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ORIGINAL ARTICLE

Xian-Ming Zhang · Jia Chen · Yu-Gui Xia · Qiang Xu

Apoptosis of murine melanoma B16-BL6 cells induced by quercetin targeting mitochondria, inhibiting expression of PKC-α and translocating PKC-δ

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Abstract *Purpose*: In our previous study, quercetin was found to induce apoptosis of murine melanoma B16-BL6 cells. The cellular and molecular mechanism of quercetin-induced apoptosis was investigated in the present study. *Methods*: Nuclear morphology was determined by fluorescence microscopy. DNA fragmentation was analyzed by electrophoresis and quantified by the diphenylamine method. The transmembrane potential of mitochondria was measured by flow cytometry. Bcl-2, Bcl-X_L, PKC-α, PKC-β, and PKC-δ were detected by Western blotting. Caspase activity was determined spectrophotometrically. *Results*: Quercetin induced the condensation of nuclei of B16-BL6 cells in a dose-dependent pattern as visualized by Hoechst 33258 and propidium iodide dying. Phorbol 12-myristate 13-acetate (PMA), a PKC activator, significantly enhanced apoptosis induced by quercetin, while doxorubicin, a PKC inhibitor, markedly decreased it. Both PMA and doxorubicin showed a consistent effect on the fragmentation of nuclear DNA caused by various dosages of quercetin. Quercetin dose-dependently led to loss of the mitochondrial membrane potential, which was also significantly reinforced or antagonized by PMA and doxorubicin, respectively. Moreover, PMA showed reinforcement, while doxorubicin showed significant antagonization, of the quercetin-mediated decrease in the expression of Bcl-2. Quercetin promoted caspase-3 activity in a dose-dependent manner, which was also regulated by PMA and doxorubicin with a pattern similar to that seen in their effect on apoptosis, mitochondrial membrane potential and Bcl-2 expression, but none of these were directly affected by PMA and doxorubicin. Free fatty acid and chlorpromazine, a PKC activator and inhibitor, respectively, did not interfere with these effects of quercetin. B16-BL6 cells expressed PKC-α, PKC-β, and PKC-δ. Quercetin dose-dependently inhibited the expression of PKC-α but not that of PKC-β and PKC-δ. Doxorubicin almost completely blocked the effect of quercetin on the expression of PKC-α. Quercetin was also involved in the translocation of PKC-δ from the cytosol to the nucleus. PMA enhanced the effect of quercetin on the translocation of PKC-δ. *Conclusions*: These results indicate that quercetin induced apoptosis of murine melanoma B16-BL6 cells by injuring their mitochondria, increasing the activity of caspase-3, inhibiting the expression of Bcl-2 and PKC-α, and inducing the translocation of PKC-δ. Doxorubicin inhibited these effects of quercetin by blocking the decreased expression of PKC-α induced by quercetin while PMA increased these effects by enhancing the translocation of PKC-δ induced by quercetin.

Keywords Quercetin · Apoptosis · Mitochondria · Murine melanoma B16-BL6 cell · Bcl-2 · PKC-α and PKC-δ · Caspase-3

X.-M. Zhang · J. Chen · Y.-G. Xia · Q. Xu (✉)
State Key Laboratory of Pharmaceutical Biotechnology,
School of Life Sciences, Nanjing University,
Nanjing, 210093, People's Republic of China
E-mail: molpharm@163.com
Tel.: +86-25-83597620
Fax: +86-25-83597620

Introduction

Apoptosis, which plays a critical role in homeostasis, development, neoplasm and some degenerative diseases including multiple sclerosis, Alzheimer's disease and Parkinson's disease, is a promising target for tumor therapy [1]. A large number of chemotherapy drugs such as cisplatin, mitomycin C, cytosine arabinoside, etoposide, and bryostatin-1 induce apoptosis of tumor cells [2–6]. The pathways leading to apoptosis mainly include mitochondria and death receptor such as Fas, TNFR, and TNF-related apoptosis-inducing ligand (TRAIL) receptor [7, 8]. Disrupting the potential and permeabilization of mitochondrial membranes cause proapoptotic proteins such as cytochrome *c* and apoptosis-inducing factor (AIF) to be released from mitochondria to the cytosol. This process is regulated by Bcl-2 family members including Bcl-2, Bcl-X_L, Bak, Bax, Bid [9–13].

Cytochrome *c* triggers a cascade from initiator to effector caspase through activating Apaf-1 whereas AIF induces caspase-independent apoptosis [14, 15]. In addition, apoptosis can also be modulated by other molecules such as IAP, cFLIP and p53, and abundant kinases including MAPK, PKB and PKC [16–19]. Moreover, subtypes of PKC play different and contrasting role in apoptosis [20].

Quercetin, a bioflavonoid widely distributed in a variety of plants, is known to have antioxidative activity and antitumor effects, and has the ability to inhibit activities of multiple kinases including PKC, PTK, ERK1/2 [21–23]. In addition, quercetin has also been found to possess antiangiogenic potential [24]. Moreover, quercetin-induced apoptosis of various tumor cells including promyeloleukemic HL-60, pancreatic tumor, breast cancer, and monoblastoid U937 cells [25–28]. In our previous study, quercetin was found to dose-dependently induce apoptosis of murine melanoma B16-BL6 cells [29].

However, the detailed molecular and cellular mechanisms involved in quercetin-induced apoptosis and the relationship between induction of apoptosis and inhibition of kinase activity by quercetin are still not completely clear. In the present study, the molecular and cellular mechanisms involved in quercetin-induced apoptosis of B16-BL6 cells and the regulative effect of PKC were investigated.

Materials and methods

Cells and reagents

Murine melanoma B16-BL6 cells were maintained as monolayer cultures in RPMI 1640 medium (Gibco, BRL) containing 2 m*M* L-glutamine, 100 U/ml of penicillin and 100 mg/ml of streptomycin supplemented with 10% fetal calf serum (FCS). The following reagents were purchased: phorbol 12-myristate 13-acetate (PMA), bovine serum albumin (BSA), Hoechst 33258, propidium iodide (PI), proteinase K, and doxorubicin from Sigma (St Louis, Mo.); 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) from Biotium (Hayward, Calif.); Ac-DEVD-pNA from Qbiogene (Carlsbad, Calif.); free fatty acid (FFA), chlorpromazine, diphenylamine from Shanghai Chemical Reagent Company (Shanghai, China); and trichloroacetic acid (TCA) from Yuanhang Chemical Factory (Shanghai, China). Anti-Bcl-2, anti-Bcl-X_L monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) and anti-PKC-α, anti-PKC-β, anti-PKC-δ antibodies and a Western blotting kit (Wuhan Boster Biological Technology Company, Wuhan, China) were also bought.

Fluorescence microscopy of nuclear morphology

Nuclear morphology was characterized by fluorescence microscopy according to a previously reported procedure [30]. B16-BL6 cells (1×10^6 ml^{-1}) were incubated with various drugs for 24 h at 37°C in RPMI 1640 medium containing 10% FCS, and stained with Hoechst 33258 at a final concentration of 5 μg/ml and PI at 10 μg/ml for 30 min in the dark. Stained cells were evaluated visually for apoptotic nuclear fragmentation and condensation. The pictures were taken with a Leica DMIRB inverted phase fluorescence microscope equipped with a ×40 objective, UV filter cube and CCD in five random fields per sample. Nuclei displaying apoptotic characteristics were counted and compared numerically with morphologically normal nuclei in the same field. The percentage of apoptotic nuclei was calculated using the formula: [apoptotic nuclei/(apoptotic nuclei + normal nuclei)] ×100. Each assay was performed in triplicate, and repeated at least three times.

Quantification of DNA fragmentation

DNA fragmentation was quantified by the diphenylamine method [31, 32]. Briefly, B16-BL6 cells (1×10^6 ml^{-1}) after 24 h incubation with various concentrations of quercetin alone or with PMA, FFA, doxorubicin, and chlorpromazine in the presence or absence of quercetin, were harvested by centrifugation at 200 *g* for 10 min at 4°C. The pellets were lysed with 200 μl hypotonic lysis buffer (10 m*M* Tris, 1 m*M* EDTA, pH 7.5) containing 0.5% Triton X-100 and 100 μg/ml proteinase K. The lysates were then centrifuged at 18,000 *g* for 10 min at 4°C to separate intact from fragmented chromatin. The supernatant, containing fragmented DNA, pooled with that from the first centrifugation, was transferred to a separate microfuge tube. Both pellet and supernatant were precipitated overnight at 4°C in 12.5% TCA and the precipitates were sedimented at 18,000 *g* for 10 min at 4°C. Then, the DNA in the precipitates was hydrolyzed by heating to 90°C for 10 min in 300 μl 5% TCA, and then 600 μl diphenylamine reagent (0.15 g diphenylamine, 0.15 ml sulfuric acid, 2.5 μl 40% acetaldehyde, 10 ml glacial acetic acid) was added to each tube. After color development overnight at room temperature, the absorbance was read at 595 nm. The ratio of DNA fragmentation was taken as the percentage of fragmented DNA in relation to total DNA. Each assay was performed in triplicate, and repeated three times.

DNA electrophoresis

DNA electrophoresis was performed according to a previously reported procedure [33]. Briefly, B16-BL6 cells (1×10^6 ml^{-1}) treated with or without various drugs were disrupted in a hypotonic lysing buffer (10 m*M* Tris, 1 m*M* EDTA, 0.5% Triton X-100, and 100 μg/ml proteinase K, pH 7.5). Fragmented DNA was extracted by a sequential treatment with phenol and phenol/chloroform and precipitated overnight in 70% ethanol con-

taining 3 *M* NaAC at −20°C. The pellets of DNA were air-dried and resuspended in TE buffer (10 m*M* Tris, 1 m*M* EDTA, pH 7.5). The loading buffer containing 1% SDS, 50% glycerol, 0.02% tylenlyanol FF, and 0.02% bromphenol blue (BPB) was then added to the DNA samples at a ratio of 1:9 (v/v). Electrophoresis was carried out on the 1.8% agarose gel in 40 m*M* Tris/acetate buffer (pH 8.0) containing 1 μ*M* EDTA for 1 h at 50 V. The DNA was visualized by staining with ethidium bromide at 0.1 μg/ml for 30 min. The appearance of a low molecular weight DNA ladder was used as an indication of DNA fragmentation and apoptosis. The experiment was repeated three times.

Determination of the transmembrane potential of mitochondria

The transmembrane potential of mitochondria was determined according to previously reported procedures [34–36]. B16-BL6 cells (1×10^6 ml^{-1}) were incubated with various drugs for 24 h at 37°C in RPMI 1640 medium containing 10% FCS, and then stained with JC-1 at a final concentration of 5 μg/ml for 30 min. The general staining characteristics of JC-1 in each sample were visually assessed using a Leica DMIRB inverted phase fluorescence microscope equipped with a ×40 objective, FITC filter cube, and CCD. Images of each sample were taken by the equipment. For flow cytometric analysis, the cells were collected and washed once with PBS. At least 10,000 events were collected per sample. For measuring the ratio of JC-1 monomers to JC-1 aggregates, a 488 nm wavelength was used in the fluorescence spectrophotometer for excitation of JC-1. In the first measurement of each sample, a 595 nm emission wavelength was used to detect total orange fluorescence (JC-1 aggregates). A second measurement was performed on each sample with a 535 nm emission wavelength to detect green fluorescence (JC-1 monomers). The ratio of the fluorescence intensities at the two wavelengths was an indication of mitochondrial membrane potential. The experiment was repeated at least three times.

Preparation of cytosol and nuclear proteins

Cytosol and nuclear proteins were prepared according to previously reported procedures [37, 38]. B16-BL6 cells were harvested and rinsed in Hank's balanced salt solution twice. Cells were suspended in 0.2 ml buffer A containing 250 m*M* sucrose, 1 m*M* EDTA, 50 m*M* Tris-HCl, 1 m*M* PMSF (pH 7.4), and homogenized with a microhomogenizer (20 strokes). The homogenate was centrifuged at 10,000 *g* for 20 min at 4°C. The supernatant was the cytosol fraction. Nuclei were extracted as described by Solovyan et al. [38]. Briefly, cells were suspended in 0.2 ml buffer B containing 10 m*M* HEPES, 10 m*M* KCl, 1 m*M* dithiothreitol, 2 m*M* $CaCl_2$, 0.1 m*M* EDTA, 0.1 m*M* EGTA, and 0.1 m*M* PMSF (pH 7.5), and kept for 20 min on ice. They were then homogenized with a microhomogenizer (15 strokes). A crude nuclear pellet was obtained by centrifugation of the cell lysate at 1000 *g* for 5 min at 4°C, and nuclei were then purified by centrifugation at 1000 *g* for 10 min through a layer of 1 *M* sucrose prepared in buffer B. Nuclei were lysed by buffer B containing 1% NP-40 for 10 min on ice. Nuclear proteins were obtained by centrifugation of nuclei lysate at 10,000 *g* for 20 min at 4°C. Cytosol and nuclear proteins were immediately used in the experiments.

Western blotting

Western blotting was carried out according to a previously reported procedure [39]. Murine melanoma B16-BL6 cells (1×10^7) were incubated for 24 h at 37°C in RPMI 1640 medium containing 10% FCS. The cells were collected and rinsed twice in PBS supplemented with 0.05% BSA. The cell pellets were lysed at 4°C for 15 min in lysis buffer (150 m*M* NaCl, 1 m*M* KH_2PO_4, 1 m*M* EGTA, 1 m*M* Na_3PO_4, 5 m*M* $MgCl_2$, 0.1 m*M* phenylmethylsulfonylfluoride, 0.15 U/ml aprotinin, 1 μg/ml pepstatin, and 10% glycerol) and centrifuged at 18,000 *g* for 20 min at 4°C. The protein concentration in the supernatant was measured by the method of Bradford. Total proteins (60 μg) were separated by SDS-PAGE using a 12.5% polyacrylamide gel. The proteins in the gel were transferred to 0.45-μm nitrocellulose membrane with a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell. Membranes were blocked for 2 h with PBST (0.05% v/v Tween-20 in PBS, pH 7.2) containing 1% nonfat milk. All additional immunostaining steps were performed in PBST at room temperature. Membranes were incubated with primary antibody (1:200) for 2 h and then with secondary antibody (1:400) for 1 h. Membranes were washed in PBST for 5 min four times between each step and were developed with diazobenzidine and enhanced with nickel chloride. The experiment was repeated three times.

Caspase-3 activity assay

The assay for caspase-3 activity was carried out according to a previously reported procedure [40]. B16-BL6 cells (1×10^6 ml^{-1}) were incubated with various drugs for 24 h at 37°C in RPMI 1640 medium containing 10% FCS, and then collected by centrifugation. The cell pellets (1×10^7) were lysed with RIPA buffer (25 m*M* Tris, pH 7.4, 150 m*M* KCl, 5 m*M* EDTA, 1% Nonidet P-40, and 0.1% SDS) on ice for 15 min, and cell extracts were obtained by centrifugation at 15,000 *g* for 15 min at 4°C. The concentration of proteins in the supernatant was determined by the method of Bradford. Cell extracts (10 μl, 20 mg/ml) were added to 90 μl protease reaction buffer (50 m*M* HEPES, pH 7.4, 75 m*M* NaCl, 0.1% CHAPS, 2 m*M* dithiothreitol) containing 25 μ*M* caspase-3 substrate of Ac-DEVD-

pNA, and then incubated for 4 h at 37°C. The extinction at 405 nm was determined in an ELISA reader. Each assay was performed in triplicate, and repeated at least three times.

Statistical analysis

One-way analysis of variance (ANOVA) for multiple comparisons was used to detect whether there were any significant differences among the different treatments. Once significant differences were detected ($P < 0.05$), Student's two-tailed *t*-test was used to evaluate the differences between two groups. All experimental results are shown as the means ± SD.

Results

Condensation of nuclei induced by quercetin in B16-BL6 cells and effects of PKC activators and inhibitors

B16-BL6 cells (1×10^6 ml^{-1}) were incubated with 10^{-6}, 10^{-5}, and 10^{-4} g/ml quercetin for 24 h. Condensation of nuclei was determined by Hoechst 33258 and PI staining. As shown in Fig. 1a, quercetin markedly induced apoptosis of B16-BL6 cells. The percentage of apoptotic cells was dose-dependent (Fig. 1b). Moreover, when B16-BL6 cells (1×10^6 ml^{-1}) were incubated with quercetin (10^{-4} g/ml) in the presence or absence of PMA (100 n *M*), a PKC activator, or doxorubicin (8.6 μ*M*), a PKC inhibitor, for 24 h, the quercetin-mediated apoptosis was significantly increased by PMA but markedly decreased by doxorubicin (Fig. 2). Treatment with PMA and doxorubicin did not directly result in apoptosis of B16-BL6 cells (Fig. 2). FFA (6.7 m*M*), a PKC activator, and chlorpromazine (14 m*M*), a PKC inhibitor, neither interfered with quercetin-induced apoptosis nor directly caused apoptosis of B16-BL6 cells (Fig. 2).

Nuclear DNA fragmentation induced by quercetin in B16-BL6 cells and effects of PKC activators and inhibitors

B16-BL6 cells (1×10^6 ml^{-1}) were incubated with 10^{-6}, 10^{-5}, and 10^{-4} g/ml quercetin for 24 h. Quercetin significantly induced nuclear DNA fragmentation of B16-BL6 cells in a dose-dependent manner, which showed a 180 bp ladder band, a typical biochemical characteristic of apoptosis [28]. Incubation in the presence of the PKC activator PMA at 100 n *M* significantly enhanced DNA fragmentation of B16-BL6 cells at each dosage of quercetin (Fig. 3a), but no enhancement was seen with FFA at 6.7 m*M*. Incubation in the presence of the PKC inhibitor doxorubicin at 8.6 μ*M* markedly reduced DNA fragmentation at each dosage of quercetin under the same conditions (Fig. 3b), but no reduction was seen with chlorpromazine at 14 m*M*. Moreover, nuclear

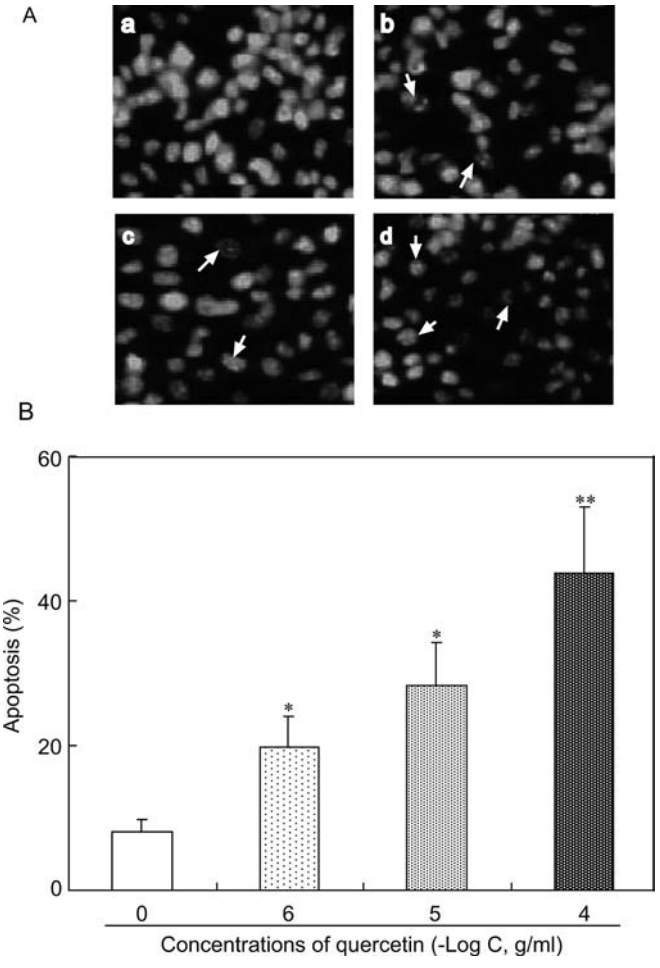

Fig. 1 Apoptosis of B16-BL6 cells induced by quercetin. **a** Condensation of nuclei induced by quercetin. B16-BL6 cells were incubated with various concentrations of quercetin for 24 h. Condensation of nuclei was assessed by Hoechst 33258 and PI staining as described in "Materials and methods." The images are representative of those of at least three independent experiments. Nuclei were amplified 40 times. *Arrows* indicated the condensed nuclei (*a* control, *b* 10^{-6} g/ml, *c* 10^{-5} g/ml, *d* 10^{-4} g/ml). **b** Quercetin-induced condensation of nuclei was dose-dependent. Apoptotic and normal cells were counted in five random fields taken by CCD. The percentage of apoptosis was calculated as described in "Materials and methods." The data presented are the means ± SD of at least three experiments, each performed in triplicate. One-way ANOVA revealed a significant effect at $P < 0.01$. *$P < 0.05$, **$P < 0.01$ versus control (Student's two-tailed *t*-test)

DNA fragmentation also showed characteristic bands on agarose gel electrophoresis indicating apoptosis, which were lightened and darkened by PMA and doxorubicin, respectively (Fig. 3c).

Mitochondrial transmembrane potential of B16-BL6 cells disrupted by quercetin and effect of PKC activators and inhibitors

B16-BL6 cells (1×10^6 ml^{-1}) were incubated with various concentrations of quercetin (1×10^{-6}, 1×10^{-5}, and

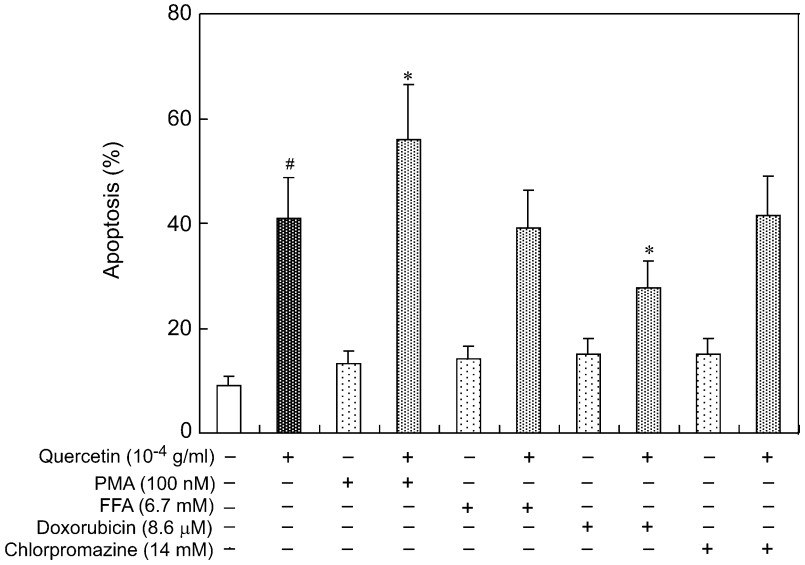

Fig. 2 Regulation by PKC of the quercetin-mediated apoptosis of B16-BL6 cells. B16-BL6 cells were incubated with quercetin (10^{-4} g/ml) in the presence or absence of PMA (100 n *M*), FFA (6.7 m*M*), doxorubicin (8.6 μ*M*) or chlorpromazine (14 m*M*) for 24 h. Apoptotic and normal cells were counted in five random fields taken by CCD. The percentage of apoptosis was calculated as described in Methods. The data are presented as the means ± SD of three independent experiments, each performed in triplicate. One-way ANOVA revealed a significant effect at $P < 0.01$. $^{\#}P < 0.01$, versus none; $^{*}P < 0.05$, versus quercetin alone (Student's two-tailed *t*-test)

1×10^{-4} g/ml) for 24 h. The cells were collected and stained with JC-1, the fluorescence of which is dependent on mitochondrial transmembrane potential. As shown in Fig. 4a, green fluorescence (JC-1 monomers) gradually increased while orange fluorescence (JC-1 aggregates) decreased as the concentrations of quercetin increased. The flow cytometric assay showed that quercetin disrupted the mitochondrial transmembrane potential of B16-BL6 cells in a dose-dependent manner (Fig. 4b). Moreover, quercetin also dose-dependently increased the JC-1 monomers/JC-1 aggregates as an indicator of disruption of the mitochondrial transmembrane potential (Fig. 5a). When B16-BL6 cells were incubated with quercetin (1×10^{-4} g/ml) in combination with the PKC activator PMA (100 n *M*) or FFA (6.7 m*M*), PMA but not FFA significantly reinforced quercetin-mediated disruption of the mitochondrial transmembrane potential, whereas the PKC inhibitor doxorubicin (8.6 μ*M*) but not chlorpromazine (14 m*M*) markedly antagonized it under the same conditions (Fig. 5b).

Expression of antiapoptotic protein Bcl-2 inhibited by quercetin in B16-BL6 cells and effect of PKC activators and inhibitors

B16-BL6 cells (1×10^{7}) were incubated with quercetin (10^{-4} g/ml) alone or with PMA (100 n *M*), FFA (6.7 m*M*), doxorubicin (8.6 μ*M*) and chlorpromazine (14 m*M*) in the presence or absence of quercetin for 24 h. The antiapoptotic proteins Bcl-2 and Bcl-X_L were measured by Western blotting in B16-BL6 cells. Quercetin alone markedly inhibited the expression of Bcl-2, but not Bcl-X_L (Fig. 6a,c, lane 2; Fig. 6b,d) [29]. PMA, but not FFA, further significantly enhanced inhibition of quercetin (Fig. 6a, lanes 4 and 6; Fig. 6b) while doxorubicin, but not chlorpromazine, nearly completely blocked quercetin-inhibited expression of Bcl-2 (Fig. 6c, lanes 4 and 6; Fig. 6d). However, they did not influence the expression of Bcl-2 and Bcl-X_L themselves (Fig. 6a,c, lanes 3 and 5; Fig. 6b,d).

Caspase-3 activity elevation by quercetin in B16-BL6 cells and effects of PKC activators and inhibitors

B16-BL6 cells (1×10^{6} ml^{-1}) were incubated with various drugs for 24 h. The cytosol proteins were extracted, and caspase-3 activity was measured as described in "Materials and methods." As shown in Fig. 7a, the activity of caspase-3 was significantly elevated by quercetin in a dose-dependent manner. When B16-BL6 cells were incubated with quercetin (1×10^{-4} g/ml) in combination with the PKC activator PMA (100 n *M*) or FFA (6.7 m*M*), PMA but not FFA significantly promoted the increase in caspase-3 activity induced by quercetin, whereas the PKC inhibitor doxorubicin (8.6 μ*M*) but not chlorpromazine (14 m*M*) markedly antagonized it under the same conditions (Fig. 7b).

Expression of PKC-α, PKC-β, and PKC-δ in B16-BL6 cells and effects of quercetin on their expression

After Bl6-BL6 cells were treated with various concentrations of quercetin for 24 h, the levels of expression of PKC-α, PKC-β, and PKC-δ in B16-BL6 cells were

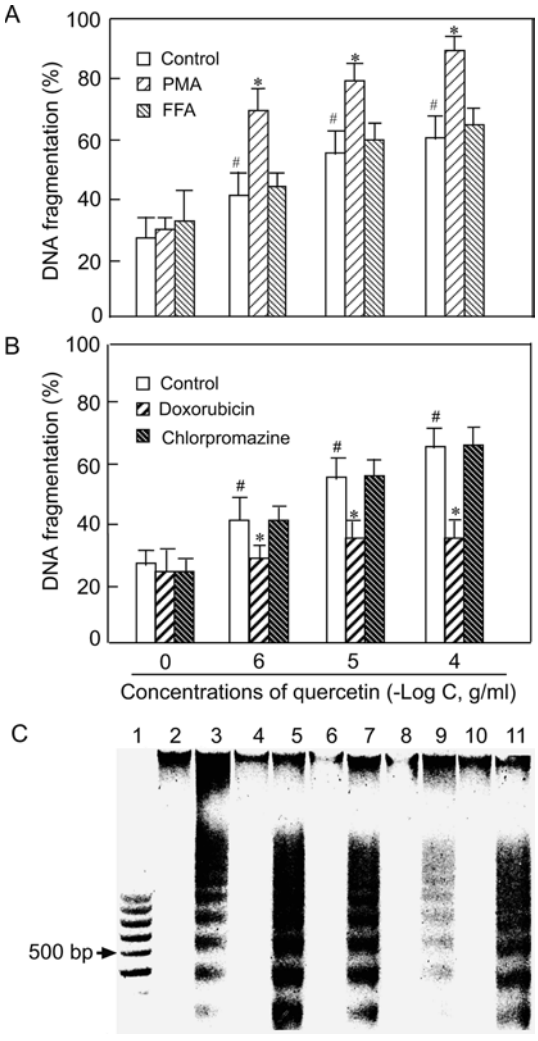

Fig. 3 Involvement of PKC in the nuclear DNA fragmentation caused by quercetin in B16-BL6 cells. B16-BL6 cells were incubated with various concentrations of quercetin in combination with PMA (100 n *M*), FFA (6.7 m*M*), doxorubicin (8.6 μ*M*) or chlorpromazine (14 m*M*) for 24 h. DNA fragmentation was determined by the diphenylamine method. **a**, **b** The data presented are the means ± SD of three independent experiments, each performed in triplicate. One-way ANOVA revealed a significant effect at $P < 0.01$. $^{\#}P < 0.01$, versus normal; $^{*}P < 0.05$, versus controls (Student's two-tailed *t* test). *Normal* neither quercetin nor PKC inhibitors were present; *control* only quercetin at different concentrations was present. DNA fragmentation in the above cells was analyzed by agarose gel electrophoresis. **c** The data presented are representative of three independent experiments. *Lane 1* marker, *lane 2* control, *lane 3* quercetin (1×10^{-4} g/ml) alone, *lane 4* PMA (100 n *M*), *lane 5* quercetin (1×10^{-4} g/ml) + PMA (100 n *M*), *lane 6* FFA (6.7 m*M*), *lane 7* quercetin (1×10^{-4} g/ml) + FFA (6.7 m*M*), *lane 8* doxorubicin (8.6 μ*M*), *lane 9* quercetin (1×10^{-4} g/ml) + doxorubicin (8.6 μ*M*), *lane 10* chlorpromazine (14 m*M*), *lane 11* quercetin (1×10^{-4} g/ml) + chlorpromazine (14 m*M*)

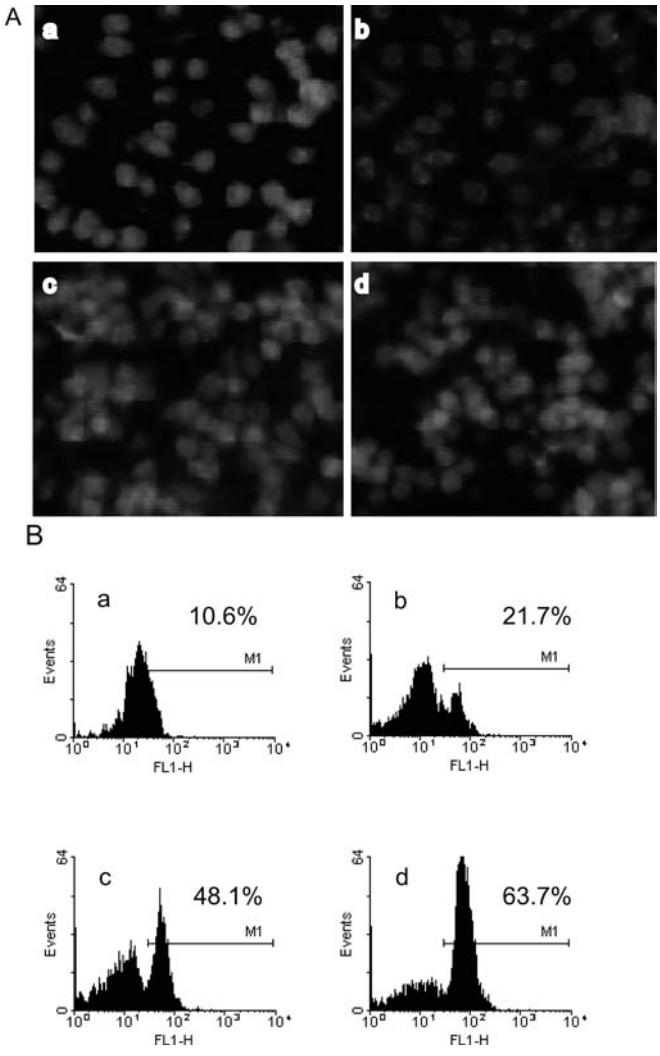

Fig. 4 Mitochondrial transmembrane potential disrupted by quercetin in B16-BL6 cells. B16-BL6 cells were incubated with various concentrations of quercetin for 24 h, and then stained with JC-1 for fluorescence microscopy or flow cytometric assay. **a** Quercetin increased JC-1 monomers and decreased JC-1 aggregates in a dose-dependent manner. The general staining characteristics of JC-1 in each sample were visually assessed using a Leica DMIRB inverted phase fluorescence microscope equipped with a ×40 objective, FITC filter cube and CCD (*a* control, *b* 10^{-6} g/ml, *c* 10^{-5} g/ml, *d* 10^{-4} g/ml). **b** Quercetin dose-dependently led to a loss of the mitochondrial transmembrane potential by flow cytometric assay. JC-1 monomers were evaluated in terms of FL-1 fluorescence (*a* control, *b* 10^{-6} g/ml, *c* 10^{-5} g/ml, *d* 10^{-4} g/ml). **a**,**b** The data presented are representative of at least three independent experiments

determined by Western blotting. As shown in Fig. 8a, PKC-α, PKC-β, and PKC-δ were all detected in B16-BL6 cells. Quercetin inhibited the expression of PKC-α in dose-dependent manner but did not affect the expression of PKC-β or PKC-δ (Fig. 8a,b).

Effects of PKC activators and inhibitors on the quercetin-induced decrease in expression of PKC-α

After B16-BL6 cells were incubated with quercetin alone or with various activators and inhibitors of PKC in the presence or absence of quercetin for 24 h, the levels of expression of PKC-α, PKC-β, and PKC-δ were determined by Western blotting. Only doxorubicin almost completely blocked the quercetin-induced decrease in

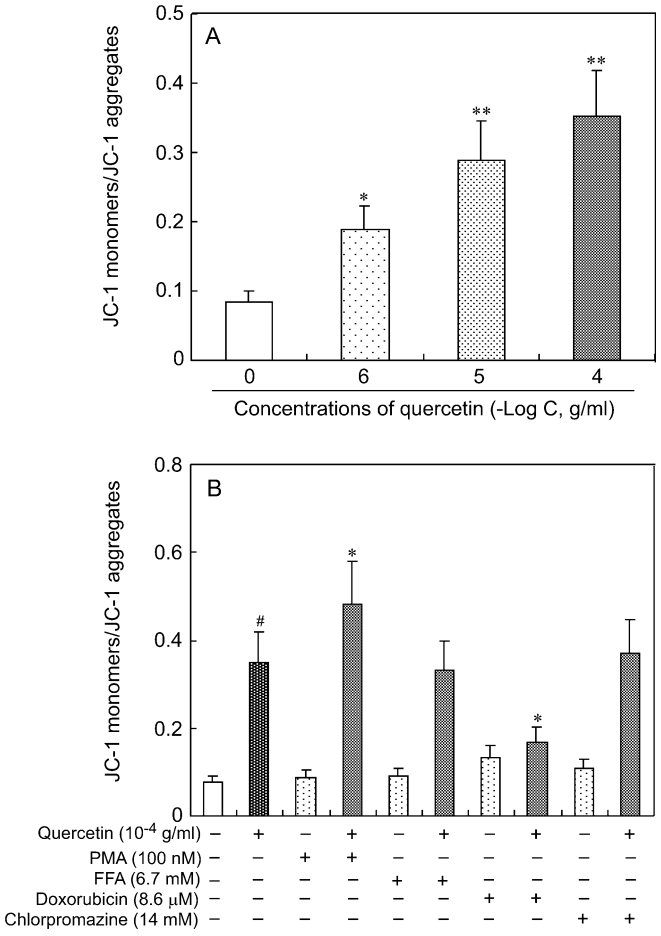

Fig. 5 Interference of PKC in quercetin-mediated disruption of mitochondrial transmembrane potential of B16-BL6 cells. **a** Quercetin enhanced the JC-1 monomers/JC-1 aggregates in a dose-dependent manner. JC-1 monomers and aggregates were measured as described in "Materials and methods." The value of JC-1 monomers/JC-1 aggregates is as an indicator of the loss of the mitochondrial transmembrane potential. One-way ANOVA revealed a significant effect at $P < 0.01$. $^{*}P < 0.05$, $^{**}P < 0.01$, versus control (Student's two-tailed *t*-test). **b** PMA further significantly reinforced, while doxorubicin almost completely blocked, the quercetin-mediated elevation of JC-1 monomers/JC-1 aggregates. B16-BL6 cells were incubated with quercetin (10^{-4} g/ml) in the presence or absence of PMA (100 n *M*), FFA (6.7 m*M*), doxorubicin (8.6 μ*M*) or chlorpromazine (14 m*M*) for 24 h. The value of JC-1 monomers/JC-1 aggregates was calculated as described in "Materials and methods." One-way ANOVA revealed a significant effect at $P < 0.01$. $^{\#}P < 0.01$, versus none; $^{*}P < 0.05$, versus quercetin alone (Student's two-tailed *t*-test). **a**,**b** The data presented are the means ± SD of at least three experiments, each performed in triplicate

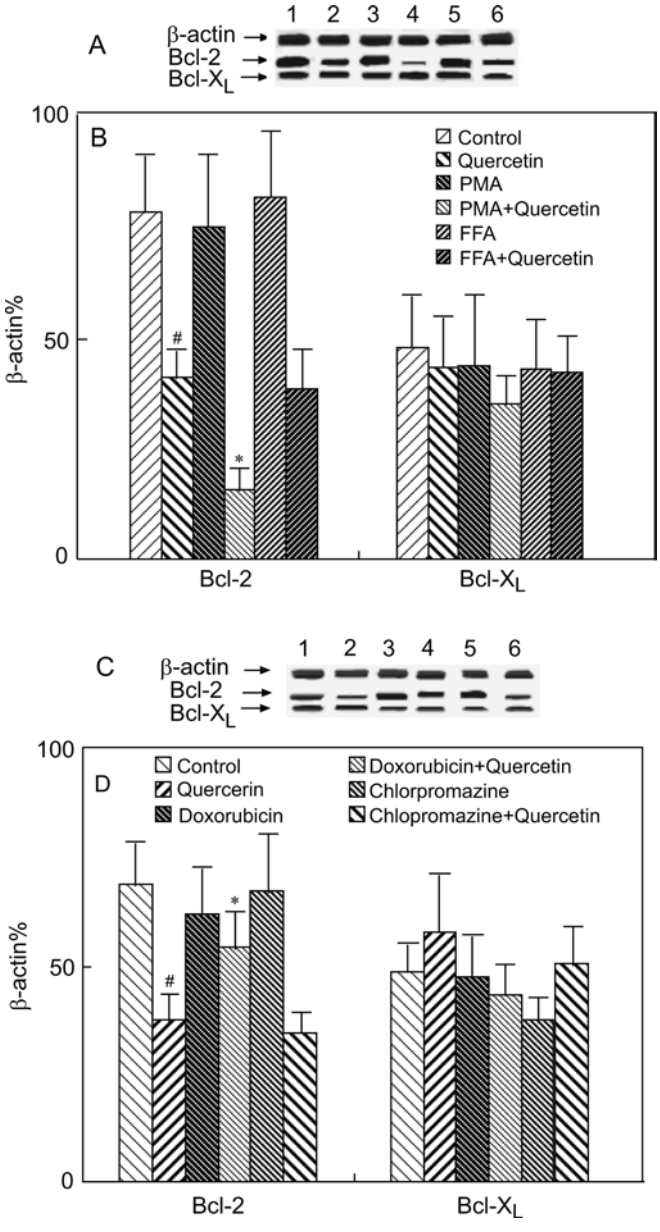

Fig. 6 The expression of Bcl-2 inhibited by quercetin and regulation by PKC of quercetin-mediated inhibition of Bcl-2 expression in B16-BL6 cells. **a** PMA strengthened the quercetin-induced decrease in the expression of Bcl-2, and (**c**) doxorubicin blocked it. B16-BL6 cells were incubated with quercetin (10^{-4} g/ml) in the presence or absence of PMA (100 n *M*), FFA (6.7 m*M*), doxorubicin (8.6 μ*M*) or chlorpromazine (14 m*M*) for 24 h. The levels of expression of Bcl-2, Bcl-X_L and *β*-actin were determined by Western blotting (**a** *lane 1* control, *lane 2* quercetin, *line 3* PMA, *lane 4* quercetin + PMA, *lane 5* FFA, *lane 6* quercetin + FFA; **c** *lane 1* control, *lane 2* quercetin, *lane 3* doxorubicin, *lane 4* quercetin + doxorubicin, *lane 5* chlorpromazine, *lane 6* quercetin + chlorpromazine). **b**, **d** The relative expression of Bcl-2 and Bcl-X_L in B16-BL6 cells was quantified densitometrically using the software LabWorks 4.0, and calculated according to the reference bands of *β*-actin. One-way ANOVA revealed a significant effect at $P < 0.01$, $^{\#}P < 0.01$ versus control; $^{*}P < 0.05$ versus quercetin alone (Student's two-tailed *t*-test). **a–d** The data presented are the means ± SD of three independent experiments

expression of PKC-α; the other reagents had no obvious effect (Fig. 9a,b).

The translocation of PKC-δ from cytosol to nuclei induced by quercetin and effects of PKC activators and inhibitors

After B16-BL6 cells were incubated with quercetin alone or with various activators and inhibitors of PKC in the

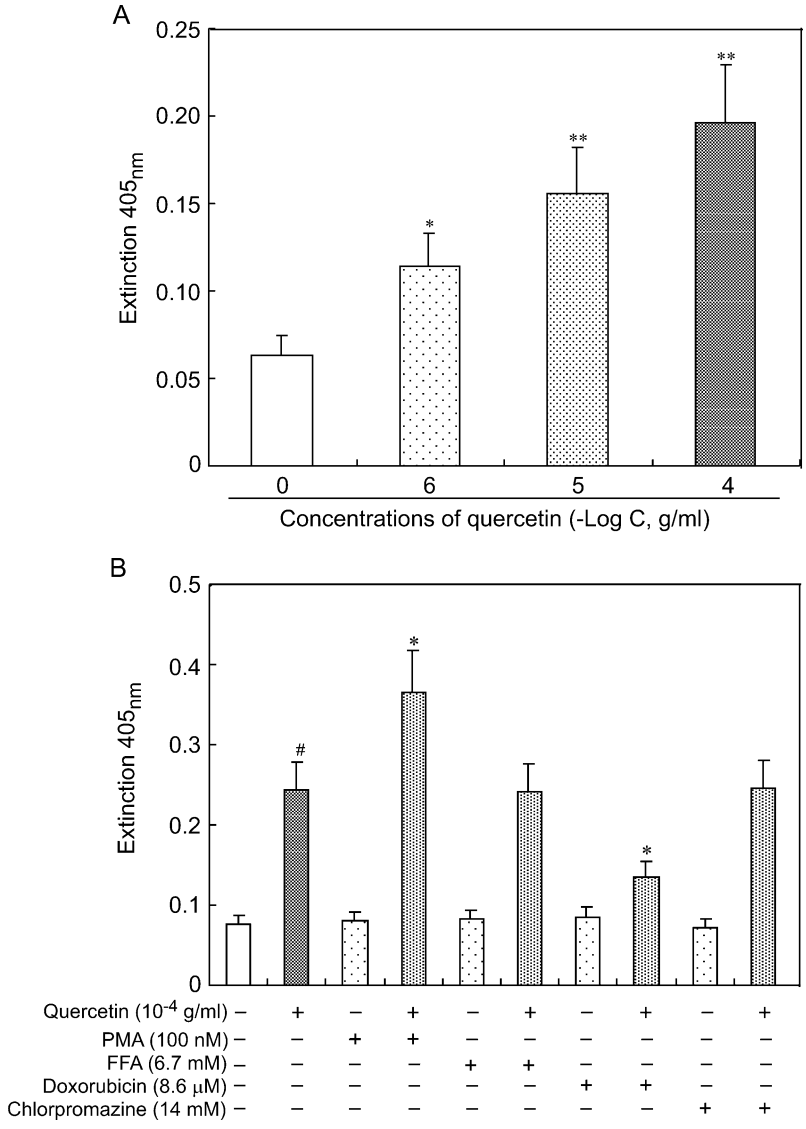

Fig. 7 Increase in activity of caspase-3 induced by quercetin and the effects of PKC on the quercetin-induced increase in caspase-3 activity. **a** Quercetin increased caspase-3 activity in a dose-dependent manner in B16-BL6 cells. **b** PMA significantly promoted the quercetin-induced activity of caspase-3 while doxorubicin antagonized the effect of quercetin. B16-BL6 cells were incubated with various concentrations of quercetin (**a**) in the presence or absence of PMA (100 n *M*), FFA (6.7 m*M*), doxorubicin (8.6 μ*M*) or chlorpromazine (14 m*M*) (**b**) for 24 h. The cytosol proteins were extracted, and the activity of caspase-3 was measured as described in Methods. The data presented are the means ± SD of at least three independent experiments, each performed in triplicate. One-way ANOVA revealed a significant effect at $P < 0.01$, $^{*}P < 0.05$, $^{**}P < 0.01$ versus control in **a**; $^{\#}P < 0.01$ versus control; $^{*}P < 0.05$ versus quercetin alone in **b** (Student's two-tailed *t*-test)

presence or absence of quercetin for 24 h, their cytosol and nuclear proteins were extracted as described in "Materials and methods." PKC-α and PKC-δ in cytosol and nuclear proteins were determined by Western blotting, respectively. Quercetin dose-dependently induced the translocation of PKC-δ but not PKC-α from cytosol to nuclei (Fig. 10a,b). Moreover, only PMA significantly enhanced the translocation of PKC-δ induced by quercetin, but other reagents had no obvious effect on it (Fig. 10c,d).

Discussion

Apoptosis can be induced by various chemotherapy drugs [41]. Quercetin, which has antitumor activity, also induces apoptosis of many kinds of cells [25–28]. In our previous study, quercetin was found to induce apoptosis of highly metastatic melanoma B16-BL6 cells [29]. Moreover, quercetin also inhibits the activity of many kinases including protein tyrosine and serine/threonine kinases [22, 23]. In the present study, we further investigated the cellular and molecular mechanisms of apoptosis induced by quercetin and the relationship between quercetin-induced apoptosis and inhibition of kinases.

Quercetin induced condensation of nuclei as well as DNA fragmentation in a dose-dependent pattern in B16-BL6 cells (Fig. 1), and also dose-dependently disrupted mitochondrial transmembrane potential (Figs. 4, 5a). These results suggest that quercetin induced apoptosis by injuring mitochondria causing them to release proapoptotic proteins such as cytochrome *c* and AIF. Bcl-2 family proteins are closely associated with membrane permeabilization and the disruption of transmembrane potential in mitochondria [9–13]. In our previous study, quercetin was found to inhibit the expression of Bcl-2, but not Bcl-X_L (Fig. 6a,c), suggesting that quercetin-mediated apoptosis is associated with inhibition of Bcl-2 expression. Proapoptotic pro-

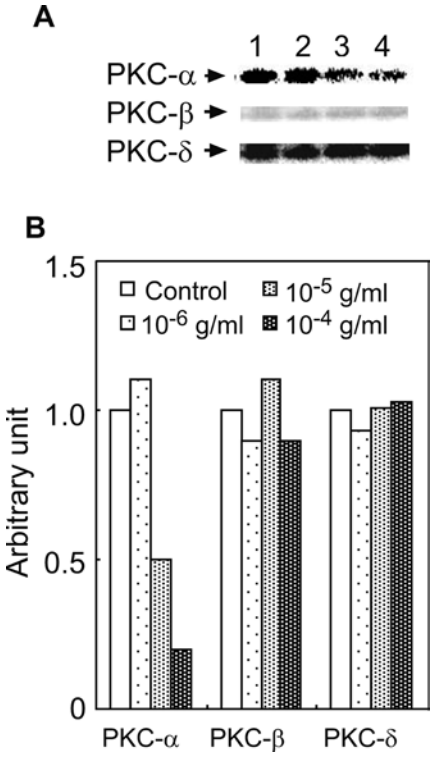

Fig. 8 The expression of PKC-α, PKC-β, and PKC-δ in B16-BL6 cells and the effects of quercetin on their expression. B16-BL6 cells were incubated with various concentrations of quercetin for 24 h. **a** The levels of expression of PKC-α, PKC-β, and PKC-δ were determined by Western blotting (*lane 1* control, *lanes 2–4* 10^{-6}, 10^{-5}, and 10^{-4} g/ml quercetin, respectively). **b** The relative expression of PKC-α, PKC-β, and PKC-δ in B16-BL6 cells was quantified densitometrically using the software LabWorks 4.0, and calculated according to the reference bands of the control. **a**,**b** The data presented are representative of three independent experiments

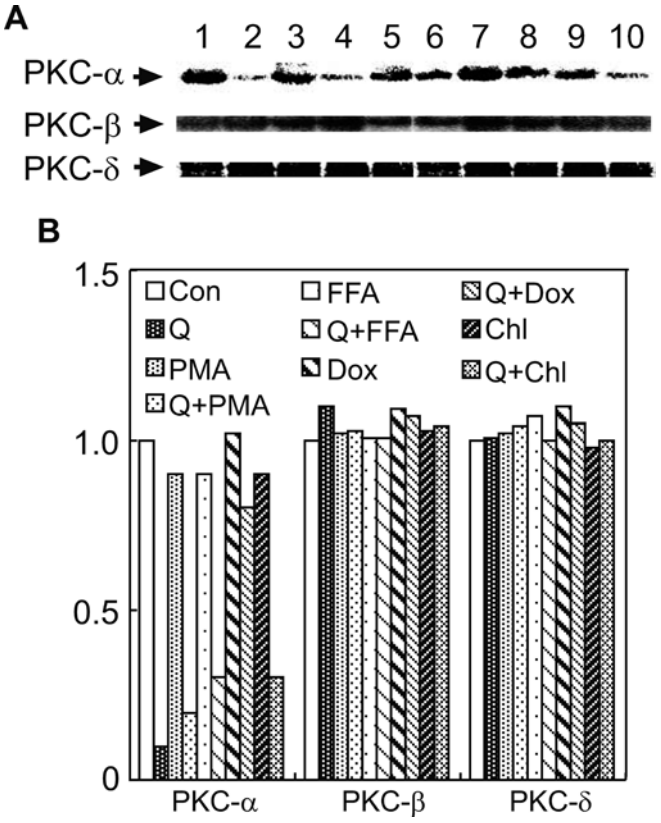

Fig. 9 Effects of PKC activators and inhibitors on the quercetin-induced decrease in expression of PKC-α. B16-BL6 cells were incubated with quercetin alone or PMA (100 n *M*), FFA (6.7 m*M*), doxorubicin (8.6 μ*M*) and chlorpromazine (14 m*M*) in the presence or absence of quercetin for 24 h. **a** The levels of expression of PKC-α, PKC-β, and PKC-δ were determined by Western blotting (*lane 1* control, *lane 2* quercetin alone, *lane 3* PMA, *lane 4* quercetin + PMA, *lane 5* FFA, *lane 6* quercetin + FFA, *lane 7* doxorubicin, *lane 8* quercetin + doxorubicin, *lane 9* chlorpromazine, *lane 10* quercetin + chlorpromazine. **b** The relative expression of PKC-α, PKC-β, and PKC-δ in B16-BL6 cells was quantified densitometrically using the software LabWorks 4.0, and calculated according to the reference bands of the control (*Con* control, *Q* quercetin, *Dox* doxorubicin, *Chl* chlorpromazine). The data presented are representative of three independent experiments

teins from mitochondria trigger a caspase cascade, which degrades proteins related to DNA repair or activate CAD, a DNA-degrading enzyme, through hydrolyzing the antiapoptotic protein ICAD [42]. Quercetin also elevated the activity of caspase-3 in a dose-dependent manner (Fig. 7a), which was perhaps related to the quercetin-mediated condensation of nuclei and DNA fragmentation. These results demonstrate for the first time that quercetin induces apoptosis of B16-BL6 cells through the mitochondrial pathway.

In addition, quercetin sensitized HPB-ALL cells to apoptosis induced by anti-Fas antibody [43]. Heat shock proteins may be involved in quercetin-induced apoptosis [44]. These results indicate that other mechanisms are involved in quercetin-mediated apoptosis. However, the investigations of the relationship between quercetin-mediated apoptosis and inhibition of kinases have rarely produced clear-cut results.

To elucidate the mechanism in the present study, PKC activators and inhibitors were used to interfere with quercetin-mediated condensation of nuclei, DNA fragmentation, disruption of mitochondrial transmembrane potential, decrease in the expression of Bcl-2 and increase in the activity of caspase-3. PMA further significantly strengthened, while doxorubicin almost completely blocked, these effects of quercetin (Figs. 2, 3, 5b, 6, 7b), suggesting that PKC is involved in quercetin-mediated apoptosis. However, FFA and chlorpromazine did not affect the effects of quercetin (Figs. 2, 3, 5b, 6, 7b). Interestingly, both PMA and doxorubicin regulate the activity of PKC through interaction with DAG, while both FFA and chlorpromazine act through modulation of cytosolic free Ca^{2+} [45–48], which suggests that quercetin exerts its effects perhaps through affecting the interaction between PKC and DAG.

PKC inhibitors synergistically increase apoptosis induced by chemotherapy drugs such as 1-beta-D-arabinofuranosylcytosine or etoposide, while PKC activators including PMA and TPA block the apoptosis [4, 5, 49]. In contrast, in our experiments, doxorubicin inhibited, whereas PMA enhanced, quercetin-mediated apoptosis,

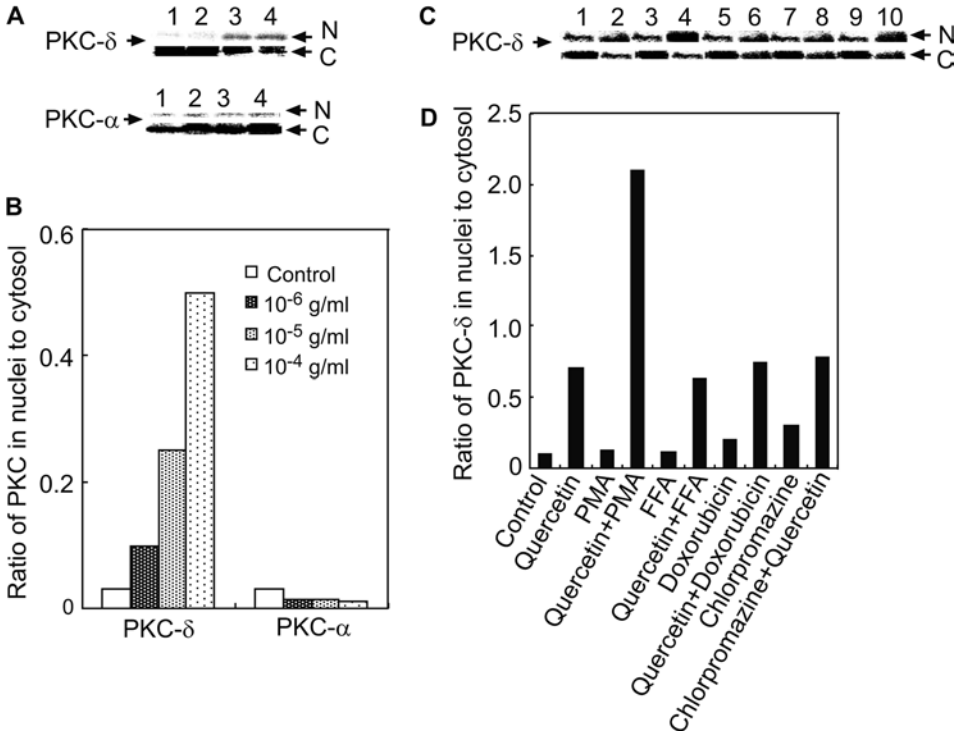

Fig. 10 The translocation of PKC-δ from cytosol to nuclei induced by quercetin and effects of PKC activators and inhibitors. After B16-BL6 cells were incubated with quercetin alone or PMA (100 n *M*), FFA (6.7 m*M*), doxorubicin (8.6 μM), and chlorpromazine (14 m*M*) in the presence or absence of quercetin for 24 h, their cytosol and nuclear proteins were extracted as described in "Materials and methods." **a** PKC-δ and PKC-α in cytosol and nuclear proteins were determined by Western blotting, respectively (*lane 1* control, *lanes 2–4* 10^{-6}, 10^{-5}, and 10^{-4} g/ml quercetin, respectively). **b** The ratio of PKC-δ and PKC-α in nuclei to cytosol. The relative levels of PKC-δ and PKC-α in cytosol and nuclei were quantified densitometrically using the software LabWorks 4.0, and calculated according to the reference bands of the control. **c** Effects of PKC activators and inhibitors on the translocation of PKC-δ from cytosol to nuclei induced by quercetin. The levels of PKC-δ in cytosol and nuclei were detected by Western blotting, respectively (*lane 1* control, *lane 2* quercetin alone, *lane 3* PMA, *lane 4* quercetin + PMA, *lane 5* FFA, *lane 6* quercetin + FFA, *lane 7* doxorubicin, *lane 8* quercetin + doxorubicin, *lane 9* chlorpromazine, *lane 10* quercetin + chlorpromazine). **d** The ratio of PKC-δ in cytosol to nuclei. The relative levels of PKC-δ in cytosol and nuclei were quantified densitometrically using the software LabWorks 4.0, and calculated according to the reference bands of the control. **a–d** The data presented are representative of three independent experiments

perhaps indicating a novel interaction between PKC and an antitumor drug. The reason might be that these PKC inhibitors and activators influenced a different subtype that plays a different role in apoptosis. For example, overexpression of PKC-α inhibits apoptosis, while overexpression of PKC-δ increases apoptosis in glioma cells [20]. Many inhibitors and activators of PKC such as PMA, TPA, sphingosine, calphostin C induce apoptosis, but neither PMA nor doxorubicin induced apoptosis of B16-BL6 cells in this study (Figs. 2, 3, 5b) [50–52]. The synergistic effect of PKC activators and inhibitors on the action of chemotherapy drugs is dependent on the properties of cells [53].

These results show that PKC is involved in apoptosis of B16-BL6 cells induced by quercetin. To further elucidate the involvement of the subtypes of PKC in quercetin-induced apoptosis of B16-BL6 and the mechanisms of the synergy of quercetin with PKC inhibitors and activators, the expressions of different subtypes of PKC were determined in B16-BL6 cells. Although PKC-α, PKC-β, and PKC-δ were expressed in B16-BL6 cells, only PKC-α was inhibited by quercetin in a dose-dependent manner (Fig. 8a,b). PKC-α inhibits apoptosis by regulating the phosphorylation of Bcl-2 and the activity of Akt [54, 55]. Moreover, only doxorubicin almost completely blocked the effect of quercetin in deceasing the expression of PKC-α (Fig. 9a,b). This result indicates that the blockage by doxorubicin of quercetin-mediated apoptosis of B16-BL6 cells is associated with PKC-α expression. PKC-δ also played an important role in apoptosis by translocating from cytosol to nuclei [56, 57]. Although quercetin did not affect the expression of PKC-δ, its effect on the translocation of PKC-δ was investigated. Quercetin had a dose-dependent effect on the translocation of PKC-δ from cytosol to nuclei in B16-BL6 cells (Fig. 10a,b). Furthermore, only PMA enhanced the quercetin-induced translocation of PKC-δ (Fig. 10c,d). Therefore the enhancement by PMA of quercetin-mediated apoptosis is related to the translocation of PKC-δ.

In conclusion, quercetin-induced apoptosis of B16-BL6 cells is related to its injurious effects on mitochondria by decreasing the expression of Bcl-2 and increasing the activity of caspase-3, which was perhaps caused by its inhibition of the expression of PKC-α and induction of the translocation of PKC-δ. In addition, PMA, which acts synergistically with quercetin, enhanced quercetin-

mediated apoptosis of B16-BL6 cells by enhancing the translocation of PKC-δ induced by quercetin, which is perhaps a novel synergistic pattern that is different from the mechanisms of action of the PKC inhibitors safingol and calphostin C which increase apoptosis induced by chemotherapy drugs. These results provide a new basis for the combination of chemotherapy drugs in the clinic.

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