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Delayed Activation of PKCδ and NFκB and Higher Radioprotection in Splenic Lymphocytes by Copper (II)–Curcumin (1:1) Complex as Compared to Curcumin

Amit Kunwar,[1] H. Narang,[2] K. Indira Priyadarsini,[1] M. Krishna,[2]* R. Pandey,[2] and K.B. Sainis[2]

[1]Radiation & Photochemistry Division, Bhabha Atomic Research Centre, Mumbai 400085, India
[2]Radiation Biology & Health Sciences Division, Bhabha Atomic Research Centre, Mumbai 400085, India

Abstract A mononuclear 1:1 copper complex of curcumin had been found to be superior to curcumin in its anti-oxidant properties. This paper describes the radio-protective effects of the complex in splenic lymphocytes from swiss mice. The complex was found to be very effective in protecting the cells against radiation-induced suppression of glutathione peroxidase, catalase and superoxide dismutase (SOD) activities. Both curcumin and the complex protected radiation-induced protein carbonylation and lipid peroxidation in lymphocytes with the complex showing better protection than curcumin. It also showed better overall protection by decreasing the radiation-induced apoptosis. The kinetics of activation of PKCδ and NFκB after irradiation in presence or absence of these compounds was looked at to identify the molecular mechanism involved. The modulation of irradiation-induced activation of PKCδ and NFκB by curcumin and the complex was found different at later time periods although the initial response was similar. The early responses could be mere stress responses and the activation of crucial signaling factors at later time periods may be the determinants of the fate of the cell. In this study this delayed effect was observed in case of complex but not in case of curcumin. The delayed effect of the complex along with the fact that it is a better free radical scavenger must be the reason for its better efficacy. The complex was also found to be less cytotoxic then curcumin at similar concentration. J. Cell. Biochem. 102: 1214–1224, 2007. © 2007 Wiley-Liss, Inc.

Key words: curcumin; radioprotection; signaling; anti-oxidant; curcumin–copper complex

Radiotherapy is one of the treatment modalities for several kinds of malignancies. The major drawback in the treatment is that normal cells, in the vicinity of the tumor, also receive radiation doses similar to the tumor. This leads to undesirable side effects and increases the risk of secondary cancers. When ionizing radiation hits the cell various reactive oxygen species (ROS) are produced leading to the activation of signaling pathways that may be cytotoxic or cytoprotective [Riley, 1994]. Therefore, there is a need for prevention of such side effects. Natural agents that have low cytotoxicity and high anti-oxidant and anti-inflammatory activities are being studied for their efficacy in preventing radiation-induced side effects and killing of normal cells. Curcumin, an important natural phytochemical, found in the rhizomes of *Curcuma longa* or turmeric is an efficient free radical scavenger and reacts with most of the ROS such as superoxide, peroxyl, hydroxyl and nitrogen oxide radicals [Priyadarsini et al., 2003; Vajragupta et al., 2004] and has been studied in great detail. In addition to its anti-radical activity, curcumin has been shown in vivo to enhance the activities of anti-oxidant enzymes in liver and kidney [Iqbal et al., 2003]. It has also been reported to modulate the PKC and NF-κB signaling pathways [Siwak et al., 2005; Rushworth et al., 2006]. Activation of PKC pathway has been implicated in cell

*Correspondence to: M. Krishna, Head, Radiation Signalling Section, Radiation Biology & Health Sciences Division, Bhabha Atomic Research Centre, Mumbai 400085, India. E-mail: malini@barc.gov.in

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survival under oxidative stress, where it has been reported to upregulate the transcription of several cytoprotective genes such as glutamyl cysteine ligase modulatory (GCLM) and homo-oxygenase-1 (HO-1) [Rushworth et al., 2006]. Activation of NF-κB by ionizing radiation has been found to protect cells from apoptosis and its inhibition enhanced radiation-induced apoptosis [Wang et al., 1996]. An additional mechanism has been said to be via upregulation of the transcription of ROS scavenging enzymes. One major class of these protective enzymes belongs to the mitochondrial superoxide dismutase (SOD) [Suresh et al., 1994], which is also the transcript of NF-κB [Murley et al., 2006]. Other important classes of anti-oxidant enzymes are glutathione peroxidase (GPx) and catalase [Matés, 2000].

Since copper chelates of anti-oxidants are well known as SOD mimics [Annaraj et al., 2004; Baum and Ng, 2004], a copper (II)–curcumin complex with the stoichiometry of 1:1 was synthesized and characterised in our laboratory [Barik et al., 2005, 2007]. The complex had high stability constant ($\log(K_f) = 15$). Studies on the superoxide radical scavenging ability and the SOD mimicking activity of the complex, showed that the complex had higher SOD like activity as compared to curcumin. Based on these studies, it was felt that the complex might be more efficient in protecting against radiation-induced damage in cells. We, therefore, investigated the mechanism of action of the complex and compared it to curcumin. Radiation-induced damage to lipids and proteins, suppression of cellular anti-oxidant defense systems, apoptosis and activation of PKC and NF-κB signaling in splenic lymphocytes were looked at.

The structures of curcumin and the copper–curcumin complex are given in Scheme 1.

MATERIAL AND METHODS

Chemicals

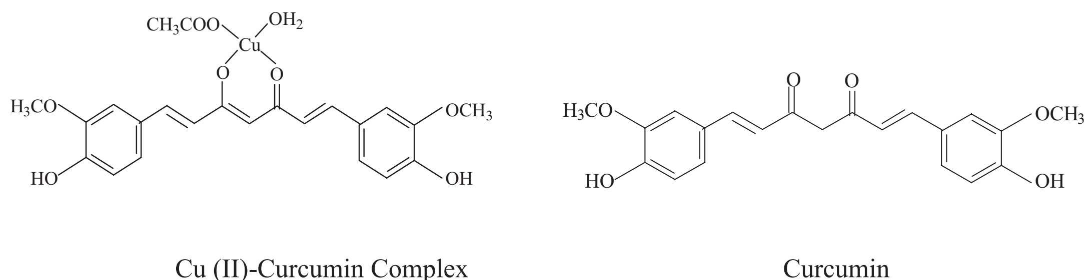
Curcumin, thiobarbituric acid (TBA), butylated hydroxy toluene (BHT), components of cell culture medium (serum free RPMI 1640 medium), hydrogen peroxide, xanthine, NADPH, EDTA, EGTA, glycerol, PMSF, Triton X-100, NP-40, Tris base, HEPES buffer and propidium iodide (PI) were obtained from Sigma–Aldrich. Xanthine oxidase was obtained from Calbiochem. All other chemicals were locally procured and were with more than 98% purity. Solutions were always made in fresh nanopure water.

Synthesis

The complex was synthesized by refluxing equimolar mixture of copper acetate and curcumin in dry ethanol. The precipitated complex was washed several times by cold ethanol and water and dried in vacuum to get dry powder. This was characterised by elemental analysis and different spectroscopic techniques. Details of the synthesis and characterisation of this complex have been reported by Barik et al. [2005].

Animals

Eight to 10-week-old inbred swiss albino male mice weighing approximately 20–25 g and reared in the animal house of Bhabha Atomic Research Centre, were used. Animals were maintained on a standard laboratory diet with water ad libitum in polypropylene cages and air-conditioned (24 ± 2°C) rooms with a 12 hourly dark and light schedule. All experiments were conducted with strict adherence to the ethical guidelines laid down by the institutional animal ethics committee of Bhabha Atomic Research Centre.



Scheme 1. Structure of Cu(II)-curcumnin complex and curcumin.

Spleen Cell Suspension

For preparation of spleen cell suspension mice were sacrificed by cervical dislocation. Spleen was removed from the mice and single cell suspension from spleen was obtained by gently teasing the organ in to RPMI 1640 medium (containing 15 mM HEPES, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 20 μM 2-mercaptoethanol) using a sterile nylon mesh. Red blood cells were eliminated by treatment with 0.83% ammonium chloride solution. Lymphocyte rich cells were further washed with medium to remove traces of ammonium chloride and the viability was assessed by trypan blue dye exclusion. This preparation is referred to as splenic lymphocytes.

Drug Preparation and Treatment of Splenic Lymphocytes

Curcumin and the complex are insoluble in water, but show fairly good solubility in alkaline water. Therefore, a stock solution of curcumin and the complex were prepared in nitrogen saturated aqueous solutions with 0.2 M NaOH. Nitrogen was used to prevent oxidation and the stability of the solutions during the experimental period was monitored by following the absorption spectra from time to time. Splenic lymphocytes suspended in serum free RPMI 1640 medium were seeded at 2.5×10^6 cells/ml to which stock solution of either curcumin or the complex was added to get final concentration of 10 μM. The pH of the culture medium after the addition of these agents was found to be 7.5. Fetal bovine serum (FBS) was added at a concentration of 5% (v/v), 30 min after addition of compounds. The incubation of lymphocytes with compounds for 30 min prior to addition of FBS was referred to as treatment of cells. Further treatment incubation was done at 37°C in humified incubator with 5% CO_2 in air for different time points (0–60 min) desired in the study.

Irradiation Protocol

The lymphocytes were incubated with curcumin or the complex for 30 min as described in earlier section followed by γ-irradiation (2 Gy) using a ^{60}Co γ-source with a dose rate of 6 Gy min^{-1} as measured by standard Fricke dosimeter [Spinks and Woods, 1990]. Further irradiation, FBS was added at a concentration of 5% (v/v) and incubation was done at 37°C in humified incubator with 5% CO_2 in air for different times (0–60 min) as desired in the study.

Assays of Anti-oxidant Enzymes Activity

Cells were collected at a time point of 1 h by centrifugation at $900 \times g$ for 4 min from irradiated and unirradiated groups, washed twice with 10 mM phosphate buffered saline (PBS), were suspended in an appropriate volume of 10 mM Tris/HCl, pH 7.4, and disrupted twice by means of a bioruptor (Cosmos Bio, Tokyo, Japan) at 200 W for 30 s each. The supernatant was collected after centrifugation at $17{,}000 \times g$ for 15 min. Protein estimation in the supernatant was carried out by DC protein assay kit (Bio-Rad) following the manufacturer's instructions. Fifty micrograms of total protein was used for the enzyme assays. SOD activity was determined as described previously [Hodges et al., 2000]. Briefly, superoxide radicals were generated by enzymatic reaction of xanthine (50 μM) with xanthine oxidase (10 mU/ml) in presence of Tris buffer (pH 7.4) and 600 μM EDTA. The superoxide radicals generated by this method were allowed to react with cytochrome *c* (Fe^{3+}) (9.5 μM) to produce reduced cytochrome *c* (Fe^{2+}), absorbing at 550 nm. The change in absorbance per unit time (A/min) was monitored up to 300 s. The concentration of xanthine oxidase was adjusted such that △A/min is ~0.025. This system was used to determine SOD activity in whole cell homogenate. In the presence of whole cell homogenate the △A/min was found to be reduced. The average difference of △A/min recorded in the absence and presence of whole cell homogenate, respectively, was calculated and represented as activity. The catalase activity was determined as described earlier [Abei, 1984] by monitoring the enzyme-catalysed decomposition of H_2O_2 at 240 nm. Reaction mixture contained 15 mM of H_2O_2 in 50 mM of potassium phosphate buffer, pH 7.0. The reaction was initiated by addition of whole cell homogenate, and the decrease in absorbance/min was recorded. The average decrease in absorbance/min, calculated from the initial linear portion of curve was presented as activity. GPx activity was measured by the method as described previously [Tappel, 1978] in which GPx activity was coupled to the oxidation of (3 mM) NADPH by glutathione reductase. The oxidation of NADPH in the

presence of whole cell homogenate was followed as decrease in absorbance at 340 nm at 37°C for 20 min. The average decrease in absorbance/min was calculated from the most linear part of the curve and presented as activity.

Assessment of Lipid Peroxidation

Cells were collected at a time point of 1 h by centrifugation at $900 \times g$ for 4 min from irradiated and unirradiated groups, washed twice with 10 mM PBS and were finally suspended in 300 μl of 10 mM PBS. To this 900 μl of TBA reagent (100 μM BHT, 0.67% (w/v) TBA in a 10% (w/v) trichloroacetic acid solution and 0.25N HCl) [Kumar et al., 2004] was added. The reaction mixture was incubated at 85°C for 20 min and cooled to ambient temperature. Samples were centrifuged at $12{,}000 \times g$ for 10 min at 25°C and thiobarbituric acid reactive substances (TBARS) in the supernatant were estimated by measuring the absorbance at 532 nm ($\varepsilon532 = 1.56 \times 105\ M^{-1}\ cm^{-1}$) [Sreejayan et al., 1997]. The results are expressed in terms of formation of TBARS/10^6 cells.

Protein Oxidation

Supernatants as obtained for enzyme activity at a time point of 1 h were used for measurement of protein carbonylation using the protocol described earlier [Oliver et al., 1987]. In brief, cell free extract containing approximately 100 μg soluble proteins in 10 mM PBS, pH 7.4 was taken and total proteins were precipitated with ice chilled 10% trichloroacetic acid (TCA). The pellet was suspended in 0.2% dinitrophenyl hydrazine (DNPH) in 2N HCl and incubated at room temperature for 2 h. Proteins were reprecipitated with TCA and excess DNPH was removed with several washes of 50% ethyl acetate in ethanol. Decolorized protein pellet was dissolved in 6N guanidine hydrochloride and the optical density was measured at 370 nm. Protein concentration was determined by DC protein assay kit (Bio-Rad) following the manufacturer's instructions.

Western Blotting

Cells were collected at different time points (0–60 min) from irradiated and unirradiated groups by centrifugation at $900 \times g$ for 4 min and washed twice with 10 mM PBS. Cells were lysed using lyses buffer as described earlier [Kurrey et al., 2005]. Proteins were estimated using DC protein assay kit (Bio-Rad) following the manufacturer's instructions. Protein samples were denatured at 95°C with sample buffer (0.125 M Tris buffer (pH 6.8), 4% SDS, 20% glycerol, 2% 2-ME, 0.03 mM bromophenol blue) for 5 min and were separated on 12% SDS-PAGE gel for MnSOD and 8% SDS-PAGE gels for IκBα and PKCδ. Proteins were transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, UK), blocked overnight with a blocking solution (5% BSA in TBS containing 0.1% Tween 20) and were exposed to the primary Ab for 2 h at room temperature. Equivalent protein loading was demonstrated by staining the membranes in 1% Ponceau S. Primary antibodies included rabbit monoclonal anti-IκBα (1:1,000), rabbit monoclonal anti-phospho (Ser40) PKCδ (1:1,000) both from Cell Signalling, sheep monoclonal anti-MnSOD (1:1,000) (Calbiochem). After washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP) linked secondary Ab anti-rabbit (1:1,500), anti-sheep (1:15,00) (Roche Molecular Bio Chemicals, Germany) and signals were detected using BM chemiluminiscence western blotting kit (Roche Molecular Bio Chemicals, Germany) as per the manufacturer's instructions. For reprobing, membranes were stripped with 100 mM 2-ME, 2% SDS and 62.5 mM Tris–HCl (pH 6.9) for 20 min at 50°C followed by immunoblotting as mentioned above. The band intensity was quantified by gelquant software (version 2.7 DNR imaging systems Ltd., Israel). Fold changes were calculated after normalization to Ponceau S.

Isolation of Nuclei for NFκB Measurement

Nuclear extracts were prepared at a time point of 1 h from irradiated and unirradiated groups as described earlier [Gao et al., 2004]. In brief, cells were washed two times with 10 mM PBS, resuspended and incubated on ice for 15 min in hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 0.6% NP-40). Cells were vortexed gently for lysis and nuclei were separated from the cytosol by centrifugation at $12{,}000 \times g$ for 1 min. Nuclei resuspended in buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and were shaken for 30 min at 4°C. Nuclear extracts were obtained by centrifugation at $12{,}000 \times g$ and protein concentration was measured by DC

protein assay kit (Bio-Rad) following the manufacturer's instructions. Nuclear extract was fractionated on 12% SDS-PAGE gel, transferred to nitrocellulose membrane, and probed with rabbit anti-NF-κB (p65) antibody using BM chemiluminiscence western blotting kit (Roche Molecular Bio Chemicals, Germany) as per the manufacturer's instructions. Equivalent protein loading was demonstrated by staining membranes in 1% Ponceau S. Fold changes were calculated after normalization to Ponceau S.

Estimation of Apoptosis

For the estimation of apoptosis irradiated and unirradiated groups were cultured for 24 h at 37°C in humified chamber with 5% CO_2 atmosphere. After 24 h, cells were collected by centrifugation at $900 \times g$ for 4 min, washed twice with 10 mM PBS, suspended in 1 ml of staining solution containing 0.5 μg/ml PI, 0.1% sodium citrate and 0.1% Triton X-100 and then analyzed by flow cytometry. PI binds to DNA and the intensity of fluorescence at 580 nm represents the total DNA content. A majority of cells was in G1 phase of cell cycle (2n DNA content). The pre-G1 phase population represented the apoptotic cells.

Statistical Analysis

Each experiment was performed at least in triplicate using a single splenic lymphocyte preparation. The entire experiment was repeated twice. Results are presented as means ± SEM, n = 6. Data were analyzed with Student's t-test and P values ≤ 0.05 were considered as a significant.

RESULTS

Effect of Curcumin and the Complex on Anti-oxidant Enzymes Status in Spleenocytes With or Without Irradiation

The activities of the different anti-oxidant enzymes (SOD, GPx and catalase) in splenic lymphocytes estimated 1 h after treatment with either curcumin or the complex under irradiated (2 Gy) and unirradiated conditions are shown in Figure 1. Treatment with the complex and curcumin alone resulted in significant ($P < 0.05$) increase in glutathione peroxidase and catalase activities, SOD activity did not show any increase rather it was reduced. GPx and catalase activities increased by 17 and 46%,

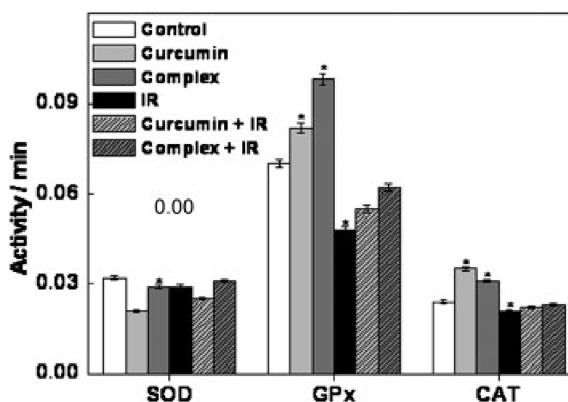


Fig. 1. Effect of curcumin and the complex on anti-oxidant enzymes activities of γ-irradiated and unirradiated (5×10^6) splenic lymphocytes. Enzymes activities were estimated using cell lysates prepared at a time point of 1 h from irradiated and unirradiated groups. Control represents splenic lymphocytes treated with only vehicle (0.2 M NaOH). *$P < 0.05$ as compared to control. IR—irradiation (2 Gy).

respectively, in curcumin treated cells and by 40 and 29%, respectively, in the complex treated cells. The decrease in SOD activity was observed to be lesser extent in the complex treated cells compared to curcumin treated cells. Irradiation alone led to a significant ($P < 0.05$) decrease in activities of all the anti-oxidant enzymes, which was reversed by pre-treatment with curcumin or complex. The complex was more effective in this respect.

Effect of Curcumin and the Complex on Irradiation-Induced Protein Oxidation and Lipid Peroxidation in Spleenocytes

The relative TBARS formation, indicative of lipid peroxidation in spleenocytes at a time point of 1 h after irradiation alone (2–8 Gy) or after pretreatment with curcumin or the complex are shown in Figure 2. Lipid peroxidation in spleenocytes increased with increase in dose of irradiation. Pretreatment with either curcumin or the complex led to reduction in the extent of lipid peroxidation at all the doses. The complex was found to be more effective in reducing the formation of TBARS at all doses compared to curcumin. The percentage inhibition of lipid peroxidation by the complex at different doses of 2, 4, 6 and 8 Gy was 75, 70, 28 and 20%, respectively, while that for curcumin under similar conditions was 50, 40, 20 and 15%, respectively.

Inset of Figure 2 shows the relative carbonyl formation due to protein oxidation induced by

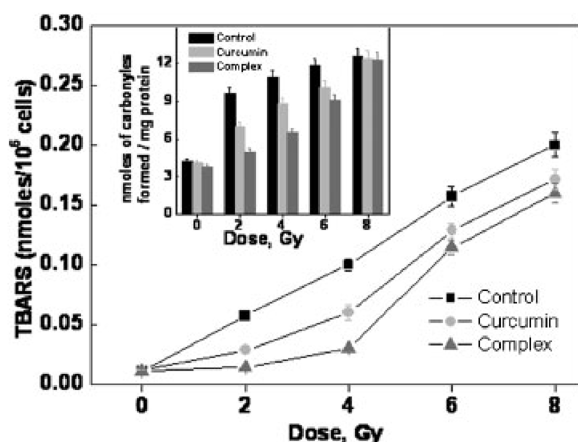


Fig. 2. Line graph showing inhibition of lipid peroxidation estimated in terms of TBARS at a time point of one hour in splenic lymphocytes (5×10^6) treated with curcumin and the complex, and exposed to varying dose of γ-radiation (2–8 Gy). Inset shows bar graph indicating inhibition of protein oxidation estimated in terms of protein carbonylation at a time point of 1 h by compounds at different absorbed doses (2–8 Gy) of γ-radiation.

γ-radiation from 2 to 8 Gy both in the absence or presence of either curcumin or the complex at identical time point. The results indicate inhibition of irradiation-induced increase in protein oxidation by these compounds, with the complex showing much stronger inhibition. The percentage reduction of carbonyl formation by the complex at different doses of 2, 4, 6 and 8 Gy was 48, 40, 24 and 3%, respectively, while with curcumin the percentage reduction was found to be 28, 19, 15 and 2%, respectively.

In order to understand the factors responsible for the protection afforded by the complex in the above studies, the signaling pathways were looked at.

Effect of Curcumin and the Complex on Protein Kinase Cδ Phosphorylation After Irradiation

The phosphorylation at Ser 40 residue of PKCδ in lymphocytes was assessed at 5, 15, 30 and 60 min after irradiation (2 Gy) alone or after pretreatment with either curcumin or the complex. Western blots and relative intensities representing the level of phosphorylated PKCδ at the above four time points are shown in Figure 3(A)–(D). Following γ-irradiation an increased phosphorylation of PKCδ was observed within 5 min, which reappeared at 60 min. When irradiation was preceded by curcumin or the complex treatment, the increased phosphorylation observed at 5 min was suppressed. Here the complex shows stronger inhibition than curcumin. However, at 60 min, pretreatment with the complex resulted in increased phosphorylation of PKCδ, as compared to irradiation alone or pretreatment with curcumin. Treatment with curcumin or the complex alone also resulted in increased phosphorylation of PKCδ but with different kinetics of activation. The

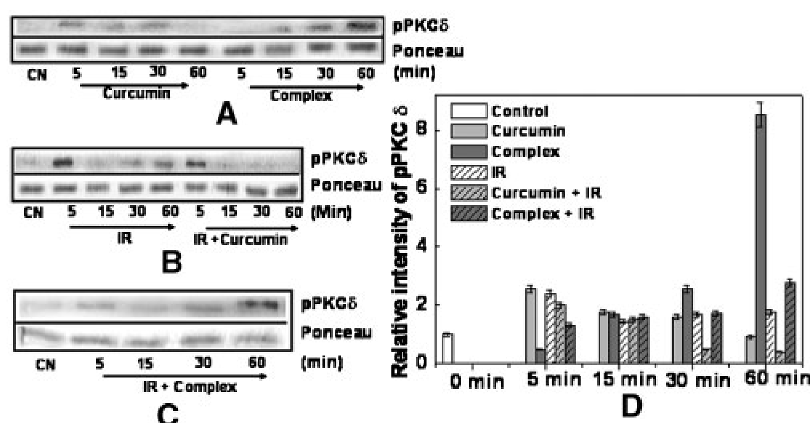


Fig. 3. Splenic lymphocytes (2.5×10^6) were treated with curcumin and the complex, irradiated with γ-radiation and then cultured. Whole cell extract was prepared at different times and western blotting was performed with phosphospecific (Ser40) antibody of PKCδ. **A**: Western blot analysis showing the comparative level of PKCδ in lymphocytes at different time points after treatment with curcumin and the complex. **B**: Western blot analysis showing the comparative level of PKCδ in curcumin pretreated lymphocytes at different time points after irradiation. **C**: Western blot analysis showing the comparative level of PKCδ in the complex pretreated lymphocytes at different time points after irradiation. **D**: Bar graph showing relative intensities of phospho PKCδ. Control represents splenic lymphocytes treated with only vehicle (0.2 M NaOH). Staining of the membrane with 1% Ponseau represents loading control. IR—irradiation (2 Gy).

complex showed time dependent increase, up to eightfold at 60 min, indicating that the complex promotes growth in normal cells since increased expression of PKCδ could be anti-apoptotic.

Effect of Curcumin and the Complex on IκBα Accumulation

The accumulation of IκBα in lymphocytes was checked at 5, 15, 30 and 60 min after irradiation (2 Gy) alone or after pretreatment with either curcumin or the complex. Western blot analysis and the relative intensities representing the level of IκBα at the above four time points are shown in Figure 4(A)–(D). Following γ-irradiation the degradation of IκBα was observed within 5 min. At 30 min there was a significant reappearance of IκBα and it accumulated to ~3 folds at 60 min. Pretreatment with curcumin or the complex prevented the initial degradation of IκBα at 5 min. The complex was also found to be more effective in significantly reversing the irradiation-induced IκBα accumulation at 60 min. Treatment with both the compounds alone reduced the levels of IκBα as compared to control although like PKCδ the kinetics of accumulation was different. IκBα after degradation is known to release NF-κB, which transports to the nucleus where it acts as a transcription factor for many anti-apoptotic genes.

Effect of Curcumin and the Complex on Nuclear Transport of NF-κB and *MnSOD* Expression

Figure 5 shows western blots (A) and relative intensities (B), respectively, of NF-κB in nuclear extract of same samples at a time point of 60 min. Following irradiation (2 Gy), the level of NF-κB decreased in the nuclear extract of untreated lymphocytes. However, when irradiated spleenocytes were pretreated with curcumin or the complex, the decrease in the level of NF-κB was reversed marginally by curcumin but very efficiently by the complex. Treatment with either curcumin or the complex alone also reduced the level of NF-κB in nuclear extract. The reduction after curcumin and the complex treatment was ~60 and 40%, respectively.

Western blot and relative intensities of MnSOD in untreated or treated lymphocytes are also shown in Figure 5. A marked increase in the level of MnSOD was observed after irradiation. Treatment with curcumin or the complex before irradiation inhibited this increase with the complex showing more profound effect than curcumin. The complex and curcumin treatment alone inhibited the expression of *MnSOD*.

Effect of Curcumin and the Complex on γ-Radiation-induced Apoptosis

Figure 6 shows percentage apoptosis in irradiated and unirradiated lymphocytes treated

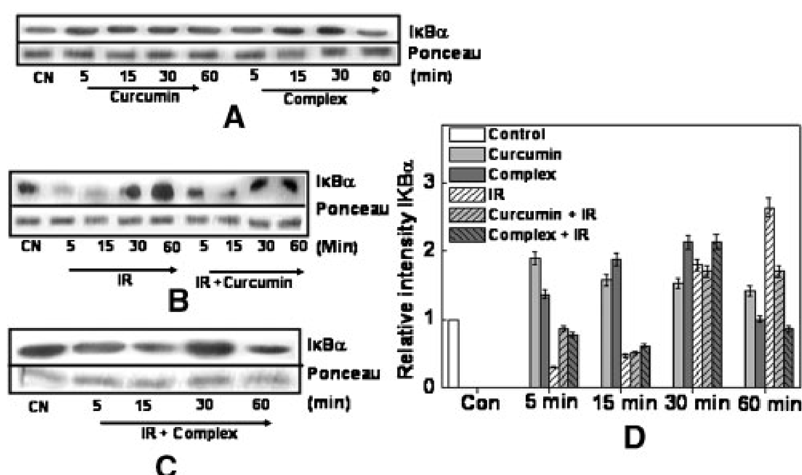


Fig. 4. Splenic lymphocytes (2.5×10^6) were treated with curcumin and the complex, irradiated with γ-radiation and then cultured. Whole cell extract was prepared at different times and western blotting was performed with IκBα antibody. **A**: Western blot analysis showing the comparative level of IκBα in lymphocytes at different time points after treatment with curcumin and the complex. **B**: Western blot analysis showing the comparative level of IκBα in curcumin pretreated lymphocytes at different time points after irradiation. **C**: Western blot analysis showing the comparative level of IκBα in the complex pretreated lymphocytes at different time points after irradiation. **D**: Bar graph showing relative intensities of IκBα. Control represents splenic lymphocytes treated with only vehicle (0.2 M NaOH). Staining of the membrane with 1% Ponseau represents loading control. IR—irradiation (2 Gy).

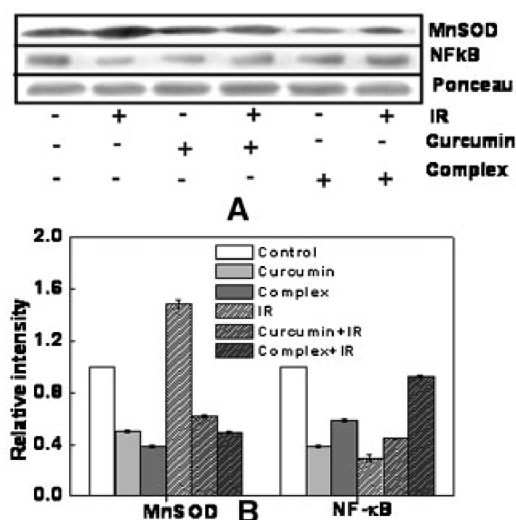


Fig. 5. Effects of curcumin and the complex on the nuclear transport of NF-κB and expression of *MnSOD* in γ-irradiated and unirradiated lymphocytes. **A**: Western blot analysis showing the comparative level of NF-κB in nuclear extract and MnSOD in cytoplasmic extract of (2.5×10^6) irradiated and unirradiated splenic lymphocytes after 1 h of treatment with curcumin and the complex. **B**: Bar graph representing relative intensities of NF-κB and MnSOD. Staining of the membrane with 1% Ponseau represents loading control. IR—irradiation (2 Gy).

with curcumin and the complex at 24 h. Although both curcumin and the complex alone led to increased apoptosis, as did the vehicle, they were able to confer protection in irradiated cells. Among the compounds the percent apoptosis was found to be significantly ($P < 0.05$) less in case of complex treated cells. Irradiation (2 Gy) alone led to substantial increase in apoptosis. The complex and curcumin were able to decrease the percentage apoptosis observed after irradiation and the complex was found to be more effective. The decrease in irradiation-induced apoptosis by the complex was 20%.

DISCUSSION

Favourable effects of curcumin on several parameters of oxidant–anti-oxidant balance have been reported by many investigators [Sharma, 1975; Strasser et al., 2005]. Its metal chelating ability has been utilized by several groups to further enhance its anti-oxidant activity [Afanas'ev et al., 2001; Vajragupta et al., 2003]. However, there are very few reports in the literature about the modulation of radiation-induced redox disturbances by curcumin [Chan et al., 2003; Krishnan et al.,

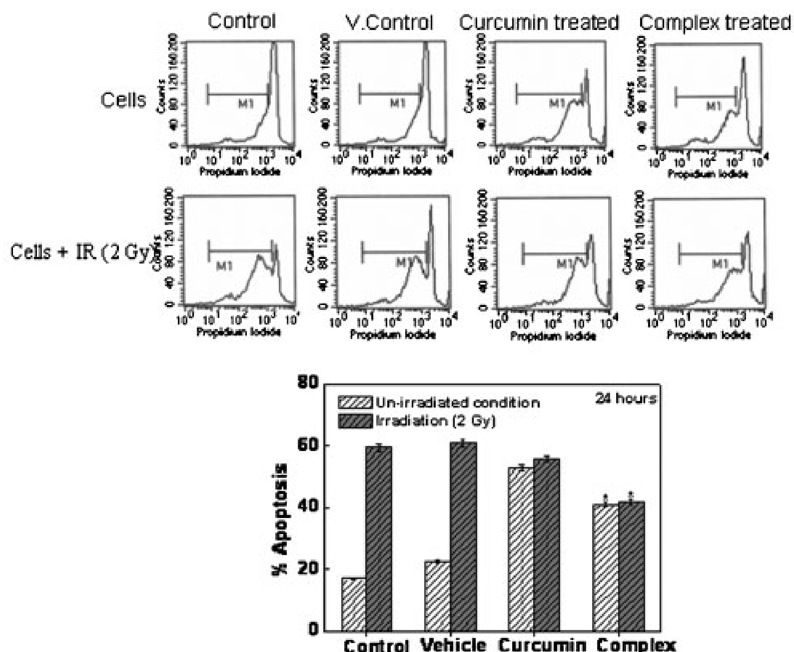


Fig. 6. Effects of curcumin and the complex on cellular cytotoxicity estimated in terms of apoptosis of spleen lymphocyte (2.5×10^6) after treatment with compounds and modulation of γ radiation-induced apoptosis. Lymphocytes were cultured for 24 h before staining with propidium iodide. The percentage apoptotic cells were calculated from pre-G1 peak as estimated through Fluorescence Activated Cell Sorter (FACS). Control represents spleen lymphocyte treated neither with any agent nor vehicle (0.2 M NaOH). *$P < 0.05$ as compared to curcumin treated lymphocytes. IR—irradiation (2 Gy).

2006; Srinivasan et al., 2006] and in fact no reports on such changes by metal chelates of curcumin. The present study therefore deals with the modulation of γ-radiation activated signaling pathways as well as other radiation-induced disturbances such as change in anti-oxidant enzymes level, damage of cellular lipid, protein and apoptosis by a copper(II)–curcumin complex in splenic lymphocytes and the results have been compared with those of curcumin.

Treatment with either curcumin or the complex resulted in increased activities of anti-oxidant enzymes after 1 h in unirradiated lymphocytes. In agreement with the previous reports on suppression of anti-oxidant defences under elevated oxidative stress [Daniel et al., 1998; Bosch-Morell et al., 1999; Han et al., 2005], a decrease in activities of anti-oxidant enzymes after γ-irradiation (2 Gy) was observed. Both curcumin and the complex prevented the decrease in GPx and catalase activities after irradiation. The complex showed very significant reversal of irradiation-induced decrease in GPx and SOD activities. The functional efficiency of GPx depends on the optimal supply of the redundant GSH [Vivancos and Moreno, 2005]. The exogenously applied anti-oxidant prevents the depletion of GSH and thus maintains its cellular concentration needed for GPx activity. Therefore the superior GPx activity associated with the complex may be because of its enhanced anti-radical activity as reported in our previous studies [Barik et al., 2005]. However the superior SOD activity under irradiated condition shown by the complex could be because of its acquiring the additional superoxide-dismutating copper centre.

Both curcumin and the complex showed considerable reduction in TBARS and carbonyls formation in lymphocytes up to a dose of 4 Gy, further increase in the dose did not cause any further decrease. At all the absorbed doses employed in these studies, the complex provided better protection than curcumin.

Apart from free radical scavenging activities, the compounds like curcumin are involved in activation or inhibition of various signaling pathways, which are crucial to the cell. Some signaling components whose activation could be beneficial to the cell in terms of its survival were hence looked at; in the presence of both the compounds and their relative efficacies against irradiation-induced stress was compared. We followed the activation of PKCδ and NFκB after irradiation in presence or absence of these compounds over a time period of 5–60 min. The inhibition of irradiation-induced phosphorylation of PKCδ at 5 min was observed by both the complex and curcumin, with complex showing stronger inhibition. Since the early response seen at 5 min could be stress responses, which occurs due to the production of reactive oxygen species, the complex therefore seems to be a better scavenger of ROS in vivo and could prevent the initial activation of PKCδ observed with irradiation. Additionally, the reduction in irradiation-induced degradation of IκBα at 5 min with the complex strengthens the fact that the complex is more effective radical scavenger and hence prevents the immediate effects of irradiation. After the initial damage response, the cell assesses the damage and activates the signaling pathways, which then determines the fate of the cell. In this study the activation, which is observed at 5 min, may be a stress response following which at later time periods (60 min) the signals that will protect the cells were initiated. PKCδ has been shown previously to activate various cytoprotective genes [Rushworth et al., 2006], The extensive phosphorylation of PKCδ at 60 min in the complex pretreated and irradiated spleenocytes indicate that the complex is more efficient than curcumin in activating cytoprotective pathways after the damage. Likewise with IκBα, after initial reduction observed at 5 min, there was an accumulation at 30 min. At 60 min there was again a decrease in the level of IκBα in the complex treated irradiated lymphocytes indicating a degradation of IκBα. This would lead to excess of translocation of NF-κB to the nucleus and the expression of cytoprotective genes [Yang et al., 2003]. These results were further supported by the content of NF-κB in the nucleus which was found to be more in the complex pretreated irradiated spleenocytes 60 min after irradiation (Fig. 5). The delayed phosphorylation of PKCδ, in the complex treated lymphocytes along with the delayed degradation of IκBα (60 min) indicate a more prolonged action of the complex as compared to curcumin. The reason for this could be the delayed biological half-life of the complex and hence its higher effectiveness. The expression of *MnSOD*, a cytoprotective gene and a transcript of NF-κB was next looked at after 60 min of treatment with the compounds. ROS are known

to induce the expression of *MnSOD* in the cell. This has also been observed in our results with irradiated spleenocytes (Fig. 5). Pretreatment with the complex or curcumin prevented this increase indicating the strong scavenging capacity of the compounds, with complex again faring better than curcumin. Comparing the translocation of NF-κB and its downstream target gene *MnSOD*, it was observed that the complex did not inhibit the translocation of NF-κB to the nucleus as much as curcumin did. Inspite of increased translocation of NF-κB to the nucleus in the complex treated spleenocytes the expression of *MnSOD* was inhibited more by the complex. The reason for this may be that *MnSOD* gene contains binding motifs for a number of transcription factors including NF-κB, activator proteins 1 (AP1) and 2 (AP2), specificity protein 1 (Sp1) and adenosine 3′,5′-cyclic monophosphate-regulator element binding factor (CREB) [Borrello and Demple, 1997; Porntadavity et al., 2001; Xu et al., 2002], Sp1 and AP2 have been linked with the process of repression of *MnSOD* rather than its constitutive expression. The differential repression caused by curcumin or the complex may be because of the differential action of curcumin or the complex on Sp1 and AP2.

The complex and curcumin have also been evaluated for their cytotoxicity on splenic lymphocytes and for their protective effect against γ-radiation (2 Gy) induced apoptosis. Curcumin is well known to induce cytotoxicity in tumor cell lines and in splenic lymphocytes [Gao et al., 2004; Siwak et al., 2005; Aggarwal et al., 2006]. When metal complexes are employed as anti-oxidants in cells, there is a possibility of their undergoing hydrolysis, thereby releasing free copper(II) [Vajragupta et al., 2004]. If this was the case, in the present study, the complex would have increased apoptosis more than that of curcumin. However the extent of apoptosis caused by the complex was found to be significantly less than that of curcumin. This suggested that the complex was stable within the lymphocytes and did not undergo any hydrolysis.

Our results thus show that the complex has lesser cytotoxicity and better free radical scavenging capability, which is reflected as maintenance of anti-oxidant enzymes level, reduction in lipid and protein damage. Complex also scores better than curcumin in activation of various signaling pathways in temporally relevant manner, i.e. PKCδ and NFκB which would then play a crucial role in determining fate of the cell.

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