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Curcumin-Induced GADD153 Upregulation: Modulation by Glutathione

David W. Scott and George Loo*

Cellular and Molecular Nutrition Research Laboratory, Department of Nutrition, The University of North Carolina at Greensboro, Greensboro, North Carolina 27402

Abstract As we reported previously, GADD153 is upregulated in colon cancer cells exposed to curcumin. In the present study, we ascertained the involvement of glutathione and certain sulfhydryl enzymes associated with signal transduction in mediating the effect of curcumin on GADD153. Curcumin-induced GADD153 gene upregulation was attenuated by reduced glutathione (GSH) or N-acetylcysteine (NAC) and potentiated by the glutathione synthesis inhibitor, L-buthionine-(S,R)-sulfoximine (BSO). Additionally, GSH and NAC decreased the intracellular content of curcumin. Conversely, curcumin decreased intracellular glutathione and also increased the formation of reactive oxygen species (ROS) in cells, but either GSH or NAC prevented both of these effects of curcumin. In affecting the thiol redox status, curcumin caused activation of certain sulfhydryl enzymes involved in signal transduction linked to GADD153 expression. Curcumin increased the expression of the phosphorylated forms of PTK, PDK1, and PKC-δ, which was attenuated by either GSH or NAC and potentiated by BSO. Furthermore, selective inhibitors of PI3K and PKC-δ attenuated curcumin-induced GADD153 upregulation. Collectively, these findings suggest that a regulatory thiol redox-sensitive signaling cascade exists in the molecular pathway leading to induction of GADD153 expression as caused by curcumin. J. Cell. Biochem. 101: 307–320, 2007. © 2006 Wiley-Liss, Inc.

Key words: curcumin; GADD153; glutathione

Curcumin induces numerous cellular effects related to chemoprevention. For example, curcumin can interfere with cell signaling events associated with colonic inflammation [Salh et al., 2003]. Curcumin is also able to inhibit the proliferation, as well as cause the death, of colon cancer cells [Moragoda et al., 2001]. Recently, it was reported [Scott and Loo, 2004] that curcumin causes upregulation of the growth arrest and DNA damage-inducible gene 153 (GADD153). Being ubiquitously expressed, GADD153 encodes a protein that functions as a leucine-zipper transcription factor [Ron and Habener, 1992]. GADD153 protein is recognized as a member of the CCAAT/EBP transcription factor family, and hence, is also known as CHOP (C/EBP homologous protein). GADD153 is induced by UV radiation [Luethy et al., 1990], DNA-damaging agents [Fontanier-Razzaq et al., 2001], oxidative stress [Guyton et al., 1996], endoplasmic reticulum stress [Nozaki et al., 2001], and nutrient deprivation [Carlson et al., 1993; Abcouwer et al., 1999]. The complete function of GADD153 is not fully known, but one particular role of GADD153 is in the initiation of apoptosis [Maytin et al., 2001].

The intracellular redox environment influences many steps in signal transduction. The glutathione/glutathione disulfide couple is the main system responsible for preserving redox homeostasis [Schafer and Buettner, 2001]. Glutathione has an especial function in protecting sulfhydryl enzymes involved in signal transduction against attack by various oxidants [Forman et al., 2004]. Depleting glutathione in cells resulted in the activation of PKCδ [Domenicotti et al., 2003a], whose catalytic and regulatory domains contain cysteine residues that are susceptible to oxidation [Gopalakrishna and Anderson, 1989]. Other sulfhydryl signaling enzymes, such as ERK

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*Correspondence to: Dr. George Loo, Department of Nutrition, 318 Stone Building, UNC-Greensboro, Greensboro, NC 27402-6170. E-mail: g_loo@uncg.edu

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[de Bernardo et al., 2004], p38 MAPK [Wu and Cederbaum, 2004], and Akt [Abdelmohsen et al., 2003] can also become activated when glutathione homeostasis has been perturbed. Interestingly, curcumin has been reported to induce activation of p38 MAPK [Balogun et al., 2003; Collett and Campbell, 2004], ERK1/2 [Balogun et al., 2003], and JNK [Collett and Campbell, 2004], which are involved in mediating GADD153 gene upregulation as induced by other test agents [Sarkar et al., 2002; Li and Holbrook, 2004]. Hence, there seems to be some commonality regarding the separate effects of curcumin and glutathione depletion on the aforementioned sulfhydryl signaling enzymes. Nevertheless, the precise relationship or interplay between curcumin, glutathione, sulfhydryl signaling enzymes, and GADD153 gene is unclear. Previously, we reported that selective MAPK inhibitors could not inhibit upregulation of GADD153 expression as caused by curcumin [Scott and Loo, 2004]. In contrast, a broad-spectrum protein kinase C inhibitor (staurosporine) was found to be inhibitory, prompting us to now focus on a specific isoform of PKC in conjunction with upstream PDK1, PTK, and PI3 kinase, in order to further clarify the upregulatory effect of curcumin on GADD153 expression.

Another most notable finding from our recent study [Scott and Loo, 2004] was that the thiol antioxidant, NAC, but not some other free radical scavengers or antioxidants, attenuated curcumin-induced GADD153 gene upregulation. Because NAC increases the intracellular concentrations of glutathione [Meister, 1991], our previous finding suggests that glutathione could be important in modulating curcumin-induced GADD153 gene upregulation. Therefore, the objective of the current study was to provide a line of evidence to support the existence of a thiol-redox signaling cascade as part of a molecular pathway leading to GADD153 gene upregulation as caused by curcumin.

MATERIALS AND METHODS

Materials

HCT-116 human colon adenocarcinoma cells and Jurkat T-lymphocytes (clone E6-1) were purchased from ATCC (Manassas, VA). LY294002, wortmannin, and rottlerin were purchased from Calbiochem (San Diego, CA). Curcumin and all other common reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise noted. Anti-GADD 153 (R20) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-PKCδ (Thr^{505}) and anti-phospho-PDK1 (Ser^{241}) were from Cell Signaling (Beverly, MA). Anti-phospho-PTK (4G10) and anti-phospho-EGFR (Tyr^{1173}) were from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cell Culture and Treatment

HCT-116 cells were propagated in McCoy's 5A medium that was supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (10 μg/ml), and 0.2% amphotericin B (250 μg/ml). Jurkat T-lymphocytes were propagated in RPMI-1640 medium containing 2 mM L-glutamine and 10 mM HEPES, but also supplemented 1with 10% FBS, 1 mM sodium pyruvate, 1% penicillin/streptomycin, and 0.2% amphotericin B. Upon reaching approximately 70% confluency, cells were treated with 0–100 μM curcumin for 0–12 h depending upon the experiment. Other experiments with curcumin included pre-incubation or co-incubation of the cells with 100 μM LY294002, 100 nM wortmannin, 5 μM rottlerin, 1 μM staurosporine, 100 μM BSO, 5–20 mM NAC, 50 μM ethacrynic acid (EA), 50 μM chlorodinitrobenzene (CDNB), or 5–20 mM GSH.

RNA Isolation and Determination of GADD153 mRNA Expression

RNA was isolated from cells, and GADD153 mRNA expression was determined by multiplex relative RT-PCR analysis, as both described previously [Scott and Loo, 2004].

Determining Expression of GADD153 Protein and Activated Sulfhydryl Signaling Enzymes

The expression of GADD153 protein and the phosphorylated or activated forms of PTK, PKCδ, PI3K, and PDK1 was determined by immunocytofluorescence microscopy. The cells were fixed with 4% formaldehyde, permeabilized with 0.2% triton X-100, and blocked with 3% bovine serum albumin. The cells were incubated at 4°C overnight with the appropriate primary antibody having the following dilutions: GADD 153 (1:500), phospho-PKCδ (1:250), phospho-PDK1 (1:250), phospho-PTK (1:250), and phospho-EGFR (1:100). Next, the cells were incubated for 2 h at room temperature

with the appropriate secondary antibody (either goat anti-rabbit or goat anti-mouse) conjugated to Alexa Fluor 488 (Molecular Probes, Inc., Eugene, OR) at a dilution of 1:500. Then, the cells were counterstained with propidium iodide.

In assessing the expression of phospho-PKCδ in some experiments, the cells were also counter-stained with MitoTracker Red CMXRos (Invitrogen, Carlsbad, CA) to visualize mitochondria and with 4′,6-Diamidino-2-phenylindole (DAPI) to visualize nuclei.

Determination of Intracellular Curcumin

Cells (seeded and propagated in phenol red-free culture media) were treated with 0–100 μM curcumin for 1 h. Additional experiments with curcumin included pre-treating cells with 5–20 mM of either GSH or NAC for 2 h, or with 100 μM BSO for 18 h. Cells were harvested and then washed thoroughly with cold Hank's buffered saline solution (HBSS) by centrifugation. The pellets were resuspended in lysis solution (0.1% Triton X-100 and 0.1% NP-40 in HBSS) for subsequent sonication and centrifugation for 10 min at 14,000*g*. The resulting supernatant fractions were stored at −80°C. For analysis, aliquots of the thawed-out supernatant fractions (50 μg protein) were diluted with HBSS to a final volume of 100 μl. The content of curcumin in the samples was determined spectrophotometrically by measuring the absorbance at 427 nm [Awasthi et al., 2000].

Determination of Intracellular Glutathione

Cells were exposed to N-ethylmaleimide (25 μM for 3 h), GSH (20 mM for 2 h), NAC (20 mM for 2 h), or curcumin (0–100 μM for 1 h). Afterwards, the cells were washed with cold HBSS, before incubation with 20 μM monochlorobimane (Molecular Probes, Inc.) for 30 min at room temperature in allowing this fluorochrome to react with glutathione (reduced form) in the cells. After washing with cold HBSS, the fluorescence of the cell samples was measured with a fluorometer (excitation/emission: 394/515 nm) and also observed by fluorescence microscopy (UV filter).

Determination of Intracellular Formation of ROS

Cells were exposed to 10 μM curcumin or 200 μM H_2O_2 for up to 1 h. Additionally, some cells were pre-treated with 20 mM of either GSH or NAC for 2 h before exposing the cells to curcumin and H_2O_2. The formation of reactive oxygen species (ROS) in the cells was detected using an Image-iT™ Live Green ROS detection kit (Molecular Probes, Inc.) containing the main reagent 5,6-dicarboxy-2′,7′-dichlorodihydrofluorescein (DCDHF).

Assessment of Chemical Interaction Between Curcumin and GSH

Potential chemical interaction between curcumin and GSH was assessed by using a slight modification of the fluorometric method described by other workers [Debiton et al., 2003]. Briefly, 80 μl of 10 mM phosphate buffer (pH 6.8) containing 0.9% NaCl but also 625 μM of GSH were mixed with 20 μl of curcumin (dissolved in absolute ethanol) to give a final curcumin concentration between 0 and 100 μM. These mixtures were allowed to incubate for up to 2 h at room temperature. Then, the mixtures were incubated for 30 min at 37°C, after mixing in 10 μl of 25 μM monochlorobimane (Molecular Probes, Inc.). The fluorescence of the samples was then measured (excitation/emission: 394/515 nm).

Statistical Analyses of Data

The data were analyzed for significant differences ($P < 0.05$) by analysis of variance (ANOVA) using JMPIN, version 4.0.3 (SAS Institute, Inc., Cary, NC).

RESULTS

Effect of GSH on Curcumin-Induced GADD153 Gene Upregulation

As shown in Figure 1A, treating HCT-116 cells with curcumin caused GADD153 mRNA expression to increase, which was prevented by GSH. This effect of GSH expands our original observation that a precursory compound to glutathione biosynthesis, namely NAC, prevented curcumin-induced GADD153 mRNA upregulation as well [Scott and Loo, 2004]. Conversely, it was next determined if lowering endogenous glutathione produces an opposite result. As shown in Figure 1B, the glutathione synthesis inhibitor, BSO, potentiated to some extent the upregulatory effect of curcumin on GADD153 mRNA, although BSO by itself did not change the constitutive expression of GADD153 mRNA.

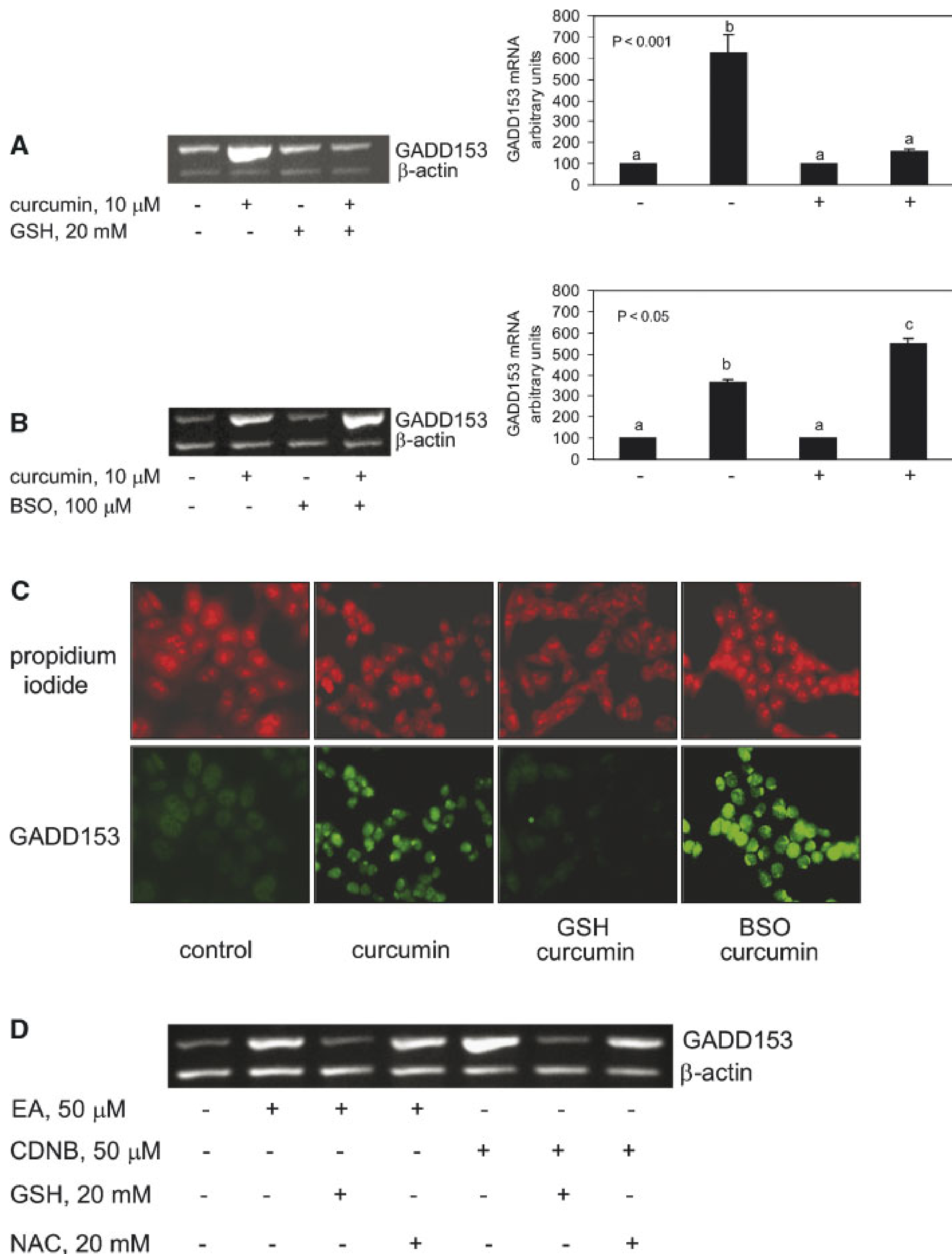

Fig. 1. Impact of glutathione on curcumin-induced GADD153 gene upregulation in HCT-116 cells. In **A**, cells were pre-treated with GSH for 2 h, and then treated with curcumin for 5 h. In **B**, cells were co-treated with BSO and curcumin for 5 h. In **C**, cells were treated with 10 μM curcumin for 12 h or co-treated with either 20 mM GSH or 100 μM BSO and 10 μM curcumin for 12 h. In **D**, cells were pre-treated with GSH or NAC for 2 h and then treated with EA or CDNB for 3 h. In A, B, and D, multiplex relative RT-PCR analysis was conducted using gene-specific primers for the target gene, GADD153, and the internal standard gene, β-actin. In C, GADD153 protein was detected by immunocytofluorescence microscopy using rabbit anti-GADD153 polyclonal antibody along with goat anti-rabbit secondary antibody conjugated to Alexa Fluor 488. Counterstaining was achieved with propidium iodide. Numerical data are expressed as the average ± SEM, n = 3. All images are representative of separate experiments performed at least three times.

A subsequent experiment was performed to see if the changes in GADD153 mRNA correspond to possible changes in GADD153 protein. Untreated HCT-116 cells do not express GADD153 protein, based on the results of both Western analysis and immunocytofluorescence microscopy [Scott and Loo, 2004]. In contrast, the expression of GADD153 protein is clearly evident in curcumin-treated HCT-116 cells (Fig. 1C). However, pre-treatment of cells with GSH inhibited, whereas co-treatment with BSO potentiated, the induction of GADD153 protein caused by curcumin. The propidium iodide counter-staining verifies that there are similar cell densities among the samples being compared.

Next, we asked the question if other compounds, besides curcumin, that are known to react covalently with glutathione could also upregulate GADD153 mRNA expression. As shown in Figure 1D, both EA and CDNB increased the expression of GADD153 mRNA, which was inhibited markedly by GSH although unexpectedly not by NAC.

Apparent Interaction Between Curcumin and Glutathione

HCT116 cells exposed to curcumin acquire the yellowish coloration of the compound itself, but this coloration was greatly reduced by pre-treatment of the cells with either NAC or GSH. Therefore, fundamental experiments were conducted to assess the relative amounts of cellular curcumin. As indicated by the incremental increase in absorbance values (Fig. 2A), cellular curcumin increased in proportion to the concentration of curcumin incubated with the cells. However, GSH and NAC decreased the relative amounts of cellular curcumin, as indicated by the decrease in absorbance values (Fig. 2B).

To complement the above findings, an additional experiment was conducted to ascertain if curcumin and GSH interact under more chemically defined conditions (Fig. 2C). Increasing concentrations of curcumin were incubated with a fixed concentration of GSH, and the relative levels of any unreacted GSH analyzed fluorometrically. As can be seen, curcumin decreased the relative levels of GSH, most likely through covalent interaction.

Effect of Curcumin on Intracellular Glutathione

HCT-116 cells were exposed to curcumin, before labeling the cells with the molecular probe, monochlorobimane, which fluoresces after conjugation with intracellular glutathione. As indicated by the reduction in fluorescence, curcumin decreased intracellular glutathione in a concentration-dependent manner as determined by fluorometry (Fig. 3A, left side of graph) and observing by fluorescence microscopy (Fig. 3B). It was next determined if pre-treating HCT-116 cells with either GSH or NAC could prevent the decrease in intracellular glutathione caused by curcumin (Fig. 3A, right side of graph). As expected, GSH and NAC increased intracellular glutathione. When GSH- and NAC-pre-treated cells were exposed to curcumin, only slight but non-significant decreases (7% and 9%, respectively) from the peak levels of intracellular glutathione induced by GSH and NAC were found. Thus, GSH and NAC largely prevented curcumin-induced lowering of intracellular glutathione, since the GSH- and NAC-pre-treated cells exposed to curcumin still had higher intracellular glutathione than the control cells.

Curcumin Causes Formation of Intracellular ROS and Impact of GSH and NAC

As shown in Figure 4, the formation of ROS, which was detected with the molecular probe DCDHF using H_2O_2-treated cells as a positive control, was evident in curcumin-treated HCT-116 cells utilizing Hoechst 33342 as the counterstain. However, GSH and NAC attenuated the capacity of curcumin to generate ROS.

Activation of Sulfhydryl Signaling Enzymes in Curcumin-Treated HCT-116 Cells

Certain sulfhydryl enzymes functioning as protein kinases in cell signaling pathways are known to become activated as a result of changes in thiol redox status associated with oxidative stress [Forman et al., 2004]. Accordingly, we asked the question if curcumin causes activation (i.e., phosphorylation) of PTK, PDK-1, and PKCδ. As can be seen in Figure 5, curcumin increased the expression of phospho-PTK, phospho-PDK1, and phospho-PKCδ. Incidentally, curcumin had no effect on the expression of PTEN and Akt (data not shown). As further seen in Figure 5, both GSH and NAC negated, whereas BSO potentiated to some extent, the curcumin-induced expression of phospho-PTK, phospho-PDK1, and phospho-PKCδ.

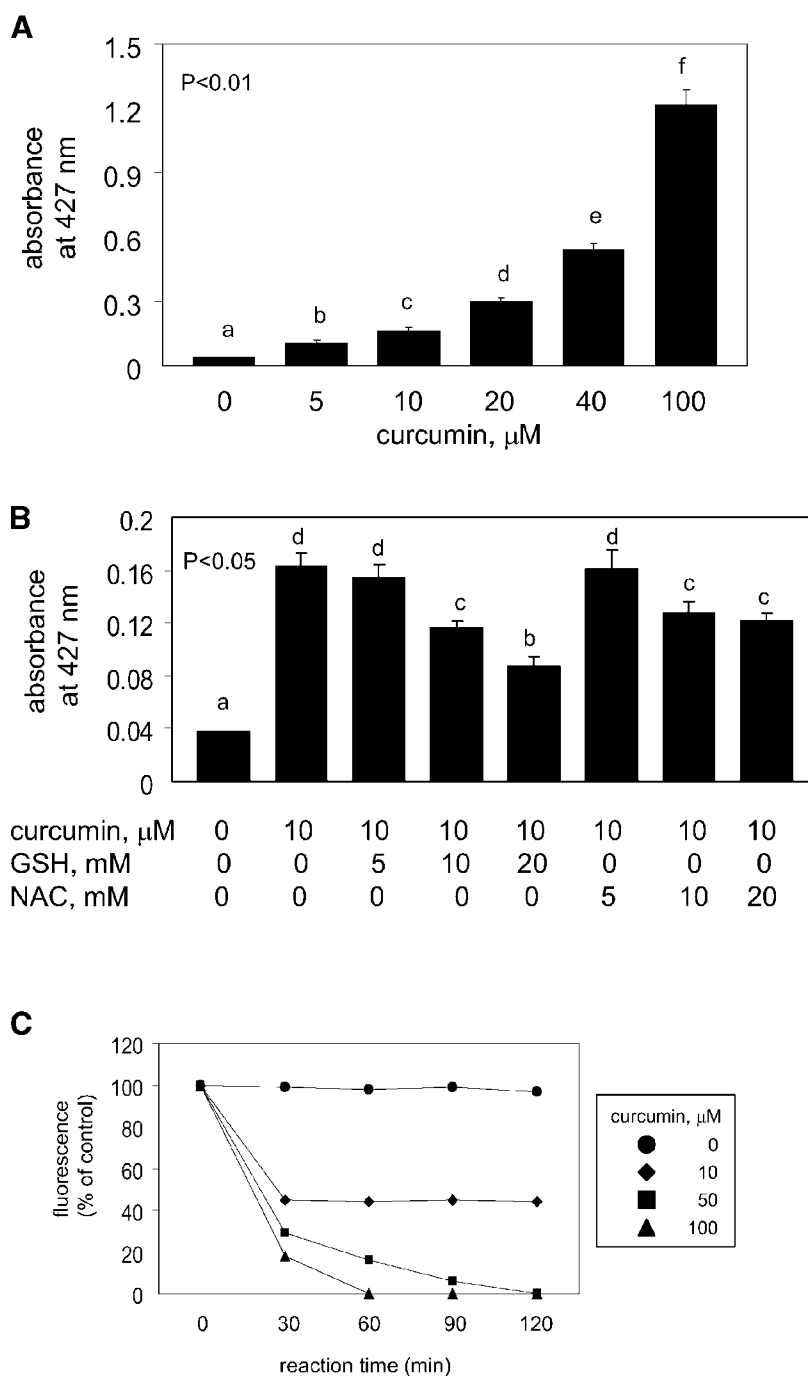

Fig. 2. Interaction between curcumin and glutathione. HCT-116 cells were treated with curcumin for 1 h (**A**). Additionally, HCT-116 cells were pre-treated with either GSH or NAC for 2 h and then treated with curcumin for 1 h (**B**). After extensive washing of the cells, whole lysates were prepared and centrifuged. The absorbance of curcumin present in the supernatant fractions was measured spectrophotometrically. In **C**, curcumin was allowed to react with 500 μM GSH for up to 2 h. Then, the residual unreacted GSH was assayed fluorometrically after addition of monochlorobimane. Numerical data are expressed as the average ± SEM, n = 3.

Effect of Selective Protein Kinase Inhibitors on Curcumin-Induced GADD153 Gene Upregulation

The results of the above experiments indicate that curcumin caused activation of certain protein kinases, raising the possibility that one or more of these enzymes might be involved in a signal transduction pathway leading to the upregulation of GADD153 gene expression. PI3K became our leading candidate to consider

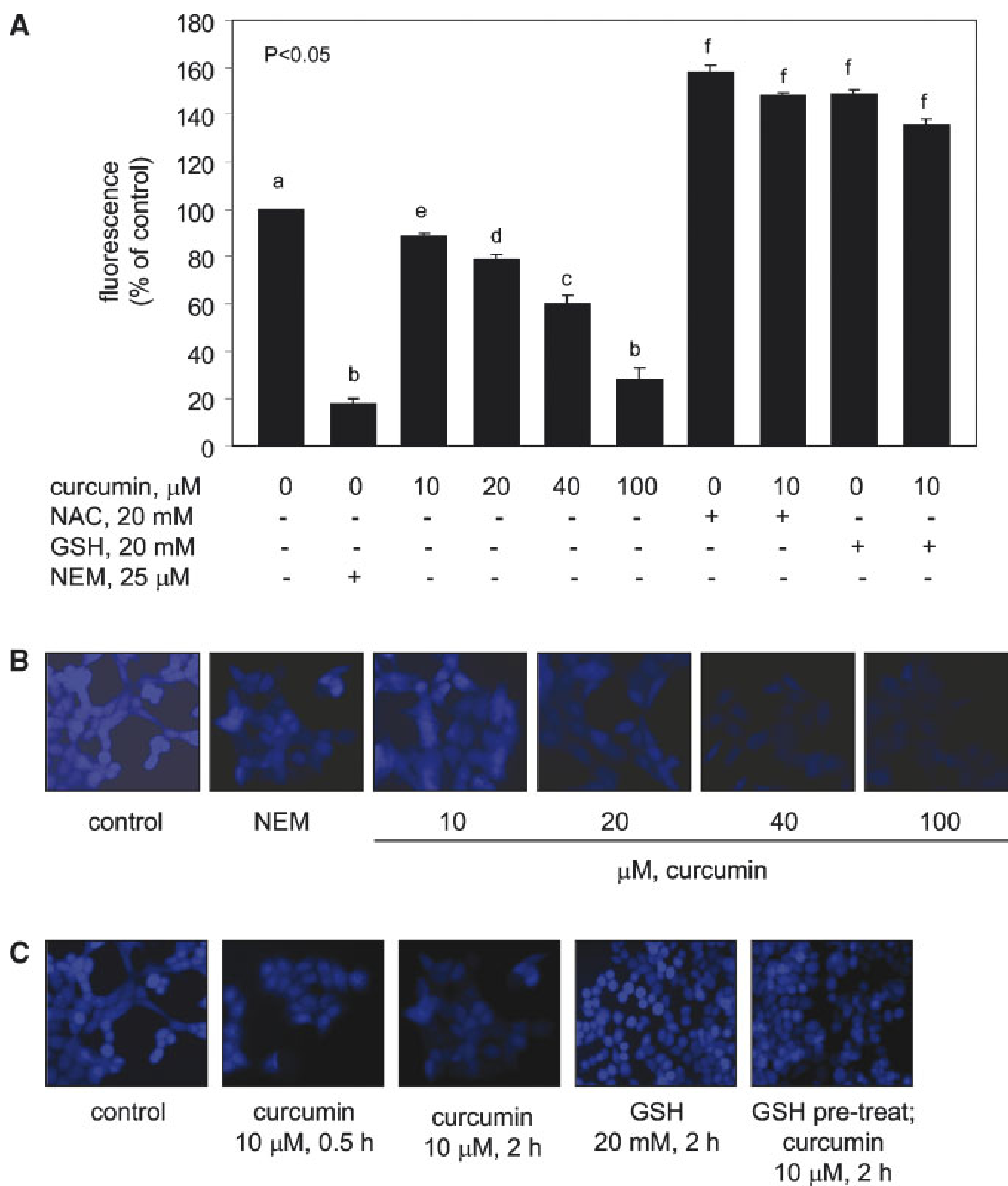

Fig. 3. Curcumin decreases intracellular glutathione. HCT-116 cells were either treated with curcumin for 1 h, or pre-treated with GSH or NAC for 2 h and then treated with curcumin for 1 h. Also, cells were exposed to NEM as a positive control. Next, all cell samples were analyzed fluorometrically (**A**) and also by fluorescence microscopy (**B** and **C**) after incubation of the cells with monochlorobimane. Numerical data are expressed as the average ± SEM, n = 3. All images are representative of separate experiments performed at least three times.

because it is centrally situated for activation by upstream protein kinases, so that activated PI3K can in turn mediate activation of downstream protein kinases such as PKCδ. As such, experiments were performed to determine if PI3K inhibitors (LY294002 and wortmannin) and an inhibitor of PKCδ (rottlerin) affect curcumin-induced GADD153 gene upregulation. LY294002 attenuated (Fig. 6A) and wortmannin prevented (Fig. 6B) the induction of GADD153 mRNA expression caused by curcumin. Furthermore, both LY294002 and wortmannin attenuated curcumin-induced GADD153 protein upregulation in HCT-116 cells (Fig. 6C).

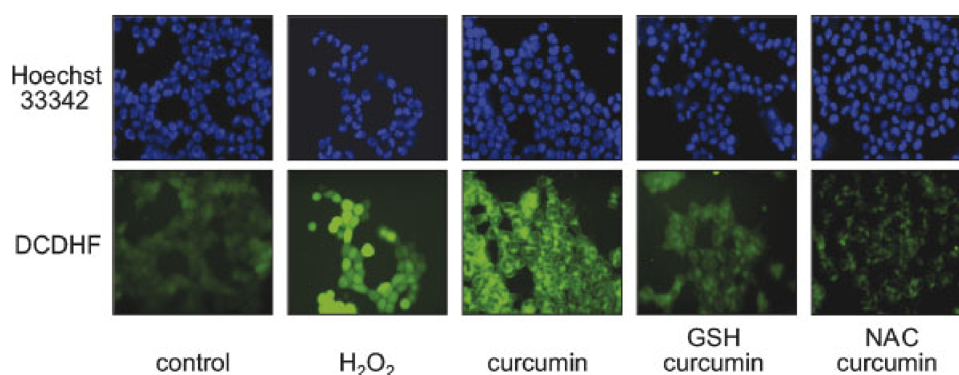

Fig. 4. Production of ROS in curcumin-treated HCT-116 cells. The cells were exposed to either 200 μM H_2O_2 (positive control) or 10 μM curcumin for 1 h. Also, cells were pre-treated with 20 mM of either GSH or NAC for 2 h before being treated with curcumin. Intracellular ROS were detected by fluorescence microscopy using the molecular probe, DCDHF, with Hoechst 33342 as a counterstain. All images are representative of separate experiments performed at least three times.

Unexpectedly, rottlerin actually induced GADD153 mRNA and also signs of apoptosis in HCT-116 cells under our experimental conditions (data not shown). Therefore, because of their lower sensitivity to being killed by rottlerin, Jurkat T-lymphocytes were consequently used out of necessity to evaluate the effect of rottlerin, on curcumin-induced GADD153 mRNA upregulation (Fig. 6D). As can be seen, rottlerin attenuated the upregulatory effect of curcumin on GADD153 mRNA expression in Jurkat cells, suggesting an involvement of PKCδ in mediating the effect of curcumin. GADD153 protein expression in such cells was not determined because of the apparent cytotoxicity of rottlerin with longer incubation periods.

Curcumin-Induced Activation of PKCδ via PI3K and Localization of Phospho-PKC-δ

The above data (Fig. 6) suggests an involvement of the PI3K pathway in curcumin-induced GADD153 gene upregulation. Activation of PKCδ is known to occur through multiple pathways, including the PI3K pathway, when cells are subjected to stress. Hence, the effect of the PI3K inhibitors on curcumin-induced activation of PKCδ was assessed in HCT-116 cells. As can be seen (Fig. 7A), curcumin increased the expression of phospho-PKCδ. The effect of curcumin on PKCδ was attenuated by either LY294002 or wortmannin. Additionally, the broad-spectrum PKC inhibitor, staurosporine, which we previously reported is able to inhibit curcumin-induced GADD153 gene upregulation [Scott and Loo, 2004], attenuated the expression of phospho-PKCδ as induced by curcumin. But, the selective PKCδ inhibitor, rottlerin, had even a greater attenuating effect than staurosporine on curcumin-induced phospho-PKCδ expression.

Upon activation, PKCδ is known to undergo translocation, such as to the mitochondria in glutathione-depleted neuroblastoma cells [Domenicotti et al., 2003b] and in H_2O_2-treated myeloid leukemia cells [Majumder et al., 2001]. To determine if curcumin caused a similar mitochondrial translocation but in HCT116 cells, the distribution of phospho-PKCδ was evaluated. As shown in Figure 7B, phospho-PKCδ seemed to be more centralized in nuclei rather than mitochondria, although it also appears that some of the phospho-PKCδ was diffusely distributed in the cytoplasm as well under these experimental conditions. Interestingly, phospho-PKCδ was present for the most part in those cells with nuclei having chromatin condensation and fragmentation, which would suggest entry of these cells (which also were shrunken in size) into a state of apoptosis. Nuclear translocation of PKCδ upon apoptotic stimuli and also a regulatory role of PKCδ in apoptosis have been reported [Brodie and Blumberg, 2003].

DISCUSSION

The initial results provide three significant findings to support the view that curcumin-induced GADD153 gene upregulation in HCT-116 cells is modulated by glutathione. First, pre-treating the cells with GSH negated the

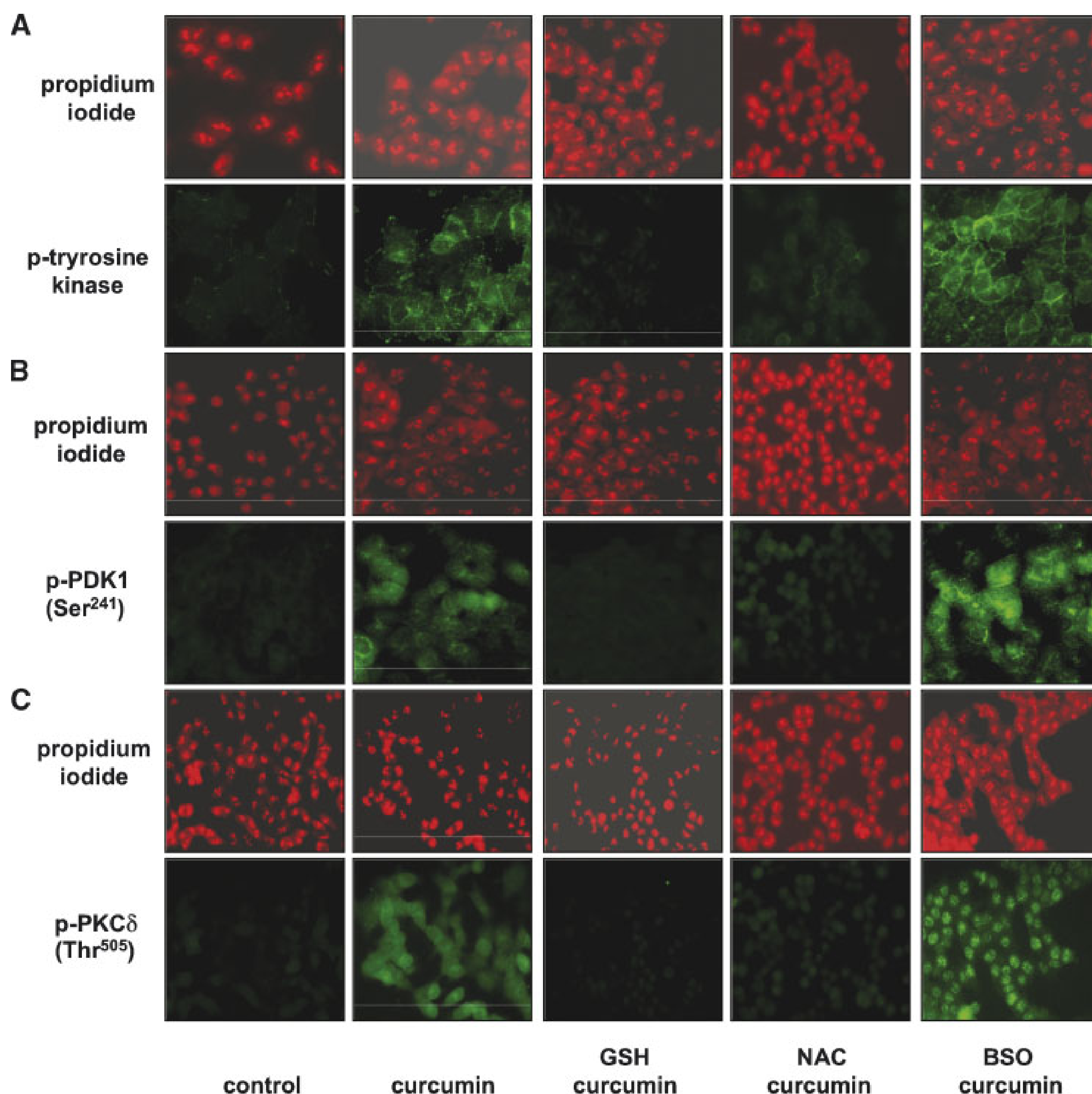

Fig. 5. Curcumin causes activation of sulfhydryl signaling enzymes in HCT-116 cells. The cells were treated with 10 μM curcumin for 30 min. Also, cells were pre-treated with 20 mM of either GSH or NAC for 2 h, or pre-treated with 100 μM BSO for 18 h, followed by treatment with curcumin. The expression of the phosphorylated forms of PTK (**A**), PDK1 (**B**), and PKCδ (**C**) was assessed by immunocytofluorescence microscopy. Counterstaining was achieved with propidium iodide. All images are representative of separate experiments performed at least three times.

ability of curcumin to increase expression of GADD153 mRNA and protein. Incidentally, our recent study [Scott and Loo, 2004] showed that NAC attenuated curcumin-induced GADD153 gene upregulation as well. Because it is known that exogenously-added glutathione is not readily transported into cells [Meister, 1991] and glutathione can react covalently with curcumin [Awasthi et al., 2000], it is possible that the GSH largely reacted with curcumin extracellularly, thereby principally negating the effect of curcumin on GADD153 gene expression in this manner. On the other hand, curcumin was able to actually decrease endogenous glutathione in HCT-116 cells. It is known that curcumin can become conjugated to intracellular glutathione [Wortelboer et al., 2003]. Second, depleting intracellular glutathione by inhibiting its

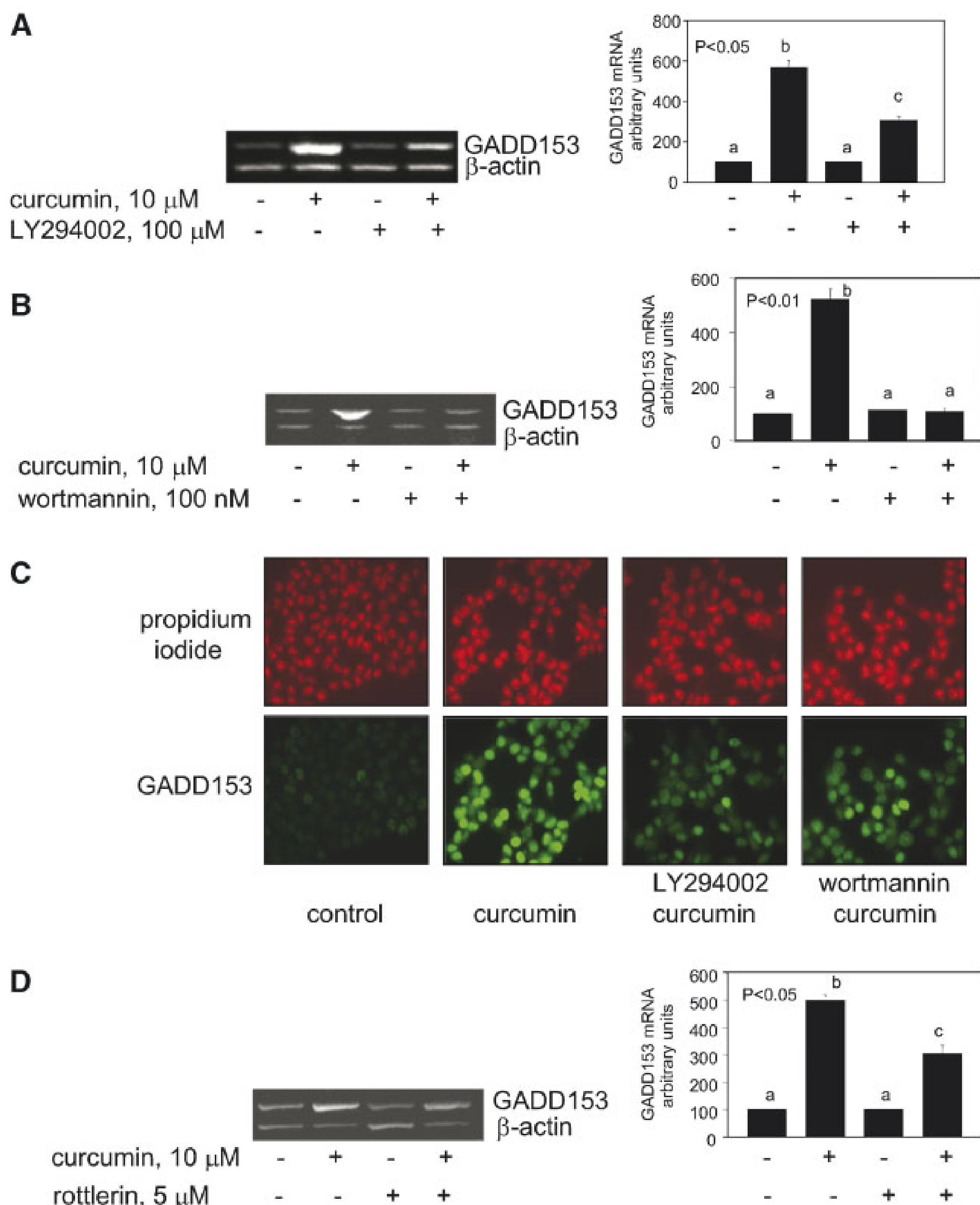

Fig. 6. Effect of selective protein kinase inhibitors on curcumin-induced GADD153 gene upregulation. HCT-116 cells were pre-treated for 30 min with either 100 μM LY294002 (**A**, **C**) or 100 nM wortmannin (**B**, **C**) to target PI3K, followed by treatment of the cells with 10 μM curcumin for 5 h (A, B) or 12 h (C). In **D**, Jurkat T-lymphocytes were pre-treated for 0.5 h with rottlerin to target PKCδ, followed by treatment of the cells with curcumin for 5 h. In A, B, and D, GADD153 mRNA expression was assessed by multiplex relative RT-PCR analysis using gene-specific primers for the target gene, GADD153, and the internal standard gene, β-actin. In C, GADD153 protein expression was assessed by immunocytofluorescence microscopy with propidium iodide counterstaining. Numerical data are expressed as the average ± SEM, n = 3. All images are representative of separate experiments performed at least three times.

biosynthesis with BSO potentiated to some extent curcumin's ability to upregulate GADD153 gene expression. It has been suggested that BSO-induced depletion of glutathione and consequent greater generation of ROS make cells more sensitive to curcumin [Syng-Ai et al., 2004]. Third, EA and CDNB, which can react covalently with glutathione, especially in the presence of glutathione-S-transferase [Awasthi et al., 2000], increased

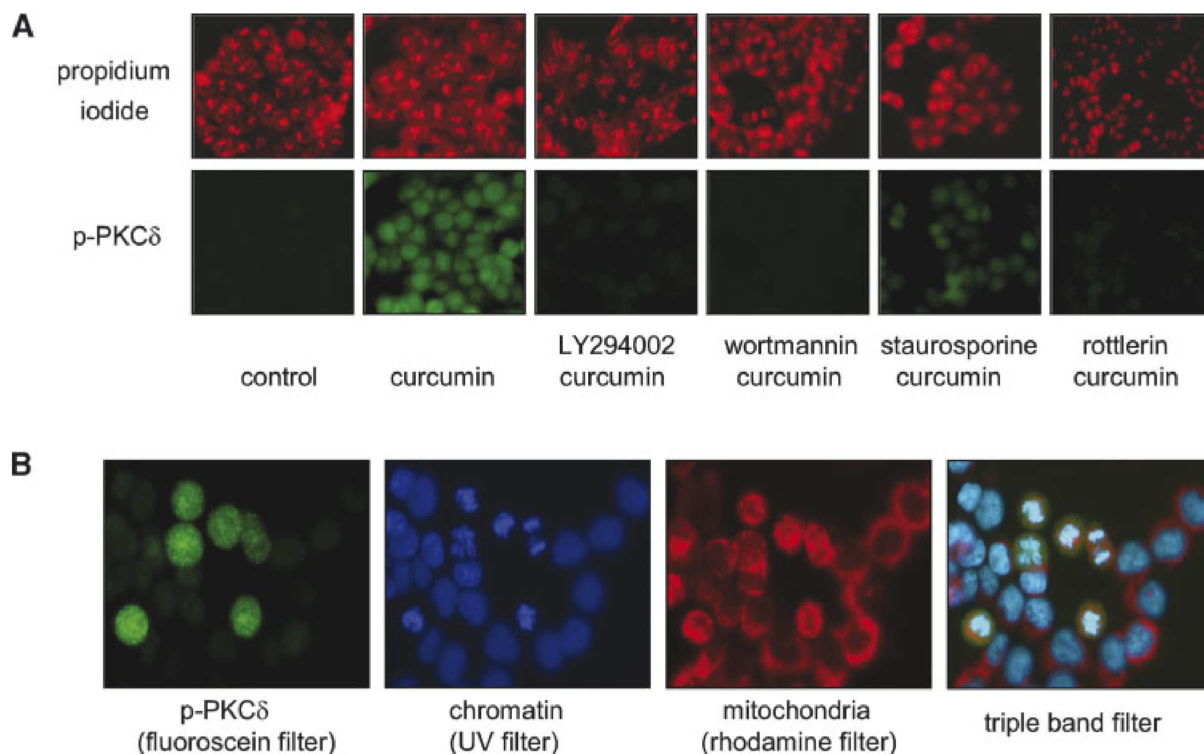

Fig. 7. Curcumin causes activation of PKCδ through the PI3K pathway and nuclear translocation of phospho-PKCδ. **A**: Effect of PI3K and PKC inhibitors on curcumin-induced PKCδ activation. HCT-116 cells were exposed to 10 μM curcumin for 30 min. Additionally, cells were pre-treated with LY294002 (100 μM), wortmannin (100 nM), staurosporine (1 μM), or rottlerin (5 μM) for 30 min before treatment of the cells with curcumin. The expression of the phosphorylated form of PKCδ was assessed by immunocytofluorescence microscopy with propidium iodide counterstaining. **B**: Subcellular localization of phospho-PKCδ in curcumin-treated HCT116 cells. Phospho-PKCδ was detected with an anti-phospho-PKCδ antibody along with a secondary antibody conjugated to Alexa Fluor 488 (green fluorescence). The cells were counter-stained with DAPI (blue fluorescence) and MitoTracker Red CMXRos (red fluorescence) to visualize nuclei and mitochondria, respectively. All images are representative of separate experiments performed at least three times.

GADD153 mRNA expression. These effects of EA and CDNB were prevented by pre-treatment of the cells with GSH.

In antagonizing glutathione, curcumin could also be inducing oxidative stress that promotes redox-sensitive gene expression. Curcumin is capable of causing the formation of ROS, such as O_2^- and H_2O_2 [Ahsan and Hadi, 1998]. It is known that ROS [Oh-Hashi et al., 2004], but also reactive nitrogen species such as peroxynitrite [Oh-Hashi et al., 2001], can initiate activation of the GADD153 gene. In accord with these facts, curcumin induced the formation of ROS in HCT-116 cells that could be almost completely inhibited by pre-treating the cells with either GSH or NAC. These thiol antioxidants also were able to prevent curcumin-induced GADD153 gene upregulation. Thus, the capability of curcumin to cause ROS to be generated, combined with the ability of curcumin to neutralize glutathione via covalent interaction, seemed to have created a cellular environment characterized by increased oxidative stress and perturbed glutathione homeostasis that were conducive for GADD153 gene activation.

The current findings suggest that ROS are central in mediating the effect of curcumin. In the appropriate amounts, ROS act as secondary messengers in initiating signal transduction [Khan and Wilson, 1995]. This particular action of ROS may reflect oxidation of susceptible cysteine sulfhydryl groups contained in certain protein kinases, resulting in conformational changes so that there is enzyme activation. PTK is thought to be activated in such a manner during oxidative stress [Nakashima et al., 2002]. Thus, in the present study, it is conceivable that the ROS induced by curcumin may have mediated the activation or phosphorylation of

PTK, but also PDK1 and PKCδ (although not EGFR, data not shown), by promoting oxidation of critical cysteine residues in these enzymes. Our finding regarding PKCδ, which has cysteine-rich motifs in its regulatory domain that are particularly susceptible to oxidation [Gopalakrishna and Anderson, 1989], is in harmony with the observation that BSO-induced depletion of glutathione and consequent stimulation of ROS production in neuroblastoma cells was associated with activation of PKCδ [Domenicotti et al., 2003b]. It is possible, however, that the ROS generated as a result of exposing HCT-116 cells to curcumin may not be the only mechanism of PKCδ activation. It has been reported [Stempka et al., 1999] that in addition to undergoing autophosphorylation brought about by conformational changes during oxidative stress, PKCδ can become activated by other distinct mechanisms such as proteolytic cleavage, translocation of the full-length peptide from cytosol to membrane, or binding to cofactors.

Our latter set of data suggests that glutathione regulates certain cell signaling steps induced by curcumin. NAC attenuated and BSO potentiated, the activating effects of curcumin on PTK, PDK1, and PKCδ, implying that glutathione protects or preserves the cysteine sulfhydryl groups of these redox-sensitive enzymes from being oxidized as a result of the ROS generated by curcumin. This critical function of glutathione is compromised when glutathione is consumed in scavenging ROS during a state of oxidative stress [Forman et al., 2004]. As such, by scavenging ROS produced by curcumin, glutathione would be capable of influencing the cascade of signaling events leading to redox-sensitive gene activation.

In initially investigating the potential involvement of protein kinases in mediating curcumin-induced GADD153 gene upregulation [Scott and Loo, 2004], we had ruled out an involvement of MAPK. However, because the broad-spectrum PKC inhibitor, staurosporine, was found to attenuate the effects of curcumin on GADD153 gene expression, we considered PKC and upstream PI3K herein. It is known that PI3K can become activated during oxidative stress [Niwa et al., 2003] and when intracellular glutathione has been perturbed [Kang et al., 2003]. Once activated, PI3K facilitates the production of PIP_3, which acts as a docking site for PDK1. Once docked, PDK1 can activate a wide variety of kinases, including protein kinase A, protein kinase G, and protein kinase C that comprise the AGC kinases [Vanhaesebroeck and Alessi, 2000]. Of primary interest is the known ability of PDK1 to activate members of the PKC family, but particularly PKCδ. Recently, it was reported that PKCδ is involved in mediating H_2O_2-induced GADD153 mRNA upregulation in Jurkat T-lymphocytes [Oh-Hashi et al., 2004].

Our data suggest some involvement of PI3K and PKCδ in curcumin-induced GADD153 gene upregulation in HCT-116 cells. Based on the substantial attenuating effects of LY294002 and wortmannin (PI3K inhibitors) on the induction of GADD153 mRNA and protein by curcumin, these results suggest that PI3K is largely involved in mediating the ability of curcumin to cause GADD153 gene upregulation. Although PI3K is known to activate Akt (protein kinase B), we eliminated Akt in being involved because the phosphorylated form of Akt was not induced in curcumin-treated HCT-116 cells (data not shown). However, in using the PKCδ inhibitor (rottlerin) in evaluating a possible involvement of PKCδ in mediating curcumin-induced GADD153 gene upregulation, our initial experiments with HCT-116 cells revealed that rottlerin alone caused GADD153 gene upregulation. Rottlerin also seemed to cause apoptosis after a short incubation period with HCT-116 cells, which is consistent with the report [Tillman et al., 2003] that rottlerin sensitizes HCT-116 cells to apoptosis, possibly by uncoupling of mitochondria respiration. Furthermore, it has been reported that mitochondrial-generated ROS, such as the ROS released by uncoupling, can upregulate the GADD153 gene [Carriere et al., 2004]. Therefore, additional experiments with rottlerin aimed at determining the involvement of PKCδ in curcumin-induced GADD153 gene upregulation were conducted in Jurkat T-lymphocytes, where rottlerin was shown to prevent H_2O_2-induced GADD153 mRNA upregulation but with no apparent cytotoxic effects [Oh-Hashi et al., 2004]. Our results show that rottlerin partially attenuated curcumin-induced GADD153 mRNA upregulation.

When cells are under stressful conditions, PKCδ can become phosphorylated by a number of protein kinases including PI3K, PDK1, and PTK [Ettinger et al., 1996; Le Good et al., 1998; Sun et al., 2000; Tapia et al., 2003]. Therefore, to

provide evidence of a signaling link between PI3K and PKCδ in the molecular cascade initiated by curcumin, we show that curcumin-induced expression of the phosphorylated form of PKCδ in HCT-116 cells could by prevented by inhibiting PI3K with either LY294002 or wortmannin. Therefore, these findings suggest that curcumin-induced phosphorylation or activation of PKCδ occurs through the PI3K pathway, implying also that such PKCδ activation may not be the result of either autophosphorylation or phosphorylation by other protein kinases. Moreover, these findings further support the notion that PI3K and PKCδ are key parts of an important thiol redox-sensitive cascade in a molecular pathway leading to curcumin-induced GADD153 gene upregulation. This new knowledge should contribute to a better understanding of the molecular mechanism of curcumin as a potential chemopreventive agent especially against colon cancer.

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