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C-Phycocyanin, a selective cyclooxygenase-2 inhibitor, induces apoptosis in lipopolysaccharide-stimulated RAW 264.7 macrophages

Madhava C. Reddy,[a] J. Subhashini,[a] S.V.K. Mahipal,[a] Vadiraja B. Bhat,[c] P. Srinivas Reddy,[a] G. Kiranmai,[a] K.M. Madyastha,[b] and P. Reddanna[a,*]

[a] *Department of Animal Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India*
[b] *Department of Organic Chemistry, Indian Institute of Science, Bangalore 560 012, India*
[c] *Chemical Biology Unit, JNCASR, Bangalore 560 064, India*

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Abstract

C-Phycocyanin (C-PC) is one of the major biliproteins of *Spirulina platensis*, a blue green algae, with antioxidant and radical scavenging properties. It is also known to exhibit anti-inflammatory and anti-cancer properties. However, the mechanism of action of C-PC is not clearly understood. Previously, we have shown that C-PC selectively inhibits cyclooxygenase-2 (COX-2), an inducible isoform that is upregulated during inflammation and cancer. In view of the reported induction of apoptosis in cancer cells by cyclooxygenase-2 inhibitors, the present study is undertaken to test the effect of C-PC on LPS stimulated RAW 264.7 mouse macrophage cell line. These studies have shown a dose dependent reduction in the growth and multiplication of macrophage cell line by C-PC. This decrease in cell number appears to be mediated by C-PC induced apoptosis as evidenced by flow cytometric and confocal microscopic studies. Cells treated with 20 μM C-PC showed typical nuclear condensation and 16.6% of cells in sub-G_o/G_1 phase. These cells also showed DNA fragmentation in a dose dependent manner. The studies on poly(ADP ribose) polymerase (PARP) cleavage showed typical fragmentation pattern in C-PC treated cells. This C-PC induced apoptosis in RAW 264.7 cells appears to be mediated by the release of cytochrome *c* from mitochondria and independent of Bcl-2 expression. These effects of C-PC on RAW 264.7 cells may be due to reduced PGE_2 levels as a result of COX-2 inhibition.

Keywords: C-Phycocyanin; Cyclooxygenase-2; Apoptosis; RAW 264.7 cells; Cytochrome *c*

C-Phycocyanin (C-PC) is one of the major biliproteins of *Spirulina platensis*, blue green algae. This water soluble protein pigment is shown to be hepatoprotective [1], antioxidant, radical scavenger [2], antiarthritic [3], and anti-inflammatory [4,5] in both in vitro and in vivo experimental models. However, little is known about its mechanism of action. Earlier, we have shown that C-PC selectively inhibits cyclooxygenase-2 (COX-2), the inducible isoform of cyclooxygenase, implicated in the mediation of inflammation, and arthritis [6]. The catalytic activity of cyclooxygenase, also called prostaglandin G/H synthase, on arachidonic acid results in the formation of prostaglandin H_2 (PGH_2). PGH_2, an unstable endoperoxide intermediate, in turn serves as a substrate for cell specific isomerases and synthases to produce prostaglandins (PGE_2, PGD_2, and $PGF_{2\alpha}$), prostacyclin (PGI_2), and thromboxane A_2 (TXA_2) [7,8]. Prostaglandins are the lipid mediators of importance in several pathological processes such as inflammation, thrombosis, and cancer in addition to normal physiological processes. Cyclooxygenase-1 (COX-1), the constitutive isoform of cyclooxygenase, is known to be cytoprotective, involved in the protection of gastrointestinal tract, maintenance of kidney ionic balance, and platelet functions [9,10]. Unlike COX-1, COX-2 expression is minimal in most tissues under basal conditions, but is significantly upregulated by

* Corresponding author. Fax: +91-40-3010-223.
E-mail addresses: prsl@uohyd.ernet.in, preddanna@yahoo.com (P. Reddanna).

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bacterial lipopolysaccharides (LPS), cytokines, growth factors, oncogenes, and carcinogens [11,12]. COX-2 activity and prostaglandin (PG) levels were found to be several folds higher in malignant tissues from colorectal cancer, human gastric, and breast tumors [13]. The increased levels of prostaglandins (PGs) in tumors provided the rational for use of non-steroidal anti-inflammatory drugs (NSAIDs) as potential chemopreventive agents. The fact that COX-2 gets induced and aspirin and other NSAIDs taken on regular basis decrease the relative risk of colorectal cancers [14], suggest a possible role for COX-2 and PGs in the induction of colorectal cancers. Although the precise mechanism for the protective effect of NSAIDs against cancers is not known, the ability of these drugs to induce cell cycle arrest and apoptosis has received more attention in recent years. A number of experimental studies demonstrate a clear positive correlation between COX-2 overexpression and down regulation of apoptosis. However, the underlying molecular mechanisms are still not fully understood.

The present study is undertaken to analyze the effect of C-PC on a mouse macrophage cell line, RAW 264.7, a cell line that expresses high levels of COX-2 upon induction with bacterial lipopolysaccharide (LPS). Our results show that C-PC induces apoptosis in mouse macrophage cell line, as indicated by characteristic nuclear condensation, DNA ladder appearance, cytochrome *c* release from mitochondria, cleavage of poly(ADP-ribose) polymerase (PARP), and FACS analysis.

Materials and methods

Chemicals and reagents. RPMI-1640 medium, fetal bovine serum, LPS (*Escherichia coli* 026: B6), propidium iodide, ethidium bromide, Trypan blue, MTT, L-NAME, leupeptin, aprotinin, pepstatin A, trypsin, Tween 20, Triton X-100, Ponceau S, and sodium orthovanadate were purchased from Sigma Chemical (St. Louis, MO, USA). Apoptotic DNA ladder kit was purchased from Roche Molecular Biochemicals, Germany. Affinity purified goat polyclonal antibodies for COX-1 and COX-2 were purchased from Santa Cruz, CA, USA. Affinity purified Goat anti-PARP and affinity purified rabbit anti-human Bcl-2 were purchased from R&D Systems, USA.

Cell culture and treatments. The mouse monocyte/macrophage cell line, RAW 264.7, was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, sodium bicarbonate (2 g/liter), 100 IU/ml penicillin, 100 μg/ml gentamycin, and 100 μg/ml streptomycin. The cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cell number and viability were determined by 0.4% trypan blue. Cells were incubated for different periods of time at 37 °C in the presence of various concentrations of C-PC (5–100 μM), LPS (1 μg/ml), and L-NAME (1 mM). In order to prevent the interference of nitric oxide (NO) in LPS stimulated mouse macrophages, L-NAME, inhibitor of nitric oxide synthase (NOS) activity, was employed throughout the study. A stock solution of 1 mM C-PC prepared in PBS was diluted in standard growth medium to the desired final concentration.

Cell viability assay. The in vitro effect of C-PC on the growth of RAW 264.7 cells was determined by quantitative colorimetric assay with MTT [15]. Cells were treated in triplicates with LPS, L-NAME, and with or without C-PC (5, 10, 20, and 50 μM) for 0, 24, 48, and 72 h. At the end of each time point, 20 μl of MTT (5 mg/ml) was added to each well and the plates were incubated for 4 h at 37 °C. Purple–blue formazan crystals formed were dissolved in dimethyl sulfoxide and the absorbance was read at 570 nm on microtiter plate reader, μ Quant Bio-Tek Instruments, Vermont, USA.

Confocal microscopic studies. RAW 264.7 cells cultured in 6 well plates were treated with LPS (1 μg/ml) and 20 μM C-PC for 48 h. The cells were harvested and fixed in 70% ethanol overnight. The fixative was removed by centrifugation, washed twice with PBS, and these cells were stained with 50 μg/ml propidium iodide. Intact and condensed nuclei were visualized using plan-apocromat 63×1.4 oil objective under Zesis confocal microscope using Zesis LSM version −150 software (Scan zoom 3.1).

DNA fragmentation analysis. DNA was extracted from both floating and attached cells after 48 h treatment with LPS, L-NAME, and C-PC (10, 20 μM), using Apoptotic DNA ladder kit as per the protocol given by the manufacturer (Roche Molecular Biochemicals, Germany). Electrophoresis of the resulting DNA was carried out in 2% horizontal agarose gel and visualized by ethidium bromide staining.

Quantitative analysis of apoptosis by flow cytometry. DNA flow cytometry was performed according to the procedure of Nicoletti et al. [16] with slight modifications. RAW 264.7 cells cultured in 6 well plates were treated with LPS, L-NAME, and C-PC (5, 10, and 20 μM) for 48 h. The cells were harvested, washed twice with PBS, and then gradually fixed by adding 1 ml of 70% ethanol and fixed overnight. The fixative was removed by centrifugation and washing twice with PBS, these cells were gently resuspended in 1 ml DNA staining reagent (containing PBS, pH 7.4, 0.1% Triton X-100, 0.1 mM EDTA, 50 μg/ml of DNase free RNase A, and 50 μg/ml propidium iodide). The cells were then incubated in the dark for over 1 h at room temperature and analyzed. Flow cytometric analysis was performed using a FACS Vantage (Becton–Dickinson). Ten thousand events were evaluated using the Cell Quest Program.

Isolation of cytosolic cytochrome c. After the cells were exposed to 20 μM C-PC for 0–24 h, both floating and attached cells were collected, washed with PBS (pH 7.2), and buffer A containing 0.25 M sucrose, 30 mM Tris–HCl (pH 7.9), and 1 mM EDTA, and pelleted by brief centrifugation. The pellets were resuspended in buffer B (buffer A plus protease inhibitors, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin) and homogenized with a glass dounce homogenizer with a B pestle (40 strokes). After the centrifugation at 14,000 rpm for 30 min, the supernatants were collected and used to detect cytosolic cytochrome *c* release by Western blotting.

Western blotting. Western blot analysis of COX-1, COX-2, Bcl-2, cytochrome *c*, and PARP were carried out by employing the respective antibodies. Cellular proteins from both floating and attached cells were isolated in lysis buffer containing 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 μg/ml PMSF, 30 μg/ml aprotinin, and 50 mM Tris–HCl (pH 8.0). The samples were then placed on ice for 30 min sonicated for 10 s and centrifuged at 14,000 rpm for 5 min. Protein concentration in the samples was measured using Bradford method. Samples containing 50 μg of protein extracted from either control or treated cells were subjected to SDS–PAGE using 10–15% (depending on the protein to be analyzed) denaturing polyacrylamide slab gels. The proteins were then transferred electrophoretically to a Hybond-C nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ) at 50 V for 3 h at 4 °C. To stain the proteins and to validate that equal amounts of protein were loaded in each lane and transferred efficiently, the membrane was immersed in 0.1% Ponceau S in 5% acetic acid. After the nitrocellulose membranes were incubated for 1 h in a blocking solution containing 5% non-fat dried milk in Tris-buffered saline–Tween (TBST—25 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20, and 0.1% sodium azide), the membranes were incubated for 1 h with anti-COX-1, anti-COX-2, anti-Bcl-2, anti-PARP,

anti-cytochrome *c*, and then with a corresponding secondary antibody. After this incubation, the membranes were washed three times with TBST and the proteins were visualized by incubating with colorogenic substrates BCIP/NBT or TMB/H_2O_2 for 15 min.

Estimation of prostaglandin E_2 production. RAW 264.7 cells were treated with LPS and different concentrations of C-PC (0–100 μM) for 24 h. The quantitative analysis of prostaglandin E_2 released into the medium was assayed by using the PGE_2 immunoassay kit as per manufacturer's instructions (Assay Designs, Ann Arbor, USA).

Statistical analysis. The results were expressed as means ± SEM of data obtained from three independent experiments. Statistical significance was determined by Student's *t* test ($P \leqslant 0.05$) and analysis of variance.

Results

Decreased cell viability by C-Phycocyanin

To test the effect of C-PC on the growth and multiplication of RAW 264.7 cells, cells were incubated with different concentrations of C-PC and the cell viability was examined by MTT assay. RAW 264.7 cells treated with four different concentrations of C-PC (5, 10, 20, and 50 μM) were examined after 0, 24, 48, and 72 h for cell viability. As shown in Fig. 1, treatment with 20 μM C-PC significantly decreased the viability of RAW 264.7 cells after 48 h (<50% control). The decrease in the cell viability was dose dependent upto 20 μM C-PC.

Effect of C-Phycocyanin on COX-1 and COX-2 protein expression

To investigate the effect of C-PC on the expression of COX-1 and COX-2, Western blot analysis was performed with whole cell lysate from C-PC treated RAW 264.7 cells after challenging with LPS. The results

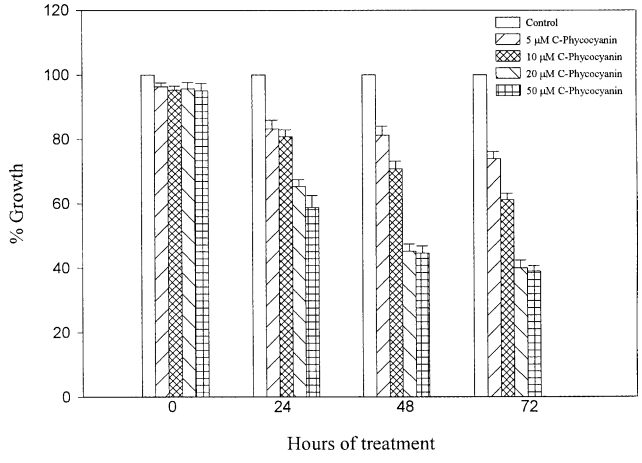

Fig. 1. Effect of C-PC on RAW 264.7 cell viability in the presence of LPS (1 μg/ml) and L-NAME (1 mM). Cell viability was determined by the MTT assay as described under materials and methods. Bars represent mean values ± SEM of three independent experiments, each performed in triplicate ($P \leqslant 0.05$).

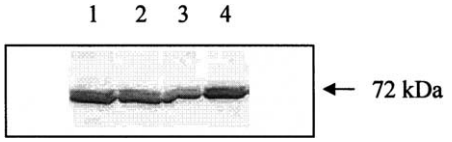

Fig. 2. Effect of C-PC on COX-1 expression in RAW 264.7 cells. Whole cell lysates (50 μg) of RAW 264.7 cells treated with 20 μM C-PC for the indicated times were analyzed by 10% SDS–PAGE and subsequently, immunoblotted with antibody against COX-1. RAW 264.7 cells treated with LPS and C-PC for 0, 12, 24, and 48 h, respectively (lanes 1–4).

presented in Fig. 2 showed no changes in COX-1 protein levels at different time intervals after treatment with 20 μM C-PC. RAW 264.7 cells treated with LPS and C-PC showed no significant changes in COX-2 protein levels in both dose dependent and time dependent manner (Fig. 3). These results indicate no effect of C-PC on the protein levels of COX-2, which are involved in mediating inflammation. Since PGE_2 is the main mediator of inflammation and C-PC is known to have anti-inflammatory effects, further studies were undertaken to estimate the levels of PGE_2 in C-PC treated macrophages by employing PGE_2 EIA kit.

Effect of C-Phycocyanin on PGE_2 levels

The activity levels of COX-2 were also measured in terms of PGE_2 formed in mouse macrophage cells, stimulated with LPS. PGE_2 secreted into the medium was measured by employing enzyme immunoassay kit supplied by Assay Designs, USA. As shown in Fig. 4, C-PC decreased the levels of PGE_2 in a dose dependent manner reaching maximum inhibition (~90%) at 100 μM concentration. These decreased PGE_2 levels in LPS stimulated macrophage cells in response to C-PC could be due to the possible inhibition of COX-2, as C-PC is a specific inhibitor of COX-2 [6].

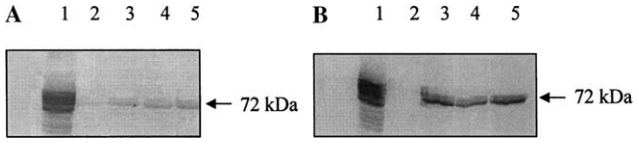

Fig. 3. Time dependent and dose dependent effects of C-PC on COX-2 protein levels in RAW 264.7 cells. Whole cell lysates (50 μg) were separated on a 10% SDS–PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with affinity purified goat polyclonal anti-COX-2. (A) Time dependent. COX-2 protein positive control (lane 1) (r hCOX-2 expressed in baculovirus infected Sf9 cells) and RAW 264.7 cells treated with LPS and 20 μM C-PC for 0, 12, 24, and 48 h, respectively (lanes 2–5). (B) Dose dependent. COX-2 positive control (lane 1), RAW 264.7 cells without LPS (lane 2) and RAW 264.7 cells treated with LPS and 5, 10, and 20 μM C-PC for 48 h (lanes 3–5).

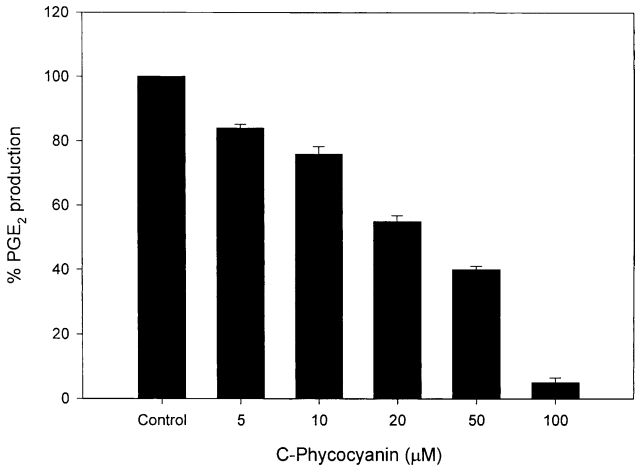

Fig. 4. Effect of C-PC on PGE2 formation in cell-culture supernatants of LPS stimulated RAW 264.7 cells treated with C-PC (5–100 μM) for 24 h. PGE2 concentration was determined by EIA kit. Each experiment was carried out in duplicates and the data are the means ± SE of two independent experiments.

Induction of apoptosis by C-Phycocyanin

To understand the possible mechanism involved in C-PC induced cell death, specific apoptotic marker studies were undertaken on the mouse macrophage cell line.

Confocal microscopic studies

A distinguishing feature of apoptosis is the condensation and fragmentation of nuclear chromatin, which can be monitored by confocal microscopy. Fig. 5 shows the confocal microscopic pictures of RAW 264.7 cells treated with (20 μM for 48 h) or without C-PC. As shown in figure, control cells showed normal chromatin with distinct large nuclei whereas C-PC treated cells showed characteristic apoptotic nuclei which are condensed and brightly fluoresced.

Assay of DNA fragmentation in C-Phycocyanin treated RAW 264.7 cells

In the present study LPS stimulated RAW 264.7 cells, on exposure to 10/20 μM C-PC for 48 h, showed generation of oligonucleosomal sized ladders of DNA fragmentation on agarose gel containing ethidium bromide. The degree of nuclear DNA fragmentation was directly proportional to the concentration of C-PC. Treatment of RAW 264.7 cells with and without LPS and L-NAME in the absence of C-PC did not induce the formation of any internucleosomal DNA fragmentation (Fig. 6). We have observed same results with TUNEL assay (data not shown).

Flow cytometric analysis

Apoptosis induced in cells cultured with LPS (1 μg/ml) and 5, 10, and 20 μM C-PC for 48 h was quantified by FACS analysis. The FACS analysis of control cells (cells treated with LPS) shows prominent G_1, followed by S and G_2/M phases (Fig. 7A). Only 1.52% of these cells showed hypodiploid DNA (sub G_0/G_1 peaks). This 1.52% hypodiploid DNA in control cells was increased to 11.84%, 14.19%, and 16.60% on exposure of cells to 5, 10, and 20 μM C-PC, respectively (Figs. 7B and D). Thus, cells treated with C-PC showed prominent peaks to the left of the main G_0/G_1 peaks (sub G_0/G_1 peaks) in C-PC treated cells (Figs. 7C and D) as compared to control untreated cells (Fig. 7A).

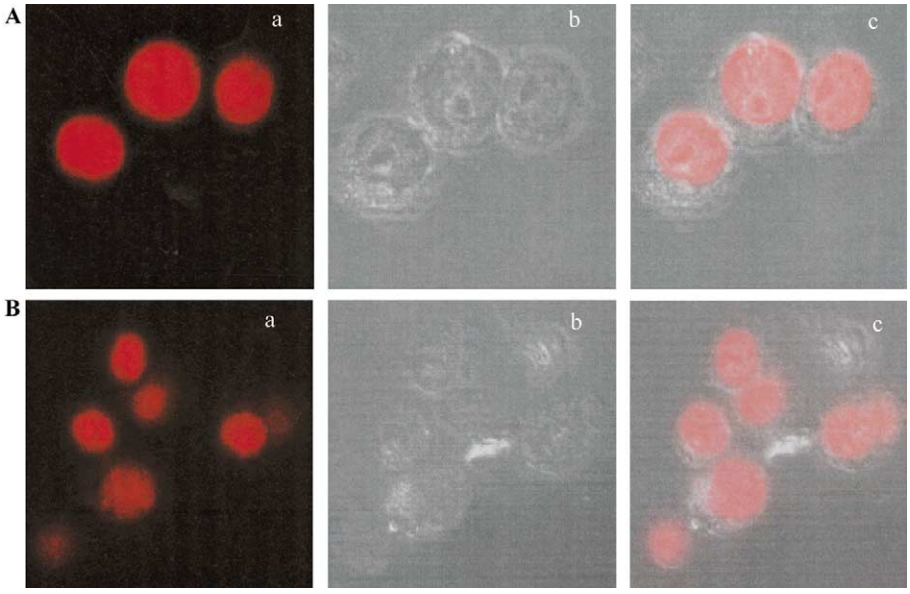

Fig. 5. Confocal photomicrographs of RAW 264.7 cells, stained with propidium iodide to view nuclear condensation. Images were taken after 48 h treatment with C-PC. (A) Control cells treated with LPS; (B) RAW 264.7 cells treated with LPS and 20 μM C-PC: a, fluorescence image; b, phase contrast image; c, overlay of fluorescence image with corresponding phase contrast image.

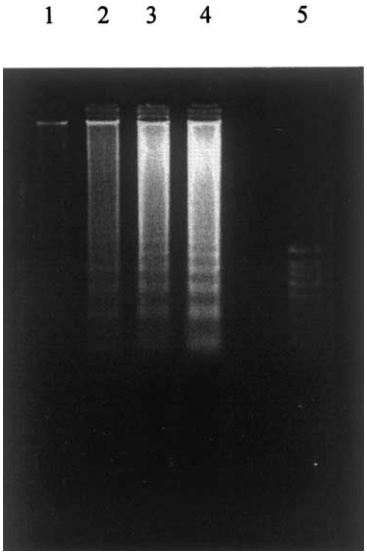

Fig. 6. C-PC induced DNA fragmentation in LPS stimulated RAW 264.7 cells. Cells were treated with and without LPS and combination of LPS & C-PC for 48 h. DNA was extracted and analyzed by 2% agarose gel electrophoresis. Both floating and adherent cells were collected and soluble DNA was extracted and electrophoresed on a 2% agarose gel containing 0.05 mg/ml ethidium bromide at 5 V/cm for 3 h. The gels were then photographed under UV illumination. RAW 264.7 cells without LPS and L-NAME (lane 1), RAW 264.7 cells with LPS and L-NAME (lane 2), RAW 264.7 cells treated with LPS and 10 μM C-PC (lane 3), RAW 264.7 cells treated with LPS and 20 μM C-PC (lane 4), and 100 bp DNA ladder (lane 5).

Cleavage of PARP

PARP, poly(ADP-ribose) polymerase, is a nuclear enzyme that is activated both during necrosis and apoptosis. However, the pattern of cleavage is variable in both modes of cell death with characteristic signature fragments of 85 and 23 kDa in apoptosis and fragments of 43 and 29 kDa in necrosis [17]. During apoptosis, PARP (116 kDa) is cleaved between amino acids Asp^{214} and Gly^{215} to yield two fragments of 85 and 23 kDa. PARP antibody specifically recognizes the 23 kDa fragment of the cleaved PARP and uncleaved 116 kDa PARP. In the present study, RAW 264.7 cells treated with 20 μM C-PC for 24 and 48 h showed 23 kDa fragment along with the uncleaved 116 kDa PARP (Fig. 8, lanes 4 and 6). In the control cells, however, no 23 kDa fragment of PARP was observed, except the uncleaved 116 kDa protein. These data clearly demonstrate the cleavage of PARP in C-PC treated RAW 264.7 cells.

C-Phycocyanin induced growth suppression is independent of Bcl-2 expression

Bcl-2 family proteins play an important role in the regulation of cell death. In the light of the recent reports that attributed COX-2 inhibitor-induced apoptosis to Bcl-2 down regulation [18,19], studies were undertaken to test whether Bcl-2 expression is affected after C-PC treatment in LPS stimulated RAW 264.7 cells. The

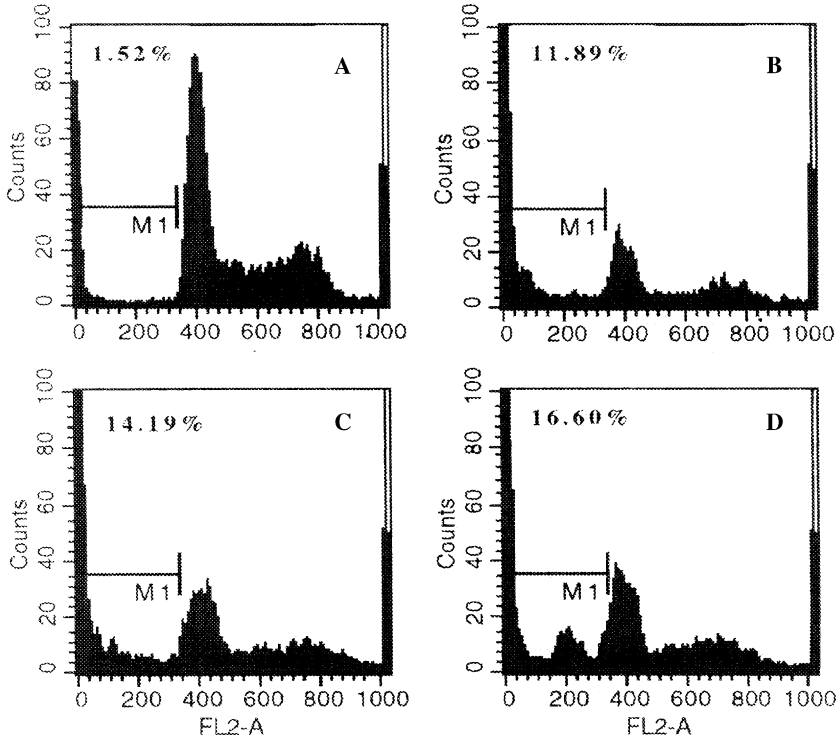

Fig. 7. Flow cytometric analysis on the effect of C-PC on LPS stimulated RAW 264.7 cells. Cells were exposed to different concentrations of C-PC for 48 h, then washed and harvested. The cells were fixed and stained with propidium iodide, and the DNA content was analyzed by flow cytometry (FACS). The number of hypodiploid (pre-G1 phase) cells is expressed as a percentage of the total number of cells. Control with LPS (A), 5 μM C-PC (B), 10 μM C-PC (C), and 20 μM C-PC (D).

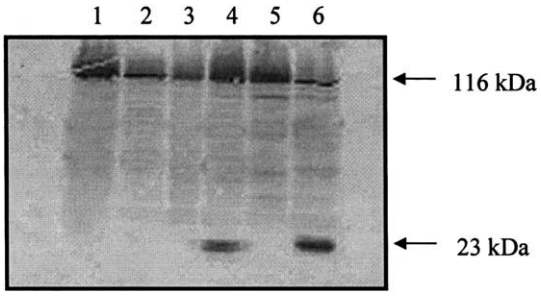

Fig. 8. Western blot analysis of time dependent degradation of PARP during apoptosis induced by C-PC in LPS stimulated RAW 264.7 cells. Whole cell lysates from RAW 264.7 cells, incubated only with LPS and with LPS and C-PC, were fractionated by 15% SDS–PAGE, and subsequently immunoblotted with antibody against PARP. This antibody recognizes both uncleaved PARP (116 kDa) and the cleaved fragment (23 kDa). RAW 264.7 cells treated with LPS for 12 h (lane 1), RAW 264.7 cells treated with LPS and 20 μM C-PC for 12 h (lane 2), RAW 264.7 cells treated with LPS for 24 h (lane 3), RAW 264.7 cells treated with LPS and 20 μM C-PC for 24 h (lane 4), RAW 264.7 cells treated with LPS for 48 h (lane 5), and RAW 264.7 cells treated with LPS and 20 μM C-PC for 48 h (lane 6).

results of Western blot analysis, employing Bcl-2 protein antibodies, revealed that C-PC did not affect the expression of Bcl-2 protein upto 48 h after treatment (Fig. 9). These results suggest that apoptosis induced by C-PC might be independent of Bcl-2 expression in LPS stimulated RAW 264.7 cells.

Cytochrome c release from the mitochondria as an early event

Cytochrome *c* is a well characterized mobile electron transport protein essential for energy conversion in all aerobic organisms. In mammalian cells, this highly conserved protein is normally localized to the mitochondrial intermembrane space. The release of cytochrome *c* into the cytosol is the hallmark of apoptosis of cells treated with certain apoptosis inducers [20,21]. Hence, in the present study the cytosolic cytochrome *c* was measured by Western blot analysis in RAW 264.7 cells, 0, 6, 12, 18, and 24 h after the treatment with 20 μM C-PC. As shown in Fig. 10, the levels of cytochrome *c* in the cytosol were elevated within 6 h of treatment with C-PC, and the levels were further increased upto 24 h. In

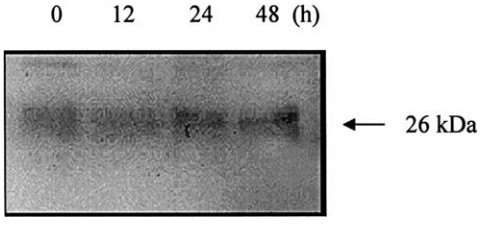

Fig. 9. Effect of C-PC on Bcl-2 protein of LPS stimulated RAW 264.7 cells. Equal amounts of protein from total cell lysates, which were treated with 20 μM C-PC for indicated times (0, 12, 24, and 48 h) were analyzed by 12% SDS–PAGE and, subsequently immunoblotted with antibody against Bcl-2.

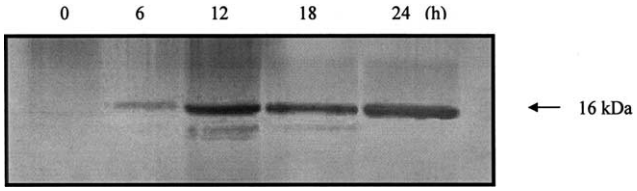

Fig. 10. Effect of C-PC on cytochrome *c* translocation from mitochondria of RAW 264.7 cells stimulated with LPS. Equal amounts of protein from the cytosolic fraction of RAW 264.7 cells, which had been treated with 20 μM of C-PC for the indicated times (0, 6, 12, 18, and 24 h) were analyzed by 15% SDS–PAGE and, subsequently immunoblotted with antibody against cytochrome *c*.

contrast to the DNA fragmentation, PARP cleavage, and FACS analysis, the release of cytochrome *c* from the mitochondria was very rapid and is an early event in C-PC induced apoptosis in RAW 264.7 cells.

Discussion

Macrophages play an important role in the regulation of inflammation and immune response. When activated, macrophages release growth factors, cytokines, and lipid mediators such as prostaglandins and leukotrienes, which promote inflammation by directing cellular migration to the site of inflammation through the production and release of proinflammatory cytokines such as IL-6 [22]. Elevated PG levels are also associated with conditions of both chronic inflammation and cancer [23,24]. Macrophages secrete PGs upon activation by the bacterial endotoxin, lipopolysaccharide, primarily due to the induction of the COX-2 gene transcription and subsequent production of the COX-2 protein [25]. The present study demonstrates that C-PC, a selective COX-2 inhibitor [6], resulted in a dose dependent decrease in the levels of PGE_2 in LPS stimulated mouse macrophage cell line, RAW 264.7. This decrease in PGE_2 with no change in COX-1 and COX-2 protein levels could be due to inhibition of COX-2 activity by C-PC as reported earlier [6].

Recent epidemiological and experimental evidences illustrate the use of NSAIDs in reducing the risk of development of colorectal cancers [26,27]. However, the signaling mechanisms responsible for the chemopreventive action of these drugs have not been clearly established [28]. The growing body of evidence suggests that modulation of the pathway for programmed cell death in tumor cells is one of the main ways through which NSAIDs exert their effects [29,30]. Elevated levels of PGE_2 produced mainly through induced COX-2 were found to cause resistance to apoptosis and thus promotion of cancer cell proliferation [31]. We have observed reduction in the growth and multiplication of LPS stimulated mouse macrophage cell line in a dose dependent manner upon C-PC treatment. Although the

mechanism behind this effect is not fully understood, based on the present studies, it is proposed that decreased levels of PGE_2 in C-PC treated cells may partially be responsible for this effect. C-PC treated cells showed pronounced morphological changes like cell shrinkage, formation of membrane blebs, and micronuclei characteristic of apoptosis as evidenced by phase contrast and confocal microscopic studies.

Several apoptosis inducing agents are known to trigger mitochondrial uncoupling leading to the rupture of outer membrane. Bcl_2 family members consisting of both antiapoptotic (Bcl_2, Bcl-X_L, and Bcl_2-W) and proapoptotic (Bcl-X_S, BAX, BAK, BAD, and BID) counter partners arbitrate these effects at mitochondrial level [32,33]. Down regulation or heterodimerization of Bcl_2 is implicated in mitochondrial transmembrane potential collapse ($\Delta\Psi m$ or permeability transition), leading to the opening of large conductance channel known as permeability transition pore, which eventually causes outer membrane rupture releasing caspase activating proteins into the cytosol [34,35]. Western blot analysis of the proteins from cells exposed to C-PC showed no significant changes in the Bcl-2 expression levels. This suggests that C-PC induced apoptosis in mouse macrophage cell line may be independent of Bcl-2 expression. In addition to C-PC induced apoptosis, similar operation of Bcl-2 independent pathways have been reported in celecoxib, selective inhibitor of COX-2, induced apoptosis in prostate cancer cells, human primitive neuroectodermal tumors, and NS-398 induced apoptosis of colon cancer cells [36–38].

Cytochrome *c* release from the mitochondria is considered as a key signal that initiates the irreversible events in cell death [20,21]. Barrier function of mitochondrial membrane is perturbed early during apoptosis, which in turn causes the release of pro-apoptotic factors such as apoptosis inducing factor (AIF), cytochrome *c*, and the apoptosis protease activating factor (Apaf-1) into the cytosol [39]. Our studies have shown the release of cytochrome *c* as early as 6 h after treatment of RAW 264.7 cells with C-PC, with later increase upto 24 h. This observation suggests that cytochrome *c* release is one of the early events of C-PC-induced apoptosis. Cytosolic cytochrome *c* forms an essential part of apoptosome composed of Apaf1, dATP, and caspase-9 which proteolytically activates effector caspases like caspase-3 [39]. Activated caspase-3 cleaves off target substrates like poly(ADP-ribose) polymerase (PARP), a nuclear enzyme that senses DNA nicks and catalyzes the ADP ribosylation of histones and other nuclear proteins in order to facilitate DNA repair [40]. PARP cleavage observed in the present study in RAW 264.7 cells exposed to C-PC provides a conclusive and definitive evidence for the induction of apoptosis upon C-PC treatment. DNA fragmentation observed in C-PC treated macrophage cells is in conformity with the existing data that cleaved PARP no longer negatively regulates the Ca^{2+} and Mg^{2+} dependent endonucleases [41–43], which thus eventually cleave DNA into oilgonucleosomal fragments. As activation of caspase-3 was shown to be the upstream event for both PARP cleavage and DNA fragmentation, a series of down stream events taking place in C-PC induced apoptosis might be mediated by caspase-3 involvement. The present studies on mouse macrophage cell line, RAW 264.7, exposed to C-PC reveal the induction of apoptosis through cytochrome *c* release, followed by PARP cleavage, and finally leading to the degradation of DNA into oligonucleosomal fragments. The entry and mechanism of action of C-PC in these cells, however, is not clear. Preliminary confocal microscopy studies employing C-PC polyclonal antibodies revealed the entry of C-PC into the cytoplasm of mouse macrophage cells (data not shown).

In summary, motivated by several claims on the therapeutic role of C-PC, particularly its anti-inflammatory, antiarthritic, and anti-cancer properties, it is planned to address the question as to whether this natural COX-2 inhibitor, C-PC, might interfere with arachidonic acid cascade. The foregoing studies on activity and mechanism of action revealed that C-PC induces apoptosis in LPS stimulated mouse macrophages, RAW 264.7 cells through inhibition of PG biosynthesis, cytochrome *c* release, and PARP cleavage.

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