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Glutathione-independent Mechanism of Apoptosis Inhibition by Curcumin in Rat Thymocytes

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ABSTRACT. Curcumin (CUR) is a natural yellow dye with antioxidant and scavenging properties present in *Curcuma* species. It is widely used as an anti-inflammatory, anti-mutagenic and chemopreventive agent. In addition to its inhibitory effect on proliferation, CUR has recently been shown to block dexamethasone-induced programmed cell death (apoptosis) of rat thymocytes. Because cellular thiols seem to play a role in redox regulation of apoptosis, the mechanism of the anti-apoptotic effect of CUR was studied by examining the levels of glutathione and acid-soluble sulfhydryl groups. CUR was shown to prevent the glutathione loss occurring in dexamethasone-treated thymocytes, enhancing intracellular glutathione content at 8 hr to 192% of that of nontreated cells. A 60% increase in acid-soluble sulfhydryl groups was also observed. In the presence of L-buthionine S,R-sulfoximine (BSO, an inhibitor of glutathione synthesis), intracellular glutathione content of thymocytes treated with dexamethasone and CUR fell to 31% and that of the acid-soluble sulfhydryl groups to 23% of control after 8 hr. Unexpectedly, the electrophoretic and flow cytometric studies of DNA fragmentation demonstrated that apoptosis did not occur even after 20 hr of incubation with buthionine S,R-sulfoximine and dexamethasone, while control thymocytes and the cells treated only with buthionine S,R-sulfoximine showed DNA fragmentation at a level corresponding to spontaneous apoptosis. These results show that CUR treatment elevated the concentrations of glutathione and nonprotein sulfhydryl groups, thus preventing their decrease in apoptotic thymocytes. Coadministration of L-buthionine S,R-sulfoximine and CUR did not affect the anti-apoptotic effect of CUR suggesting a glutathione-independent mechanism of cell protection. BIOCHEM PHARMACOL **56**;8:961–965, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. apoptosis; curcumin; glutathione; thiol groups

The yellow dye CUR‖ [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-di-one] extracted from the rhizome of *Curcuma* species has been widely used for the treatment of inflammatory conditions for many years. Recently, this antimutagenic and anticarcinogenic [1] compound has been shown to prevent apoptosis (programmed cell death) of rat thymocytes stimulated by DEX [2]. The inhibition was accompanied by partial suppression of the AP-1 transcription factor, classified as an antioxidant responsive factor [3] which is activated in apoptotic cells [4].

Intracellular balance of reactive oxygen species and antioxidants seems to determine cell survival [5]. Thiols in particular are considered to be involved in defence mechanisms [6, 7]. Direct exposure of cells to oxidants results in apoptosis, which can be inhibited by free radical scavengers and thiol reductants [8]. Many agents capable of apoptosis induction evoke production of reactive oxygen intermediates [9]. Apoptotic thymocytes after DEX stimulation show a significant decrease in cellular thiols and a higher peroxide level [8, 10]. On the other hand, both glutathione and N-acetylcysteine can prevent cell death [5, 10].

Interestingly, curcumin not only exhibits antioxidative and scavenging properties [11] but also enhances the activities of other antioxidants such as superoxide dismutase, catalase and glutathione peroxidase [12]. CUR was found to modulate the activity of glutathione S-transferase [13–15] and change the glutathione content [15, 16]. The aim of this study was to investigate whether curcumin blocks DEX-induced apoptosis via induction of glutathione synthesis.

MATERIALS AND METHODS

Cell Culture and Drug Exposure

Thymocytes prepared as previously described [4] were routinely cultured with or without additives such as 10^{-7} M DEX (Sigma) to induce apoptosis, 50 μM CUR (Sigma, 10 mM stock in methanol, protected from light, stored at

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‖ *Abbreviations:* BSO, L-buthionine S,R-sulfoximine; CUR, curcumin; DAPI, 4′,6-diamidino-2-phenylinole; DEX, dexamethasone; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; GSSG, glutathione disulfide; and TCA, trichloroacetic acid.

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−20°) to block apoptosis, and 100 μM BSO (Sigma, 10 mM stock in RPMI 1640, stored at 4° for no longer than 1 week) to inhibit GSH synthesis. Cells were harvested at 2, 4 and 8 hr of incubation in a humidified atmosphere of 5% CO_2 in air at 37°. At each time point, the cells were supravitally exposed to Trypan blue to distinguish necrosis.

Apoptosis Measurements

Apoptosis was estimated by DNA fragmentation monitored by agarose gel electrophoresis [17] and flow cytometry [18]. For flow-cytometry analysis, cells were washed with PBS and fixed in cold 70% ethanol. 1×10^6 cells after washing were stained at 4° with 1 μg/mL of DAPI (Molecular Probes) and with 20 μg/mL of sulforhodamine (Molecular Probes) and analyzed for the amount of DNA and protein on FACS Vantage (Becton Dickinson) using Cell-Quest software (Becton Dickinson).

Measurement of Nonprotein Sulfhydryl Groups

DTNB (Sigma) reduction by sulfhydryl (SH) groups results in the chromophoric product (2-nitro-5-thiobenzoic acid, Ellman's anion) being detected spectrophotometrically at 412 nm [19]. The method was standardized with known GSH concentrations. Aliquots of 2×10^7 cells washed with PBS were lysed in water by freezing and thawing. TCA-precipitated pellet was solubilized in 0.5 M NaOH and the amount of protein determined according to Lowry *et al.* [20]. One hundred μL of supernatant was mixed with 800 μL of 0.1 M sodium phosphate, pH 8.0 (blank), combined with 100 μL of 0.04% DTNB (w/v in buffer), and analyzed at 412 nm after 15 min. CUR did not interfere in thiol determination with DTNB because of its low solubility and degradation in aqueous solutions at pH above 7 [15].

Determination of Total (Reduced plus Oxidized) Glutathione [21]

Glutathione measurement utilizes the continuous reduction of Ellman's regent by GSH, leading to the chromophoric product (Ellman's anion) monitored at 412 nm, mixed disulphide as intermediate product, and oxidized glutathione (GSSG), which is then reduced by NADPH in the presence of glutathione reductase. Quantitation was performed by comparison with a standard curve of known GSSG concentrations. Cells were prepared as described above. Perchloric acid-precipitated pellet was solubilized in 0.5 M NaOH and protein content measured according to Lowry *et al.* [20]. Protein-free supernatant was neutralized with 2 M KOH in 0.3 M Tris, and 100 μL of sample was mixed with 1 mL of sodium phosphate, pH 7.0, containing 1 mM EDTA; 50 μL of 4 mg/mL NADPH in 0.5% $NaHCO_3$ (freshly prepared); 20 μL of 1.5 mg/mL DTNB in 0.5% (w/v) $NaHCO_3$ and 20 μL of 6 U/mL glutathione reductase (Sigma).

Statistical Analysis

The data are presented as the means ± SEM from three separate experiments, each performed in triplicate. The significances of differences between mean thiol concentrations after indicated treatments were calculated using Student's *t*-test and were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Recently, CUR has been shown to block DEX-induced apoptosis of rat thymocytes [2]. Because oxidative stress is considered as a mediator of apoptosis and thiol antioxidants seem to play a key role in redox regulation of cell death [5–10], the influence of CUR on levels of glutathione and TCA-soluble SH groups was studied in an attempt to gain further insight into the mechanism of this process.

Following the exposure of cells to DEX, the intracellular content of glutathione (GSH + GSSG) progressively decreased with time (Fig. 1: DEX), representing 36% of the level observed in control cells (Fig. 1: CON) at 8 hr. The corresponding level of TCA-soluble SH groups (Fig. 2: DEX) was 43% of that in control cells (Fig. 2: CON). These results confirm those obtained by Beaver et al. [10]. Interestingly, GSH loss occurred independently of the apoptosis-inducing stimulus and may either be due to extrusion outside the cell [22] or be a consequence of oxidative stress accompanying the onset of apoptosis [8, 9].

On the other hand, the preservation of the intracellular glutathione level was found to be correlated with a prevention of oxidative stress in thymocytes and with a reduction in apoptotic DNA fragmentation [5, 8, 10]. In the present study, 50 μM curcumin enhanced the glutathione content of the DEX-stimulated cells by 92% (Fig. 1: CUR + DEX) and increased the TCA-soluble SH group level by 60% (Fig. 2: CUR + DEX) at 8 hr, while in the absence of curcumin a 64% decrease in glutathione and a 57% decrease in the SH groups was observed in DEX-treated cells (Fig. 1 and 2: DEX, respectively). In nonstimulated thymocytes, the corresponding CUR-induced 107% increase in glutathione (Fig. 1: CUR) and the 98% increase in nonprotein SH group levels (Fig. 2: CUR) were not significantly higher than in DEX-stimulated cells.

The mechanism of GSH synthesis induction by curcumin is not clear. Several mechanisms of γ-glutamylcysteine synthetase regulation are possible, including GSH depletion, formation of GSH conjugates, and production of reactive oxygen species. For example, a sublethal concentration of menadione caused a sustained elevation of intracellular GSH content following a transient GSH depletion via rapid conjugation with menadione and excretion as menadione-glutathione conjugates. This transient decrease could release the feedback inhibition of γ-glutamylcysteine synthetase activity [23]. CUR was reported to conjugate with GSH [24], and formation of such conjugates catalyzed by glutathione S-transferase was proposed to cause depletion of SH groups in liver, lung, stomach, small

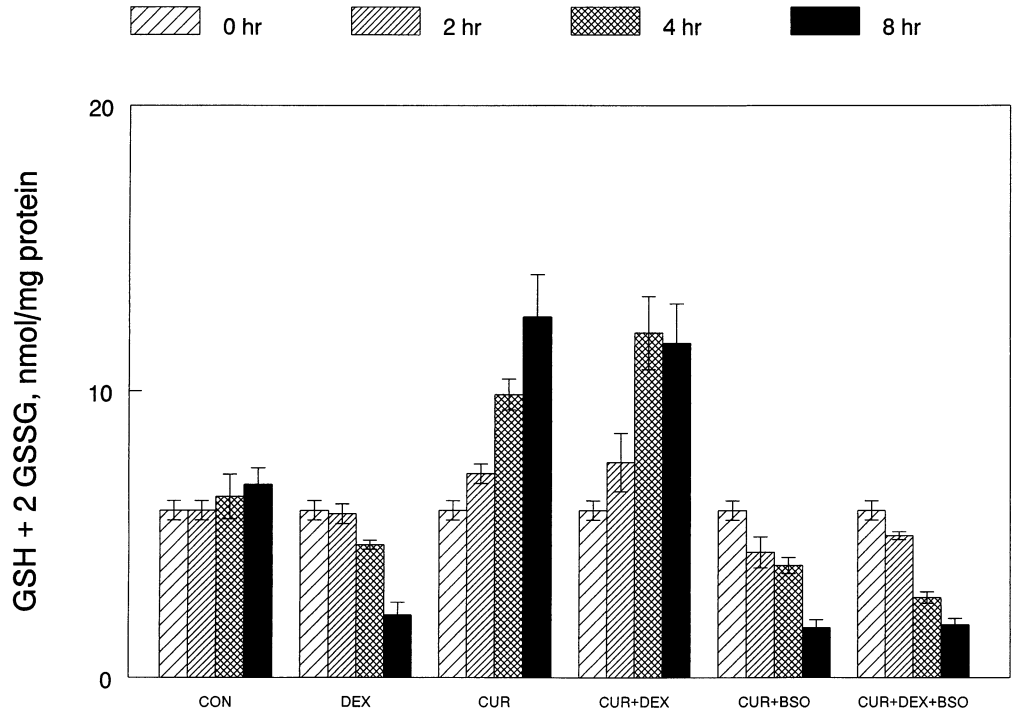

FIG. 1. Effect of DEX (10^{-7} M), CUR (50 μM) and BSO (100 μM) on the total glutathione content (nmol GSH + 2 GSSG/mg of protein) in thymocytes incubated for 2, 4 and 8 hr at 37°. CON, control, nontreated cells. Means ± SEM, N = 9.

intestine and kidney [16]. In contrast, Oetari *et al.* [15] were unable to observe either spontaneous or enzymatic conjugation of GSH to CUR. In view of this controversy, biotransformation of CUR and its effect on phase I and II enzymes still remains to be clarified.

To test the notion that the induction of GSH synthesis

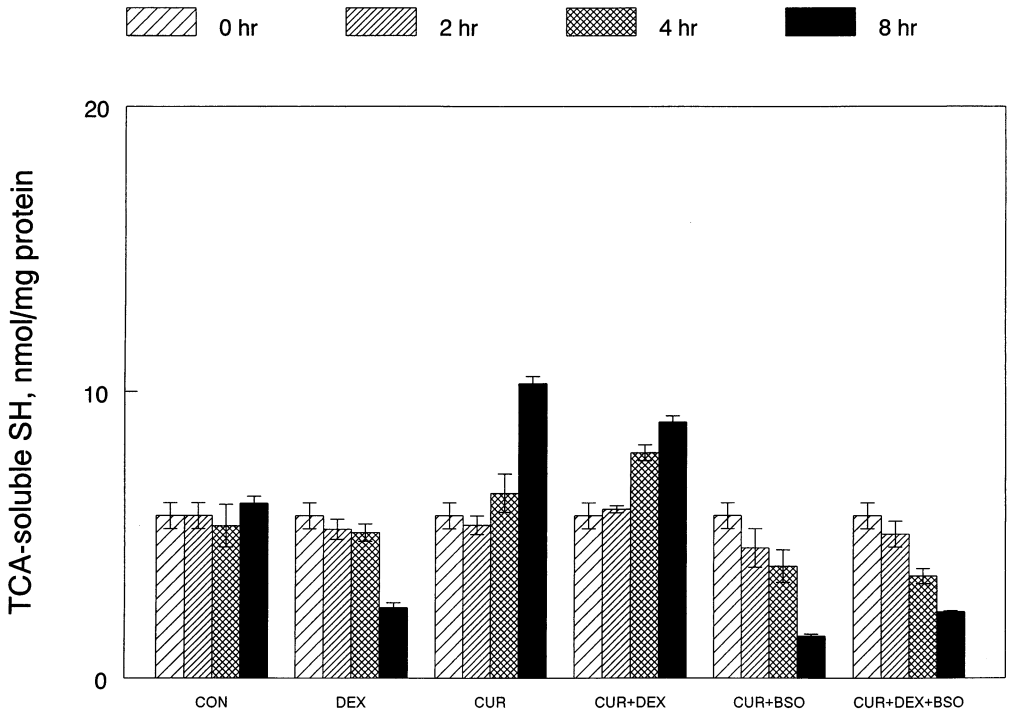

FIG. 2. Effect of DEX (10^{-7} M), CUR (50 μM) and BSO (100 μM) on TCA-soluble thiol group content (nmol SH groups/mg of protein) in thymocytes incubated for 2, 4 and 8 hr at 37°. CON-control, non-treated cells. Mean ± SEM, N = 9.

TABLE 1. Hypo-diploid fractions were harvested at 4, 8, 12 and 20 hr after the indicated treatments.

TIME [hr]	HYPO-DIPLOID FRACTION OF THYMOCYTES [%]											
	CON			BSO			CUR/BSO			CUR/DEX/BSO		
4	1.9	6.8	8.	10.4	8.4	11.5	1.1	0.3	2.8	1.1	0.6	1.3
8	12.3	8.0	12.0	10.3	13.5	15.3	1.0	0.9	2.5	1.1	1.5	2.0
12	11.2	8.3	16.4	14.8	16.5	18.9	1.6	2.6	4.5	1.6	3.2	2.7
20	12.9	16.0	21.8	20.8	20.8	24.0	2.2	3.8	6.3	2.5	4.7	3.7

CON, control (nontreated) cells; DEX (10^{-7} M); CUR (50 μM); BSO (100 μM). Thymocytes were stained with DAPI and sulforhodamine for DNA and protein analysis. The results are from three separate experiments.

is a key factor in apoptosis prevention by CUR, the effect of BSO, a selective inhibitor of γ-glutamylcysteine synthetase, was examined. Thymocyte exposure to 50 μM CUR in the presence of 100 μM BSO resulted in a progressive decrease in total glutathione reaching 70% at 8 hr in either DEX-stimulated (Fig. 1: CUR + DEX + BSO) or nonstimulated cells (Fig. 1: CUR + BSO). The corresponding levels of TCA-soluble SH groups decreased at 8 hr by 76% in both DEX-stimulated (Fig. 2: CUR + DEX + BSO) and nonstimulated cells (Fig. 2: CUR + BSO).

The analysis of DNA fragmentation by agarose gel electrophoresis and measurement of hypo-diploid fraction by flow cytometry showed a spontaneous DNA degradation of 17% in nontreated thymocytes at 20 hr (Table 1: CON and Fig. 3, lower panel: CON), which did not significantly differ from the value of 22% degradation observed in BSO-treated cells (Table 1: BSO and Fig. 3, lower panel: BSO). BSO-mediated glutathione depletion alone was reported to be insufficient to induce apoptosis of Jurkat T cells and human peripheral blood lymphoblasts [6]. However, DEX-treatment (Fig. 3, upper panel: DEX) led to a significantly higher extent of apoptotic fraction than control (45%, data not shown). CUR alone did not cause any DNA fragmentation, and hypo-diploid fraction in CUR-treated thymocytes did not exceed 8% after 24 hr [2]. Curcumin prevented both spontaneous DNA degradation and DEX-induced laddering [2]. We found that BSO coadministered with CUR resulted in thiol depletion corresponding to that observed in apoptotic cells after DEX-stimulation (Fig. 1 and 2: DEX). Unexpectedly, this did not cause the appearance of a sub-G_1 peak on the DNA histogram or DNA laddering on agarose gel in either DEX-treated (Table 1: DEX/CUR/BSO, Fig. 3, upper panel: DEX + CUR + BSO) or nonstimulated cells (Table 1: CUR/BSO, Fig. 3, lower panel: CUR + BSO). This would imply a GSH-independent protection against apoptosis by CUR. Such a mechanism of prevention was also proposed for nerve growth factor and forskolin, which promoted the survival of PC12 cells exposed to hydrogen peroxide. Interestingly, the increase in glutathione level was observed in H_2O_2-treated PC12 cells even without apoptosis suppression by epidermal growth factor [25]. Moreover, sulfhydryl oxidation by diamide could still induce apoptosis in glutathione-depleted cells [6].

In conclusion, the present results indicate that CUR treatment elevated the concentrations of glutathione and nonprotein SH groups, thus preventing the decrease observed during DEX-treatment in apoptotic thymocytes. Nevertheless, this increase in glutathione level did not seem to be necessary for the rescue of cells from apoptosis by CUR because coadministration of BSO and CUR did not affect the antiapoptotic effect of CUR. Taken together, these results favour the hypothesis that changes in glutathione level observed during induction or inhibition of apoptosis are not pivotal in the signaling pathway to

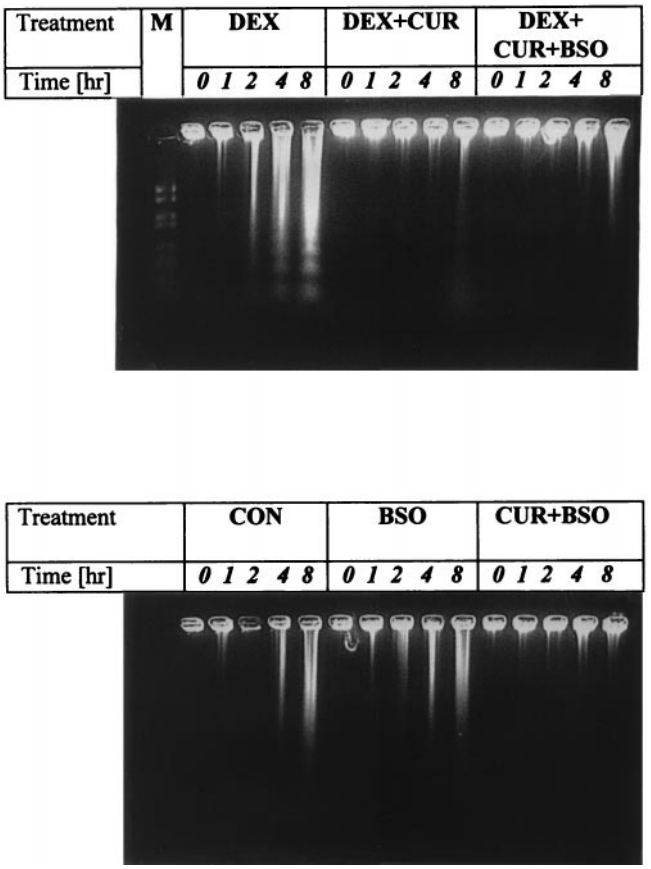

FIG. 3. Agarose gel electrophoresis of DNA extracted from 5 × 10^6 thymocytes harvested at 1, 2, 4 and 8 hr after the indicated treatments. M, DNA fragment marker; CON, control (nontreated) cells; DEX (10^{-7} M); CUR (50 μM); BSO (100 μM). The electrophoregram is representative of at least five separate experiments.

DEX-induced apoptosis. The inhibitory effect of CUR on apoptosis remains to be further investigated.

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