytic internalization of lectin during the labeling period.

Electron Microscopy

In all experiments, leukocytes (4×10^6 cells in 0.5 ml PBS) were incubated at 37°C for 5 min with (in the case of treated cells) or without (in the case of untreated cells) 100 μg/ml Con A. Diamide and BHP were given at a concentration of 50 nmol/10^6 cells either during a preincubation period (5 min at 37°C for diamide, 10 min at 37°C for BHP) or after Con A treatment. For cell suspensions pretreated with drugs, Con A was added without immediate washing of the cells. For cell suspensions post-treated with drugs, Con A was removed by centrifugation before addition of fresh medium containing BHP and diamide. After exposure to Con A and drugs, the cells were pelleted and resuspended for 30 min at 37°C in fixative consisting of 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The samples were washed twice in buffer alone, postfixed in 2% aqueous OsO_4 for 30 min, washed in distilled water, and stained en bloc for 30 min at 60°C in 2% uranyl acetate (aqueous). Cell pellets were dehydrated through a graded series of cold ethanols and embedded in an Epon-Araldite mixture. Thin sections (silver) cut on a diamond knife were picked up on 300-mesh copper grids, stained with uranyl acetate and lead, and examined in a Philips 300 electron microscope.

Morphometric determinations of centriole-associated MT were performed in two ways. The method of Hoffstein et al. (10) was adapted as follows: sections of PMN containing a centriole were photographed at a stage magnification of $\times$ 14,200. MT were not visible at this magnification, thus precluding sample bias. They were made visible by printing at a final magnification of $\times$ 53,000. MT, recognized by their straight walls separated by 24–27 nm and coursing for at least 40 nm, were counted within 4 μm^2 containing a centrally disposed centriole. In addition, direct MT counts were made on sections viewed in the electron microscope at a stage magnification of $\times$ 57,000. An area of approximately 4 μm^2 surrounding the centriole was again analyzed for the presence of MTs. This method of analysis permitted examination of a large number of cells within each experimental group. However, only long (>80 nm) MT profiles were readily identified, and the numerous short profiles emanating from the centriolar area and associated satellite structures were not easily discernible under these conditions. For each experimental treatment, silver sections from three separate pellets were examined and analyzed by both direct counting and counting on micrographs.

MT Assembly In Vitro

Tubulin was isolated from fresh bovine brain by the following modification of the method of Weingarten et al. (32). After a cold (4°C), high-speed centrifugation (at 90,000 *g* for 30 min), initial MT polymerization was performed by warming the supernate to 37°C for 30 min in 0.1 M 2[*N*-morpholino]ethane sulfonic acid (MES) buffer, pH 6.8, containing 1 mM GTP, 1 mM GSH, 0.5 mM $MgCl_2$, 1.0 mM EGTA, 0.1 mM EDTA, and 2 M glycerol. MT were pelleted by high-speed centrifugation (90,000 *g*, 30 min, 25°C), resuspended in cold buffer containing 1.0 mM GTP and GSH, and briefly homogenized in a loose-fitting Dounce homogenizer (Kontes Co., Vineland, N.J.).

The rate and extent of MT polymerization under various experimental conditions were evaluated by measuring fluorescence resonance energy transfer between mixtures of tubulin labeled separately with donor and

acceptor fluorochromes as previously described (1). Two populations of tubulin were labeled separately with either FITC or rhodamine isothiocyanate (RITC) and chromatographed over Sephadex G-25 equilibrated with MES buffer to remove unconjugated dye. Dye to protein molar ratios for the protein conjugates used here were 0.11 for RITC-tubulin and 0.013 for FITC-tubulin. The labeled tubulin was carried through a second depolymerization-polymerization cycle under the conditions described above and used immediately for energy transfer experiments in a Perkin-Elmer MPF-4 spectrofluorimeter (Perkin-Elmer Ultek Inc., Palo Alto, Calif.). Assembly was followed by the increase in the emission peak for rhodamine after exciting the sample near the absorption maximum for the donor, fluorescein (480 nm). Samples from energy transfer experiments were routinely examined by negative staining with uranyl acetate in the electron microscope.

GSH Measurement

The levels of GSH in leukocytes were determined by a modification of the method of Beutler et al. (2). Cells ($12\text{-}15 \times 10^6$ in 1 ml PBS) were collected by 3-s centrifugation in an Eppendorf microcentrifuge. Medium was removed and immediately replaced by 0.3 ml of precipitating solution (1.67% [wt/vol] metaphosphoric acid, 30% [wt/vol] NaCl, 0.2% [wt/vol] EDTA) plus 0.2 ml of 10 mg/ml bovine serum albumin (BSA) in 0.25% Triton X-100. The tubes were vortexed for 20 s and held at room temperature for at least 10 min. Protein was pelleted by 2-min centrifugation in the Eppendorf microcentrifuge. GSH assay mixtures contained protein-free supernate (0.2 ml), 0.3 M Na_2HPO_4 (0.7 ml), and 0.04% 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in 1% sodium citrate (0.1 ml). GSH concentrations were calculated from the absorbance of the solutions at 412 nm. A blank value was obtained in parallel mixtures in which all GSH was oxidized by addition of 5 μl of 10 mM diamide to the supernate before addition of the DTNB.

The rate of diamide utilization was calculated by measuring its rate of removal from the incubation medium. Medium diamide was titrated by adding known amounts of GSH to aliquots of medium and determining the excess GSH by the DTNB procedure as described above. 2 mol of GSH are consumed by 1 mol of diamide.

Oxidation

Leukocyte suspensions (5×10^6 cells in 2 ml of PBS with or without added drugs) were incubated with gentle shaking for 10 min at 37°C in closed Warburg flasks. The medium contained 0.1 mM glucose with [1-^{14}C]glucose at approximately 1 μCi/ml. The center well held 0.2 ml of 0.25 M NaOH and a paper wick. The reaction was terminated by tipping in 0.2 ml of 1 N HCl from the sidearm. An additional 2-h incubation with shaking was allowed, to trap the radioactive CO_2 liberated by the HCl. The radioactivity in the center well was then counted by liquid scintillation.

RESULTS

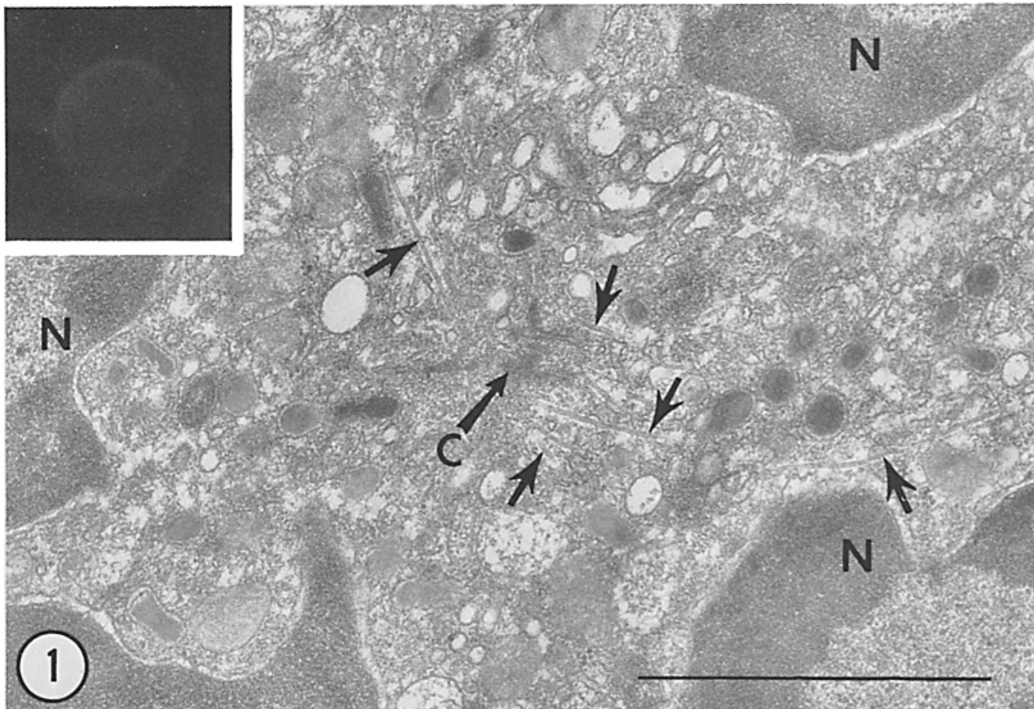
Con A Cap Formation in Human PMN Induced by Diamide and BHP

The typical distribution patterns of FITC-Con A on the surface of untreated, diamide-treated, and BHP-treated PMN are shown in the insets to Figs. 1 and 2. The majority of untreated cells show a homogeneous labeling pattern (Fig. 1, *Inset*). By contrast, the majority of cells preincubated for 5 min with diamide (50 nmol/10^6 cells) followed by 5-min labeling with FITC-Con A show movement of the surface-bound lectin into a tight knob-like extension at one pole of the cell (Fig. 2, *Inset*). BHP (50 nmol/10^6 cells) also promotes movement of Con A into a surface cap at one pole of the cell. BHP-induced caps are often more diffuse than those that develop on diamide-treated cells, and capping is less frequently accompanied by formation of a tail or uropod.

Dose-response data for capping, induced after a 5-min preincubation of 10^6 leukocytes in 0.5 ml of PBS containing various amounts of diamide and BHP, are given in Fig. 3 A and B. Both agents oxidize GSH by a stoichiometric reaction. Therefore, although their doses are plotted for convenience in molar concentrations, they are discussed in terms of nanomoles per 10^6 cells. Diamide promotes an extreme degree of capping when present in amounts between 25 and 250 nmol diamide/10^6 cells. Lower doses are ineffective and amounts of diamide above 500 nmol/10^6 cells are inhibitory. This inhibition at high concentrations of diamide may reflect direct oxidation of surface or intracellular protein sulfhydryl residues required for the capping process. BHP shows a broader range of effective doses, promoting capping between 25 and 500 nmol/10^6 cells. Doses above 500 nmol BHP/10^6 cells appear to be toxic, since cells show increased fragility, decreased surface binding, enhanced diffusion of FITC-Con A into cells, and inhibition of cap formation.

The data in Table I established that the extent of Con A capping induced by a maximally effective dose of diamide (established above) is equal to the maximum capping response that can be induced by colchicine or R17934 (24). The maximum capping response to BHP is consistently lower by 15-25% than the maximal response to diamide.

The data plotted in Fig. 4 show the time-course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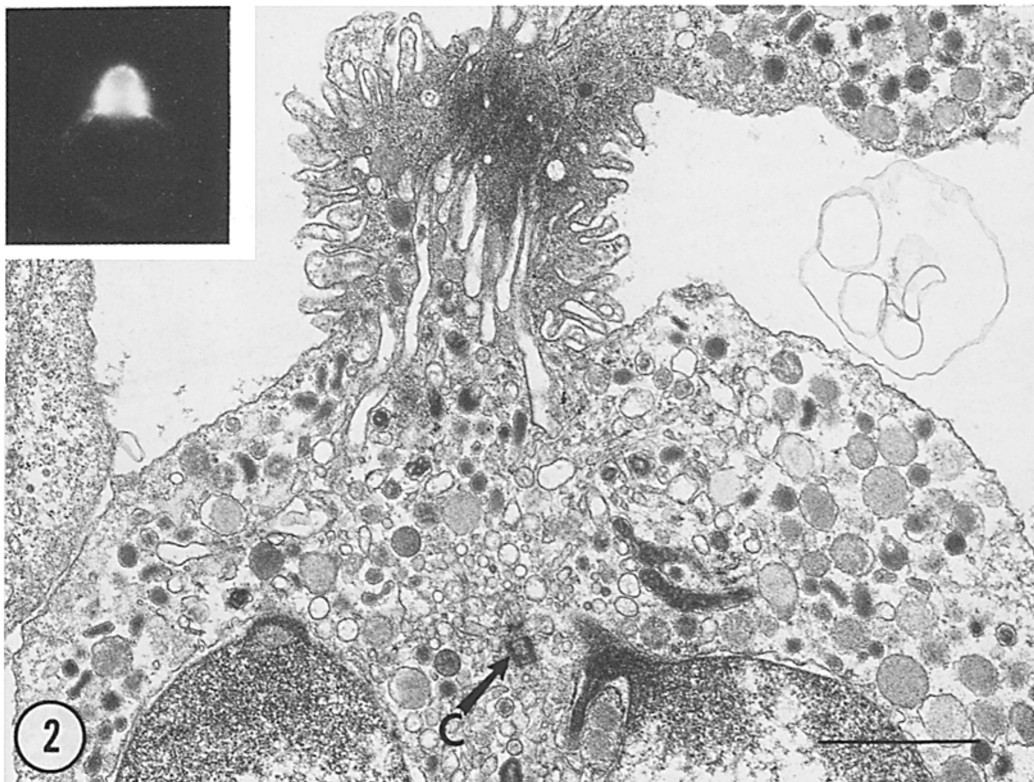
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of Con A capping induced by the lowest maximally effective doses of diamide and BHP. In the upper curves (A), 25 nmol/10^6 cells diamide or BHP were added at time zero, and the medium was left unchanged during the entire incubation period. Both agents induced a high degree of capping during the first 20 min of incubation, after which the proportion of cells that capped decreased rapidly. Addition of a second portion of either drug after 60 min restored Con A capping on the majority of cells.

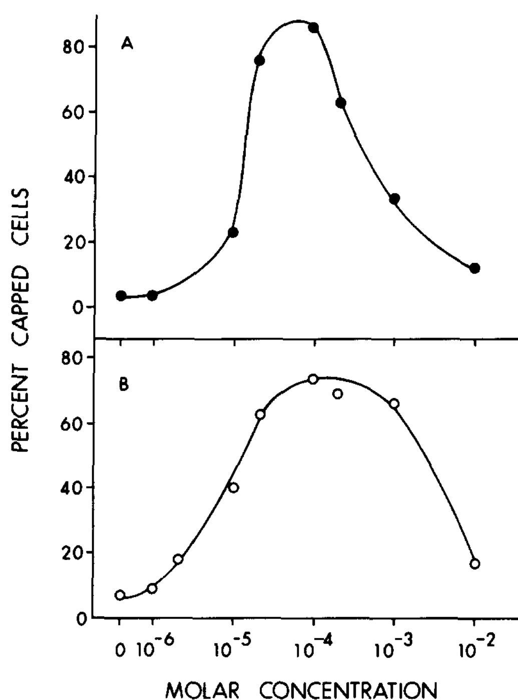

FIGURE 3 Concentration dependence of Con A capping induced by diamide and BHP. Cell suspensions (10^6 leukocytes in 0.5 ml PBS) were preincubated for 5 min with amounts of diamide (A) or BHP (B) between 0.5 nmol/10^6 (10^{-6} M) and 5 nmol/10^6 cells (10^{-2} M) before 5-min labeling with FITC-Con A (10 μg/ml). Drug concentrations are plotted on a logarithmic scale. Results are the average of two separate experiments.

In the lower curves (B), 25 nmol/10^6 cells diamide or BHP were present only during the first 10 min of incubation. Further incubation was carried out in fresh medium without drugs. As anticipated, the duration of the increased capping response was shorter than in A where drugs were present for longer periods. Addition of fresh drug restored cap formation to high levels.

It was established that diamide and BHP promote capping not only in cells pretreated with drug before Con A labeling, but also in cells that are prelabeled with Con A and subsequently incubated with GSH-oxidizing agents (Table II). This suggests that the drugs cause disassembly of preformed MT as well as preventing their assembly.

The effect of exogenous GSH on Con A capping induced by diamide and BHP was also studied. GSH alone does not alter the distribution of FITC-Con A on human PMN. Simultaneous addition of excess GSH with diamide blocked the action of diamide by exhausting it in direct chemical reaction. However, pretreatment of cells with diamide (5 min) followed by GSH (5 min) led to a full diamide-induced capping response. This indicates that once diamide has entered the cells, its action cannot be reversed by addition of the relatively impermeable tripeptide. By contrast with diamide, capping induced by BHP is not affected by the simultaneous presence of GSH, indicating that there is little or no direct interaction between BHP and GSH, at least during short incubation periods.

During the course of these experiments, it was noted that lymphocytes, which form 15–20% of the cell suspensions, are also extensively capped by diamide and BHP. Monocytes are also capped.

FIGURE 1 Fluorescence and electron micrographs of normal human PMN exposed to Con A (10 or 100 μg/ml, respectively) for 5 min. *Inset* shows the typical uniform surface distribution of FITC-Con A. Ultrastructurally, PMN treated with Con A typically display many longitudinal microtubular profiles (arrows) emanating from the centriole (*C*). *N* = nuclear lobes. × 26,400. Scale bar = 2.0 μm.

FIGURE 2 The inset illustrates the concentration of FITC-Con A on a capped PMN pretreated with diamide (50 nmol/10^6 cells). An electron micrograph of a PMN similarly treated with diamide and subsequently exposed to Con A; note the absence of centriole (c)-associated microtubules and the pronounced accumulation of dense material beneath the capped cell protrusion. The cap is extensively plicated in contrast to the evenly-contoured noncapped membrane. *N* = nuclear lobes. × 10,600. Scale bar = 2.0 μm.

TABLE I

Effects of Agents Known to Inhibit MT assembly and Agents that Oxidize GSH on Con A Capping on Human PMN

Line	Pretreatment	Time, min	Distribution of fluorescence		
			Random	Patched	Capped
1	PBS	10	86	8	6
2	PBS	30	84	9	7
3	Colchicine (10^{-6} M)	30	8	4	88
4	R17934 (5×10^{-7} M)	30	9	4	87
5	Diamide*	10	10	3	87
6	BHP*	10	24	3	73

Cells were pre-incubated with drugs at 37°C for the times indicated. FITC-Con A (10 μg/ml) was present during the last 5-min period of incubation. Results are the average of five separate determinations.

* 50 nmol/10^6 cells.

Effects of Diamide and BHP on GSH Levels

It was not possible to measure GSH levels in the small numbers of leukocytes (10^6 cells in 0.5 ml PBS) used in the capping assay. Therefore, larger numbers of cells were employed (12–15 × 10^6 cells in 1 ml of PBS) and a proportional increase in the amounts of added diamide and BHP was made, to maintain the ratio of nanomoles anti-GSH reagent to cell number identical to the ratio employed for the capping experiments.

The GSH levels in cell suspensions incubated at 37°C with diamide and BHP at 25 nmol/10^6 cells are shown in Fig. 5, A and B. Diamide causes an abrupt decrease in GSH levels from a control value of 1.7 nmol/10^6 cells to about 30% of control in 5 min. This new steady-state level of GSH is maintained for approximately 20 min. After 30 min, no diamide remains in the medium and GSH levels are restored to normal. Since exhaustion of 25 nmol of diamide implies oxidation of 50 nmol of GSH, the GSH pool in these cells must have turned over 50/1.7 or about 30 times in 20 min. Similar data (not shown) were obtained with 50 nmol diamide/10^6 cells, except that the GSH level remained at about 27% of control until 30 min.

BHP also causes a rapid decline followed by a rise in intracellular GSH levels. However, the decrease is smaller with BHP than with diamide – to between 55 and 65% of control after 10 min with 25 nmol/10^6 cells. Using 50 nmol BHP/10^6 cells, GSH levels decreased to 49% of control after 10 min and began to rise towards normal after 30 min.

Although lower concentrations of BHP and diamide (2 nmol/10^6 cells) caused no measureable decrease in GSH levels during a 5-min incubation at 37°C, a similar incubation at 4°C decreased GSH to between 0 and 10% of normal. This

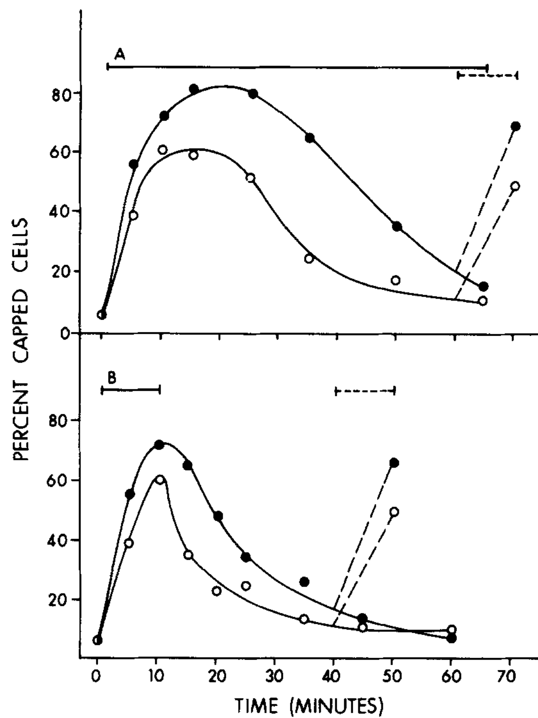

FIGURE 4 Time-course of Con A capping induced by diamide (●) and BHP (○). Cell suspensions (10^6 cells in 0.5 ml PBS plus glucose [5×10^{-3} M]) were incubated at 37°C in the presence of 25 nmol of diamide or BHP. The solid bars above each curve show the total length of incubation without change of medium. Addition of fresh drug to cell suspensions is indicated by the dotted bars. FITC-Con A (10 μg/ml) was present during the last 5 min of incubation.

Table II

Cap Formation Induced by Diamide and BHP on PMN Prelabeled and Postlabeled with FITC-Con A

			Distribution of Fluorescence, %		
Line	First incubation (10 min)	Second incubation (10 min)	Random	Patched	Capped
1	PBS	FITC-Con A	80	16	4
2	FITC-Con A	PBS	60	30	10
3	Diamide*	FITC-Con A	11	1	88
4	FITC-Con A	Diamide*	7	12	81
5	BHP*	FITC-Con A	21	12	67
6	FITC-Con A	BHP*	22	20	58

Cells were collected by rapid centrifugation and resuspended in PBS between the first and second incubations. Cells exposed to FITC-Con A (10 μg/ml) during the first incubation period (lines 2, 4, 6) showed more patch formation than cells exposed to lectin only during the second incubation period (lines 1, 3, 5). This reflects internalization of Con A during extended incubation. Results are the average of two separate determinations.

* 50 nmol/10^6 cells.

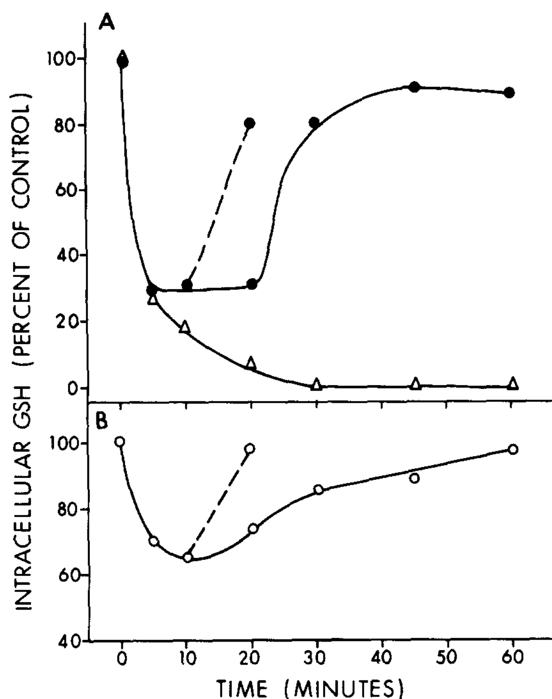

Figure 5 GSH levels in diamide- and BHP-treated leukocytes. Cell suspensions (12.9 × 10^6 cells in 1.0 ml PBS-glucose in A; 15.8 × 10^6 cells in 1.0 ml PBS-glucose in B) were incubated at 37°C in the presence of 25 nmol/10^6 cells of diamide (A) or BHP (B). At various times, the cells were collected by centrifugation and GSH levels determined in acid extracts. The solid lines show GSH levels in cells treated continuously with diamide or BHP. The dotted line shows the change in GSH levels after removal of drug and 10-min incubation in fresh PBS. The line joined by triangles in A shows the amount of diamide present in the medium. All values are given as percent of values at time zero. In A, resting cells contain 1.7 nmol GSH/10^6 cells, and in B, 1.4 nmol GSH/10^6 cells. Assuming 0.347 μl cell water/10^6 cells (9), these values correspond to GSH concentrations of 4.9 and 4.0 mM in cells from two separate donors. These results are from single representative experiments. The variation between different donors in GSH levels was from 1.2 to 1.8 nmol/10^6 cells (3.6-5.1 mM). Slight variability in the percent reduction in GSH levels and in the duration of the depression was observed in cells from different donors. However, the data were qualitatively similar and the rate of GSH regeneration was the same within 5 min between experiments.

suggested that the mechanism for GSH regeneration is active only at the higher temperature.

The most obvious pathway for the high rate of GSH regeneration in leukocytes involves activation of the hexose monophosphate shunt (HMS) and of the GSH reductase catalyzed reduction of GSSG by NADPH. HMS activity, estimated by the production of labeled CO_2 from [1-^{14}C]glucose was increased markedly and nearly equally (5 to 10-fold) by diamide or BHP during a 10-min incubation at 37°C.

Con A (100 μg/ml) increased [1-C^{14}]glucose oxidation two- to threefold under the same conditions (10-min exposure of cells to Con A plus isotope) but had little effect on GSH levels: in five experiments with 10–100 μg/ml Con A, GSH levels ranged from 10% below control values to 5% above control.

Electron Microscopy

Untreated human PMN characteristically display several short MT profiles associated with a juxtanuclear centriole complex (10, 25) (Table III, line 1). Brief exposure to Con A (5 min)

results in a marked stimulation of MT assembly (Fig. 1 and Table III, line 2). The number of MT visible by direct counts in the microscope was increased four- to fivefold after Con A binding, and the increase measured by micrograph counts was approximately sevenfold. In addition to an increased number of centriole-associated MT the length of these MT was also greater in Con A-treated cells, and more MT were observed, but not quantified, in the peripheral cytoplasm (Fig. 1). Qualitatively similar observations have been reported by Hoffstein et al. (10).

Compared to cells treated with Con A alone, addition of Con A to PMN previously exposed to diamide for 5 min or BHP for 10 min produces striking changes in cellular organization (Fig. 2). First, the marked increase in MT observed in control cells after Con A does not occur with drug treatment (Table III, lines 3 and 4). MT are also extremely rare in cells previously exposed to Con A to induce assembly and then given diamide or BHP (not shown). Second, the cells show a dramatic change in shape. Whereas control cells are rounded with uniformly ruffled surfaces, diamide-treated cells show a prominent capped region (Fig. 2). The cap is observed as a knob-like extension of the cell surface which is highly plicated in contrast to the smooth contour of the remaining cell membrane. BHP followed by Con A produces a similar effect. The cytoplasm beneath the cap is extremely dense, containing numerous 6- and 10-nm filaments (not shown) while generally excluding membrane-bounded organelles. Caps of similar morphology accompanied by a marked diminution in MTs were also observed in lymphocytes and monocytes in diamide or BHP- and Con A-treated samples.

Inhibition of MT assembly, like Con A capping, was readily reversible. Thus, PMN exposed to diamide for 10 min, and then in PBS alone for 30 min before Con A labeling, were indistinguishable from cells treated with Con A alone in terms of cell shape. The number of centriole-associated MT under these conditions was slightly elevated over control (Table III, line 5).

MT Assembly In Vitro

The effects of diamide and BHP on MT assembly in vitro were studied by the fluorescence energy transfer method developed by Becker et al. (1). Basically, FITC- and RITC-tubulin are combined under polymerization conditions, the mixture is excited near the absorption maximum for FITC (donor), and MT assembly is determined from the increase in emission from RITC (acceptor). Emission from RITC can occur only when RITC- and FITC-tubulins are closely approximated, as when MT are assembled. We have previously shown that, under polymerizing conditions, energy transfer (MT assembly) is evident at 5 min and maximal at 15 min. Energy transfer fails to occur at 4 or 37°C in the presence of $CaCl_2$ (4 mM) or absence of GTP.

Using this sensitive technique, we found that pretreatment of labeled tubulins with 1 mM diamide for 1 h at 4°C or addition of 1 mM diamide just before incubation at 37°C totally inhibits MT polymerization. The effect of diamide is not reversed by addition of 4 or 10 mM GSH to the polymerizing mixture 5 min after addition of diamide, but GSH (2 mM) added simultaneously does protect against inhibition, suggesting that GSH is a preferred substrate for oxidation by diamide. Diamide is also capable of depolymerizing MT. Rapid disappearance (within 7 min) of energy transfer follows addition of diamide (1.0 mM) to suspen-

TABLE III
Microtubule Counts in Untreated and Con A-Treated Human Neutrophils

Line	Treatment	Counting procedure A	Counting procedure B
		MT ± SEM	
1	PBS	1.1 ± 0.4 (52)	2.6 ± 0.4 (11)
2	Con A	4.9 ± 0.3 (48)	17.7 ± 2.5 (17)
3	Diamide-Con A	0.6 ± 0.2 (17)	2.8 ± 0.6 (8)
4	BHP-Con A	2.3 ± 0.5 (18)	3.6 ± 1.1 (9)
5	Diamide-PBS-Con A	6.3 ± 0.6 (15)	25.0 ± 1.4 (7)

MT were counted by (A) direct examination of centriolar regions in the electron microscope or (B) from micrographs of the centriolar area as described in the text. MT, mean number of MT profiles. Number of cells examined is given in parentheses.

sions of assembled MT. These data are consistent with the effects of diamide on MT assembly determined by Mellon and Rebhun (20) using light scattering to follow MT polymerization, except that the latter workers observed a reversal of diamide inhibition after addition of 10 mM GSH to the polymerization mixture.

BHP (0.1–1.0 mM), which effectively causes dissolution of MT in vivo, is without effect on the in vitro assembly of MT as determined by the energy transfer technique.

Negative staining of samples used for energy transfer studies showed abundant MT in control and BHP-treated mixtures. No recognizable MT were present in diamide-treated mixtures.

DISCUSSION

The importance of free SH groups for the polymerization of MT in vitro was first indicated by Kuriyama and Sakai (17). Reaction of *N*-ethylmaleimide, *p*-chloromercuribenzenesulfonate (PCMBS) or DTNB with 2 mol of SH per mole of tubulin dimer blocked tubulin polymerization and depolymerized preformed MT. GSH, which accounts for over 90% of the nonprotein SH groups of the cell and participates in a variety of SH-exchange reactions, is a likely candidate for protecting these essential residues against oxidation in vivo.

The first evidence for a role of GSH in MT assembly in vivo was obtained by studies in fertilized sea urchin embryos. Rebhun and co-workers (21, 28) showed that treatment of dividing sea urchin embryos with diamide prevented mitotic spindle formation and caused rapid dissolution of existing spindles. This response to diamide was presumed to be a direct result of GSH oxidation. The present data indicate that maintenance of GSH levels is required for the assembly and preservation of not only spindle but also cytoplasmic MT. Several mechanisms which may resolve the relationship of GSH to MT are discussed below.

We first established that treatment of cells with diamide or BHP promotes Con A cap formation on human PMN. Con A capping on PMN has so far been observed only in cells treated with anti-MT agents and in cells from mice and patients with Chediak-Higashi syndrome in which Con A binding fails to stimulate MT assembly (22–27). These data immediately suggested that agents that oxidize GSH inhibit cytoplasmic MT assembly. Both reagents promoted capping on cells prelabeled with Con A and subsequently exposed to diamide or BHP. This suggested not only inhibition of MT assembly but also depolymerization of assembled MT.

Electron microscope examination of control and treated PMN established a direct relationship between Con A capping induced by diamide and BHP and inhibition of MT assembly. Untreated normal PMN have few assembled MT. Con A binding is followed after 5 min by the appearance of many MT emanating from centrioles and coursing towards the cell periphery. Cells pretreated with diamide followed by Con A showed prominent uropods (caps) and an almost complete absence of MT. Similarly, very few MT were present after Con A labeling in BHP-treated cells.

It is apparent that the anti-MT effects of diamide and BHP are observed only when sufficient drug is present to reduce GSH levels and that a highly active mechanism for maintenance of GSH levels operates in human PMN. The rapid regeneration of GSH after oxidation to GSSG is most likely a consequence of the highly active HMS in these cells. As noted, both diamide and BHP stimulated the HMS between 5- and 10-fold during a 10-min incubation. The HMS provides NADPH required for the reduction of GSSG by GSH reductase.

By using BHP as well as diamide to oxidize GSH, we were able to minimize the possibility, recognized by Rebhun and co-workers (20) in their studies of the role of GSH in assembly of the mitotic apparatus, that MT disruption is a result of direct oxidation of tubulin-SH by the reagents. Although diamide can directly inhibit MT assembly and cause depolymerization of MT in vitro, BHP has no direct effect on MT assembly in vitro or presumably in vivo. Thus, the effects we observe are most likely secondary to GSH oxidation and not a result of direct tubulin oxidation by the drugs. Several other lines of evidence also argue against a direct action of diamide in vivo upon cytoplasmic MT in PMN. It was observed that GSH completely protects against inhibition of MT assembly by diamide in vitro. Diamide-induced capping and MT disassembly in PMN in vivo occur when substantial amounts of GSH (at least 30% of normal) are present so that a protective effect of GSH against direct diamide-tubulin interaction should persist. In addition, the inhibition of MT assembly in vitro by diamide is not reversible in our hands by GSH or other treatment. By contrast, MT integrity is restored as soon as GSH

levels rise (by removal or exhaustion of diamide) in vivo.

The sum of these data leads to the conclusion that GSH is essential for MT assembly and preservation in vivo. However, it is clear that various stimuli which induce MT polymerization do not affect the levels of intracellular GSH. This is true both for the Con A-treated PMN in our experiments and for mitosis in the fertilized sea urchin egg (11, 12, 20, 28). Therefore, we do not propose that changes in GSH levels necessarily initiate MT assembly but rather that maintenance of GSH levels is required to permit or to preserve polymerization of MT.

This point becomes significant when we consider that the MT assembly which accompanies Con A binding and phagocytosis in human PMN and fertilization in the sea urchin egg occurs simultaneously with the production of oxidants. In the case of PMN, it is established that both stimuli of MT assembly also promote the production of substantial amounts of superoxide and H_2O_2 (3, 29). H_2O_2 would be expected to promote GSH oxidation either by direct chemical reaction or as a substrate for GSH peroxidase. Indeed, we find that PMN treated for 5 min with Con A plus exogenous H_2O_2 (250 nmol/10^6 cells) show a 50% reduction in GSH levels and that approximately 65% of cells are capped (J. M. Oliver, D. F. Albertini, and R. D. Berlin, unpublished results). The intriguing possibility exists that tubulin itself may act as an oxidant by consuming reduced SH equivalents (as GSH) during MT polymerization. No direct in vivo data are available on this point, but several older reports claim that oxidative responses are depressed when PMN phagocytize in the presence of colchicine (4, 7, 18, 19). Thus, MT assembly is dependent on the ability of cells to maintain GSH levels in the face of the oxidative challenge from endogenous H_2O_2 and perhaps from tubulin. Suitable mechanisms are available in human PMN. It is established that the activity of GSH reductase is increased within 15 s of initiation of phagocytosis (31) and that the HMS (turned on most effectively by decreased $NADPH/NADP^+$ ratios in the presence of GSSG [5]) is stimulated after phagocytosis or Con A binding (3, 14). Fertilization in sea urchin eggs has qualitatively the same metabolic accompaniments—increased O_2 consumption (6), and concomitant HMS activation (13) and GSH reductase activation (11, 12). These latter responses represent elements of a mechanism for GSH homeostasis. Thus, MT assembly can be initiated in response to surface-binding events (in PMN) or after fertilization (in sea urchin eggs) despite the well-documented increase in oxidative metabolism. We suppose that the oxidative challenge induced by diamide, BHP, and high concentrations of exogenous H_2O_2 is greater than that induced by surface binding or fertilization so that GSH levels cannot be maintained and MT assembly is inhibited.

The overwhelming oxidation of GSH by diamide or BHP could lead to oxidation of tubulin and thus inhibition of MT assembly by one of two mechanisms. The first is that GSSG itself is the immediate tubulin oxidant. If this were so, one would predict that GSSG per se would inhibit MT assembly in vitro. We have confirmed the report of Mellon and Rebhun (20) that indeed 10 mM GSSG prevents polymerization of isolated tubulin. However, we find that 4 mM GSSG (equivalent at least to four times the GSSG concentration of PMN where diamide or BHP are used to reduce GSH levels by 50%) neither alters the assembly of MT nor effects the disassembly of MT in vitro (D. F. Albertini and R. D. Berlin, unpublished observations). Thus, the in vivo effect of decreasing GSH (increasing GSSG) does not appear to be mimicked in vitro by a direct action of GSSG at well above the physiologically attainable concentration range for PMN. Of course, an enzymatic couple between GSH, GSSG, and tubulin SH may exist in vivo but be missing from the purified tubulin used in our in vitro studies.

The second mechanism puts the process into the perspective of GSH homeostasis and the physiologic oxidative challenge. According to this mechanism, GSH normally serves as a trap for the H_2O_2 generated after Con A binding. When GSH levels are depressed, some H_2O_2 remains free to oxidize tubulin SH. The presence of GSSG serves only to indicate that the defense mechanism has been overwhelmed. In support of this, we find that H_2O_2 oxidizes tubulin SH residues and is a potent inhibitor of MT polymerization in vitro (D. F. Albertini and R. D. Berlin, unpublished results). Either mechanism would suggest potential pathways for the physiological regulation of MT assembly.

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