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Studies on the Inhibitory Effects of Quercetin on the Growth of HL-60 Leukemia Cells

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ABSTRACT. Quercetin, a naturally occurring flavonoid, has been shown to exert multiple pharmacological effects and to be an anticancer agent or a supplementary anticancer agent. In this report, the human HL-60 promyelocytic leukemia cell line was used to study the effects of quercetin on the growth, cell cycle, activities of cytosolic and membrane protein kinase C (PKC) and tyrosine protein kinase (TPK), and phosphoinositide production of the tumor cells. The results showed that quercetin inhibited the growth of HL-60 cells in a concentration-dependent manner, with an IC_{50} value of about 7.7 μM after 96 hr of treatment; when the concentration of quercetin was 10 μM, the percent inhibition on the growth of HL-60 cells was 17.1, 27.3, 40.1, and 52.7% after 24, 48, 72, and 96 hr of treatment, respectively. Flow cytometric analyses showed that quercetin caused an increase in cells in the G_2/M phase and a decrease in cells in the G_0/G_1 phase of the cell cycle in a concentration-dependent manner; these effects were reversed when quercetin was removed from the culture medium. Quercetin strongly inhibited the activities of cytosolic PKC and membrane TPK from HL-60 cells *in vitro*, with IC_{50} values of about 30.9 and 20.1 μM, respectively, but did not affect membrane PKC or cytosolic TPK activity from HL-60 cells *in vitro*. Quercetin markedly inhibited in a concentration-dependent manner the production of phosphoinositides in intact HL-60 cells. The results provide evidence that the inhibitory effect of quercetin on the growth of HL-60 cells may be related to its inhibitory effects on PKC and/or TPK *in vitro* and/or on the production of phosphoinositides. BIOCHEM PHARMACOL **54**;9:1013–1018, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. human HL-60 promyelocytic leukemia cell; flavonoids; quercetin; cell cycle; protein kinase C; phosphoinositides; tyrosine protein kinase

Flavonoids are found in many plants. Various pharmacological activities of flavonoids have been studied extensively [1–5]. It has been shown that quercetin (3,3′,4′,5,7-pentahydroxyflavone) inhibits the growth of cancer cell lines [6–8] and of acute lymphoid and myeloid leukemia cells [9]. The antiproliferative activity of this compound could be mediated by type II EBS† [6–10] and PKC [11]. It has been reported that quercetin markedly inhibits the growth of human gastric and colon cancer cells by inhibiting cell cycle progression at the G_1-S boundary [12, 13], and that quercetin arrests the cell cycle in late G_1 prior to the beginning of the S phase in T-cells [14] and at the G_2/M phase in human MDA-MB468 breast carcinoma cells [15]. However, Matsukawa *et al.* [16] reported that genistein specifically arrests the cell cycle of HGC-27 cells at G_2/M and suggested that the effect of genistein may be related to its specific inhibition of TPK. Worland *et al.* [17] showed that the flavone L86-8275 delayed the progression of aphidicolin-synchronized MDA-468 breast carcinoma cells through S phase and prevented progression through G_2. In summary, the mechanism of the action of flavonoids on the growth and cell cycle of tumor cells has remained unclear [11–17].

Considerable evidence is available to implicate PKC, TPK, and phosphoinositide signaling systems in the growth and proliferation of cells and the development of cancer [18–23]. An enormous amount of work has shown that quercetin is capable of inhibiting PKC and TPK [11, 24–27]. Recently, an overview of a systematic study demonstrated that quercetin inhibits the proliferation of OVCAR cells and human breast carcinoma cells [28]. The decline of PI-4K activity precedes the inhibition of cell proliferation, whereas PIP-K activity is inhibited to only a minor extent [28]. In the present report, we describe the effects of quercetin on the proliferation and cell cycle of HL-60, and on the activities of cytosolic and membrane PKC and TPK from HL-60 leukemia cells, and on the

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† *Abbreviations:* DTT, dithiothreitol; FBS, fetal bovine serum; PI, phosphatidylinositol; PI-4K, phosphatidylinositol 4-kinase; PIP-K, phosphatidylinositol 4-phosphate 5-kinase; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-triphosphate; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; pNPP, *p*-nitrophenyl phosphate disodium salt; TCA, trichloroacetic acid; TPK, tyrosine protein kinase; and type II EBS, type II estrogen binding sites.

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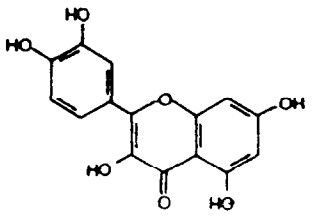

FIG. 1. Structure of quercetin.

production of phosphoinositides in intact HL-60 cells *in vitro*.

MATERIALS AND METHODS

Chemicals

Quercetin, whose structure is shown in Fig. 1, leupeptin, $NaVO_3$, EGTA, diolein, histone III-S, PI, PIP, ATP, RNase, poly Glu · Tyr (4:1), PIP_2, and propidium iodide were purchased from Sigma (St. Louis, MO). RPMI-1640 medium was obtained from GIBCO (Grand Island, NY), and Triton X-100 was purchased from FARCO Chemical Supplies (Hong Kong). DTT and EDTA were obtained from the SERVA Co. (Hong Kong), and pNPP and PMSF were purchased from the Institute of Biochemistry (Shanghai, China). FBS was obtained from the Hangzhou Bioch Tech Co. (Hangzhou, China); [γ-^{32}P]ATP was purchased from the Beijing Yahui Bioch Tech Co. (Beijing, China). All other chemicals were of reagent grade.

Cell Culture

HL-60, a human promyelocytic leukemia cell line that has a doubling time of 24 hr, was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and antibiotics in a 5% CO_2 atmosphere at 37°.

Growth Inhibition of Cells

Cells were seeded at a density of 5×10^4 cells/mL RPMI-1640 medium in 60-mm diameter dishes. Quercetin was dissolved in dimethyl sulfoxide and diluted to its final concentration in each culture dish. An equivalent volume of dimethyl sulfoxide added to the control dishes was found to have no measurable effect on HL-60 cells. At 24, 48, 72, and 96 hr, the number of viable cells was ascertained, using the trypan blue dye exclusion test.

Analysis of Cell Cycle Progression

HL-60 cells were plated at a density of 5×10^5 cells/mL of medium in 60-mm diameter dishes. Cells were removed at the indicated times from culture dishes by centrifugation. After washing with ice-cold PBS, cells were suspended in about 0.5 mL of PBS, 0.8 mL of solution containing 0.1% Triton X-100, 20 μg/mL of RNase, and 50 μg/mL of propidium iodide were added, and the cells were kept for 30 min at 4°. The suspension was filtered through 50 μm nylon mesh, and the DNA content of stained nuclei was analyzed by a flow cytometer (EPICS XL, Coulter Co., Miami, FL). The cell cycle was analyzed using Multicycle—DNA Cell Cycle Analysis Software. To elucidate the reversibility of the effects of quercetin, medium containing quercetin was removed by aspiration 24 hr after addition, and cells were washed with fresh medium and then refed with an equal volume of fresh medium. After incubating for another 24 hr, cells were harvested and treated as above.

Partial Purification and Assay of TPK

Partial purification and assay of TPK activity were accomplished by the method of Kong and Wang [29] as modified by us [30]. HL-60 cells (about 1×10^7 cells/mL) were washed with ice-cold PBS, suspended in buffer A (20 mM Tris–HCl, pH 7.5, 0.25 M sucrose, 20 mM DTT, 2 mM EDTA, 0.5 mM PMSF, and 5 μg/mL of leupeptin), and homogenized in an ice bath. All subsequent operations were carried out at 0–4°. The homogenate was centrifuged at 280 × *g* for 10 min; the supernatant was centrifuged at 100,000 *g* for 1 hr, and used for the assay of cytosolic TPK. To the particulate fraction was added buffer A containing 0.5% Triton X-100, which was then homogenized in an ice bath and extracted for 1 hr. The homogenate was centrifuged at 100,000 × *g* for 1 hr, and the supernatant was used for analysis of membrane TPK activity. The TPK assay reaction mixture (50 μL) contained 50 mM Tris–HCl, pH 7.5, 50 mM $MgCl_2$, 5 mM $MnCl_2$, 50 μM $NaVO_3$, 7 mg/mL of pNPP, 840 μg/mL of poly Glu · Tyr (4:1), 50 μM [γ-^{32}P]ATP (containing 1 μCi), enzyme preparation (13 μL, about 50 μg enzyme protein), and the required concentration of quercetin. It was necessary for the assay to measure the basal enzyme activity, which was carried out in the absence of poly Glu · Tyr (4:1) and was subtracted from the experimental values. After incubation for 10 min at 30°, the reaction was terminated by application of the entire 50 μL onto 1.5-cm diameter Xinhua No. 3 filter paper, which was added to 10% TCA. After being washed extensively with TCA, the filter paper was washed with acetone, and radioactivity was measured using an LS600C Beckman Scintillation Spectrometer.

Partial Purification and Assay of PKC

Partial purification and assay of PKC activity were accomplished by the method of Krug and Tashjian [31] as modified by us [30]. HL-60 cells (about 1×10^7 cells/mL) were washed with ice-cold PBS, suspended in buffer B (20 mM Tris–HCl, pH 7.5, 0.25 M sucrose, 10 mM EGTA, 0.5 mM PMSF), and homogenized in an ice bath. All subsequent operations were carried out at 0–4°. The cytosolic PKC was obtained in the same manner as TPK. The particulate fraction was added to buffer B containing 0.5% Triton X-100. Then the analysis of membrane PKC was the same as that of TPK. The PKC reaction mixture (100 μL) contained 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.5

mM $CaCl_2$, 40 μg/mL of phosphatidylserine (PS), 0.8 μg/mL of diolein, 0.2 mg/mL of histone III-S, 50 μM [γ-^{32}P]ATP (containing 1 μCi), enzyme preparation (20 μL, about 75 μg of enzyme protein), and the required concentration of quercetin. Measurement of the basal enzyme activity was carried out in the presence of 0.5 mM EGTA instead of PS, diolein, and $CaCl_2$ and was subtracted from the experimental values. After incubation for 10 min at 30°, the reaction was terminated by applying 90 μL onto 1.5-cm diameter Xinhua No. 3 filter paper disks, which were added to 10% TCA. All subsequent operations were the same as those for TPK.

Determination of Phosphoinositides in Intact HL-60 Cells

Extraction, separation, and autoradiography of PI, PIP, and PIP_2 were performed as described previously [32, 33]. HL-60 cells (about 1×10^6 cells/mL) were put into tubes (0.5 mL/tube, containing 10 μCi/mL [γ-^{32}P]ATP and 10 μg/mL of saponin), and incubated with various concentrations of quercetin for 30 min at 30°. Reactions were stopped with 1.5 mL of chloroform:methanol (2:1, v/v), and 1.5 mL of 3.1 M HCl and 1 mL of chloroform were added. After extensive vibration and centrifugation at 4000 × *g* for 10 min, the organic phase was removed and washed two times with 1 mL of 0.75 M NaCl containing chloroform:methanol:0.1 M HCl (3:48:47, by vol.), and the organic phase was dried under air or N_2. The lipids containing PI, PIP, and PIP_2 were redissolved in 50 μL of chloroform:methanol (2:1, v/v), applied to TLC plates coated with silica gel, which were impregnated with 1% potassium oxalate and activated at 110° for 1 hr, and developed with chloroform: acetone:methanol:36% acetic acid (8.0:3.0:6.0:3.4, by vol.). The ^{32}P-labeled lipids were visualized by autoradiography, and the specific areas of PI, PIP, and PIP_2 were located by iodine staining, with standards of the lipids as markers. The quantitation of phosphoinositide production was accomplished by densitometric scanning (Imaging densitometer, Model GS-670, Bio-Rad, Hercules, CA) of the autoradiogram.

Data are expressed as means ± SD, and statistical significance was analyzed by a *t*-test.

RESULTS

Effect of Quercetin on the Growth of HL-60 Cells

Figure 2 shows the growth curve of HL-60 cells in the various concentrations of quercetin. A concentration-dependent inhibition of growth was observed between 10 and 80 μM quercetin. On day 4, the IC_{50} was 7.7 μM; when the concentration of quercetin was 10 μM, the percent inhibition on the growth of HL-60 cells was 17.1, 27.3, 40.1, and 52.7% after 24, 48, 72, and 96 hr of treatment, respectively.

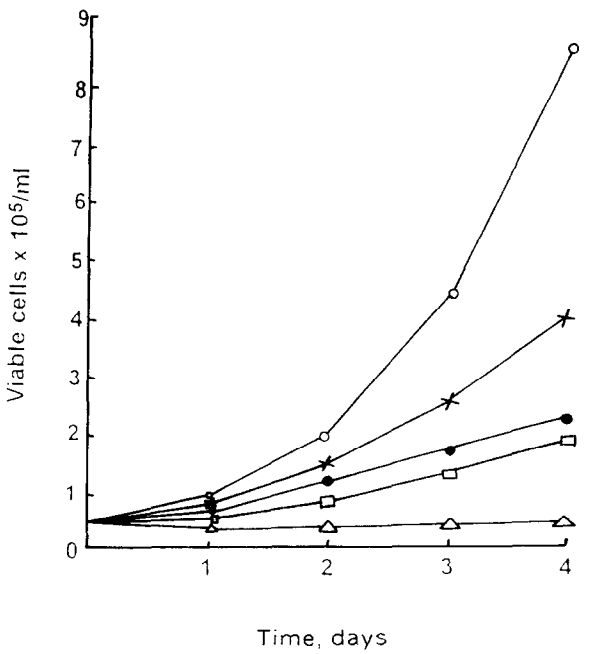

FIG. 2. Effects of various concentrations of quercetin on the growth of HL-60 cells. The number of viable cells was ascertained using the trypan blue dye exclusion test. Key: (○——○) control; (×——×) 10 μM; (●——●) 20 μM; (□——□) 40 μM; and (△——△) 80 μM.

Effects of Quercetin on the Cell Cycle of HL-60

To investigate the effects of quercetin on the cell cycle of HL-60, the DNA content of HL-60 nuclei was measured by flow cytometric analysis after 24, 48, and 72 hr of exposure to quercetin. In Fig. 3, DNA histograms show that quercetin (20, 40, and 60 μM) increased the number of cells in G_2/M from 7.6% to 12.4, 19.1, and 23.5%, respectively, and decreased the population of G_0/G_1 cells from 46.2% to 40.2, 32.1, and 34.5%, respectively, while it had no significant effect on the percentage of cells in S phase after 24 hr of treatment. The results after 48 hr of treatment were similar to the findings after 24 hr of exposure. Figure 3 also shows that quercetin (20, 40, and 60 μM) not only increased the number of cells in G_2/M from 3.6% to 10.7, 12.4, and 13.3%, respectively, and but also comparatively decreased the population of G_0/G_1 cells from 38.1% to 31.3, 30.9, and 27.8%, respectively. Subploidy cells, whose percentage was 9.1, 5.8, and 8.3%, respectively, also existed after 72 hr of treatment. The effects of quercetin on the cell cycle of HL-60 were reversible after 24 hr of treatment and after replacement of the agent for another 24 hr with fresh medium (data not shown).

Effect of Quercetin on the Activities of Cytosolic and Membrane TPK from HL-60 Cells In Vitro

Quercetin inhibited the activity of membrane TPK from HL-60 cells with an IC_{50} value of 20.1 μM *in vitro*. At 120 μM, the inhibitory rate of quercetin on TPK activity was 95% (Table 1). In contrast, quercetin had no effect on the

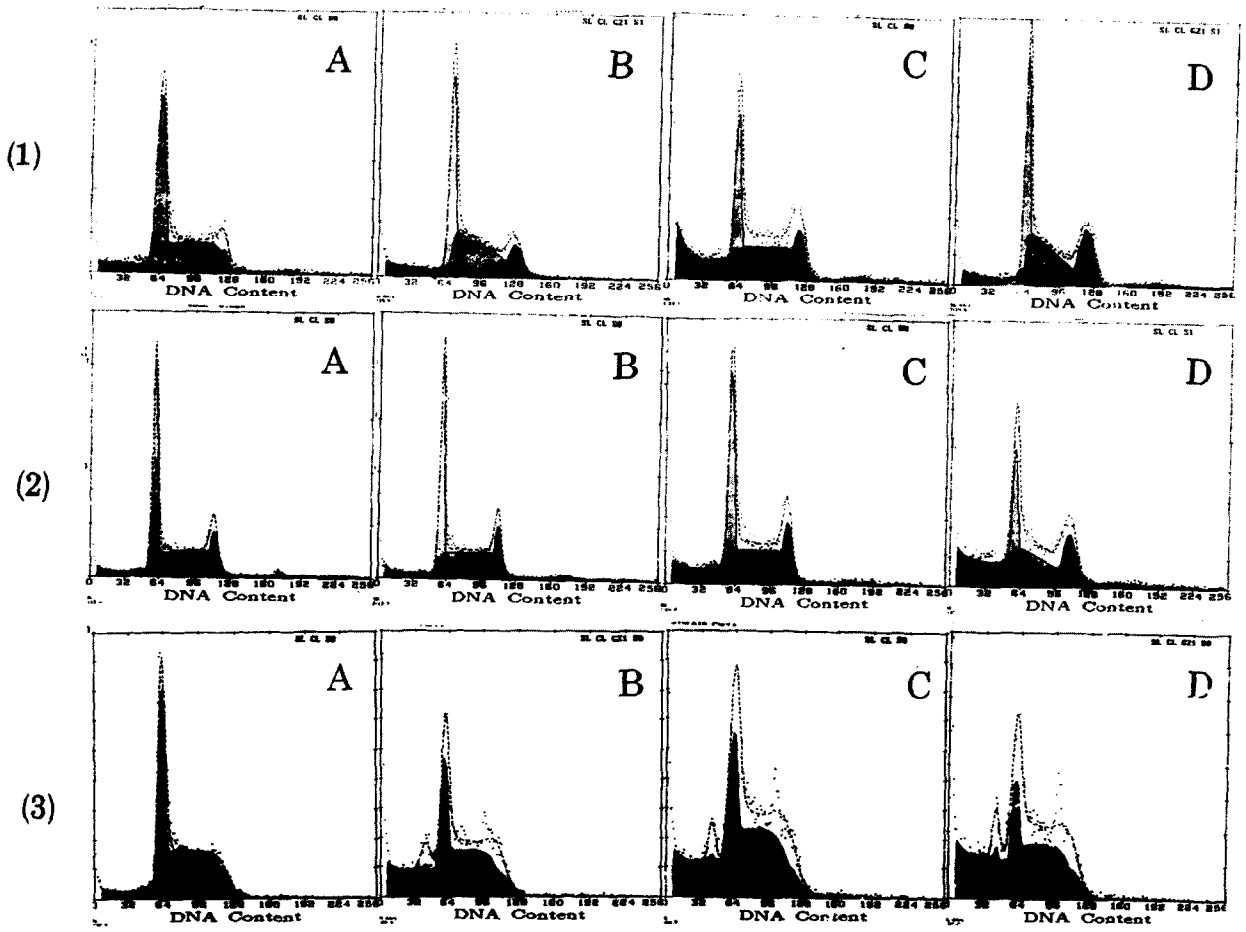

FIG. 3. Effects of quercetin on DNA content of HL-60 cells after (1) 24 hr, (2) 48 hr, and (3) 72 hr of treatment as determined by flow cytometry following staining with propidium iodide. (A) control; (B) 20 μM; (C) 40 μM; and (D) 60 μM.

activity of cytosolic TPK from HL-60 cells (data not shown).

Effects of Quercetin on the Activities of Cytosolic and Membrane PKC from HL-60 Cells In Vitro

Quercetin inhibited the activities of cytosolic PKC from HL-60 cells with an IC_{50} of 30.9 μM *in vitro*. When quercetin was at 120 μM, the activity of cytosolic PKC was 11.1% of control (Table 2). However, quercetin did not affect the activity of membrane PKC from HL-60 cells (data not shown).

Effects of Quercetin on the Production of Phosphoinositides in Intact HL-60 Cells

Quercetin markedly inhibited in a concentration-dependent manner the production of phosphoinositides in intact

TABLE 1. Effects of quercetin on the activity of membrane TPK from HL-60 cells *in vitro*

Quercetin (μM)	cpm	Inhibitory rate (%)
Control	353 ± 8.7	0
7.5	330 ± 45.9	7 (3.5)
15.0	216 ± 2.9*	39 (4.7)
30.0	131 ± 4.0†	64 (5.4)
60.0	63 ± 19.8†	82 (5.9)
120.0	17 ± 1.8†	95 (6.6)

The cpm values are means ± SD, N = 4. Numbers in parentheses represent the values of the corresponding inhibitory rate in probit scale.
*,† Significantly different from control: * $P < 0.05$, and † $P < 0.01$.

TABLE 2. Effects of quercetin on the activity of cytosolic PKC from HL-60 cells *in vitro*

Quercetin (μM)	cpm	Inhibitory rate (%)
Control	981 ± 73.7	0
7.5	876 ± 64.8	12 (3.8)
15.0	683 ± 22.8*	30 (4.5)
30.0	502 ± 28.4†	49 (5.0)
60.0	350 ± 26.8†	64 (5.4)
120.0	109 ± 34.9†	89 (6.2)

The cpm values are means ± SD, N = 3. Numbers in parentheses represent the values of the corresponding inhibitory rate in probit scale.
*,† Significantly different from control: * $P < 0.05$, and † $P < 0.01$.

TABLE 3. Effects of quercetin on the production of phosphoinositides in intact HL-60 cells

Quercetin (μM)	PI	PIP	PIP_2	PIP_3(?)
Control	12.8 ± 1.3	8.1 ± 0.9	7.1 ± 0.8	4.3 ± 0.4
20	12.5 ± 1.0	8.3 ± 0.9	7.2 ± 0.7	3.9 ± 0.3
40	7.3 ± 0.8*	6.5 ± 0.7†	4.7 ± 0.6*	3.2 ± 0.4†
60	6.0 ± 0.3*	3.9 ± 0.5*	4.6 ± 0.5*	3.5 ± 0.4
80	3.2 ± 0.3*	1.8 ± 0.2*	2.2 ± 0.3*	3.8 ± 0.2

Values are means ± SD, N = 3. The quantitation of phosphoinositide production was accomplished by densitometric scanning of the autoradiogram. Numbers shown here are densitometric scales (no units).

*,† Significantly different from control: * $P < 0.01$, and † $P < 0.05$.

HL-60 cells. When quercetin was used at 80 μM, the production of PI, PIP, and PIP_2 was inhibited almost completely (Table 3 and Fig. 4).

DISCUSSION

Various pharmacological activities of quercetin have been described [1–5]. In the present study, we have demonstrated that quercetin strongly inhibits the growth of HL-60 cells. This led to an increase in cells in the G_2/M phase of the cell cycle and a decrease in the population of G_0/G_1 cells, without altering the percentage of cells present in the S phase. We found that quercetin markedly inhibited the activities of membrane TPK and cytosolic PKC from HL-60 cells, but had no effect on cytosolic TPK and membrane PKC from HL-60 cells *in vitro*, as well as markedly inhibiting the production of phosphoinositides in intact HL-60 cells.

It has been reported that quercetin inhibits the growth of a variety of cancer cell lines [4–15] and of acute lymphoid and myeloid leukemia cells [9]. Our finding that quercetin strongly inhibited the growth of HL-60 cells confirms and extends these previous reports. The fact that quercetin

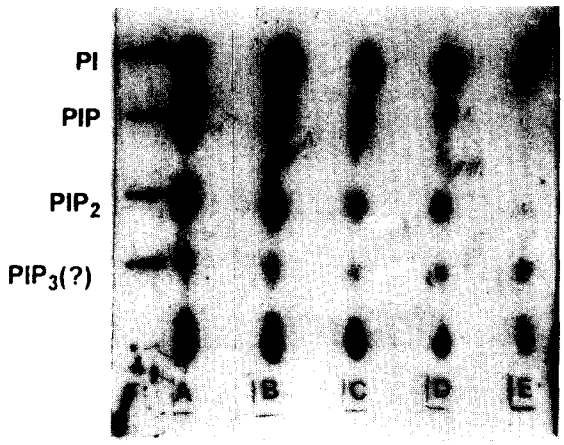

FIG. 4. Concentration-dependent inhibitory effects of quercetin on the production of phosphoinositides in intact HL-60 cells. PI, PIP, PIP_2, and PIP_3(?) were separated by TLC and autoradiography as described in "Materials and Methods." PI, PIP, and PIP_2 were located by iodine staining with standards of the lipids as markers. The experiment was repeated three times. (A) control; (B) 20 μM; (C) 40 μM; (D) 60 μM; and (E) 80 μM.

increased the number of cells in the G_2/M phase of the HL-60 cell cycle was consistent with the report that quercetin causes an analogous accumulation in the human MDA-MB468 breast cancer cell line [15]. Furthermore, the findings with HL-60 were similar to the demonstration that genistein arrested HGC-27 cells in G_2/M [16], which suggested that the effects of genistein may be related to its inhibition of TPK. Our results were also similar to those of a report showing that the flavone L86-8275 delayed the progression of aphidicolin-synchronized MDA-468 breast carcinoma cells through S phase and prevented progression through G_2 [17]. However, the results in HL-60 cells were not consistent with several reports which showed that quercetin inhibited the cell cycle progression of gastric cancer cells at G_1 [12–14]. These findings suggest that quercetin may have different effects on the cell cycle of different cells, and that the effects of quercetin on the HL-60 cell cycle may be related to its strong inhibition of HL-60 membrane TPK. When HL-60 cells were treated for 72 hr with quercetin, some subploidy cells were found. We believe that this may be the result of the apoptosis of HL-60 cells, and that the induction of apoptosis by quercetin may be one of the mechanisms of its antitumor activity; this concept is consistent with the report of Wei *et al.* [5].

The antiproliferative activity of quercetin could be mediated by type II EBS [6–10] and PKC [11]. Many studies have shown that quercetin inhibits PKC and TPK activities [11, 24–27]. TPK and PKC play important roles in cell growth, especially in the proliferation of tumor cells [18–21]. In the present study, we found that quercetin markedly inhibited the activities of membrane TPK and cytosolic PKC from HL-60 cells *in vitro*, suggesting that the antiproliferative activity of quercetin on HL-60 cells may be related to its inhibitory effects on membrane TPK and/or cytosolic PKC.

Recently, Weber and coworkers [28, 34–37] reported that PI kinase and PIP kinase activities are increased in some tumor cell lines, such as in OVCAR-5 cells and in MDA-MB-435 human breast carcinoma cells, and that quercetin inhibited the proliferation of OVCAR cells with an IC_{50} of 63 μM and of human breast carcinoma cells with an IC_{50} of 55 μM. Furthermore, they suggested that the inhibitory effects of quercetin on tumor cells might be due to a decline in the activities of PI kinase and PIP kinase. In the present study, we found that quercetin in the range of 20 to 80 μM produced a pronounced inhibition of the production of phosphoinositides by direct determination of the contents of these metabolites in intact HL-60 cells.

Since there is no PIP_3 standard, the location of PIP_3 was estimated. The finding was consistent with those of Weber and coworkers [28, 34–37]. PIP and PIP_2 are produced from the phosphorylation of PI and PIP, catalyzed by PI kinase and PIP kinase, respectively; thus, the effects of quercetin may be due to inhibition of PI kinase and/or PIP kinase activities.

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