Cholesterol starvation decreases P34^{cdc2} kinase activity and arrests the cell cycle at G2

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ABSTRACT **As a major component of mammalian cell plasma membranes, cholesterol is essential for cell growth. Accordingly, the restriction of cholesterol provision has been shown to result in cell proliferation inhibition. We explored the potential regulatory role of cholesterol on cell cycle progression. MOLT-4 and HL-60 cell lines were cultured in a cholesterol-deficient medium and simultaneously exposed to SKF 104976, which is a specific inhibitor of lanosterol 14-α demethylase. Through HPLC analyses with on-line radioactivity detection, we found that SKF 104976 efficiently blocked the [^{14}C]-acetate incorporation into cholesterol, resulting in an accumulation of lanosterol and dihydrolanosterol, without affecting the synthesis of mevalonic acid. The inhibitor also produced a rapid and intense inhibition of cell proliferation (IC_{50} = 0.1 μM), as assessed by both [^{3}H]-thymidine incorporation into DNA and cell counting. Flow cytometry and morphological examination showed that treatment with SKF 104976 for 48 h or longer resulted in the accumulation of cells specifically at G2 phase, whereas both the G1 traversal and the transition through S were unaffected. The G2 arrest was accompanied by an increase in the hyperphosphorylated form of p34^{cdc2} and a reduction of its activity, as determined by assaying the H1 histone phosphorylating activity of p34^{cdc2} immunoprecipitates. The persistent deficiency of cholesterol induced apoptosis. However, supplementing the medium with cholesterol, either in the form of LDL or free cholesterol dissolved in ethanol, completely abolished these effects, whereas mevalonate was ineffective. Caffeine, which abrogates the G2 checkpoint by preventing p34^{cdc2} phosphorylation, reduced the accumulation in G2 when added to cultures containing cells on transit to G2, but was ineffective in cells arrested at G2 by sustained cholesterol starvation. Cells arrested in G2, however, were still viable and responded to cholesterol provision by activating p34^{cdc2} and resuming the cell cycle. We conclude that in both lymphoblastoid and promyelocytic cells, cholesterol availability governs the G2 traversal, probably by affecting p34^{cdc2} activity.—Martínez-Botas, J., Suárez, Y., Ferruelo, A. J., Gómez-Coronado, D., Lasunción, M. A.**

Cholesterol starvation decreases P34^{cdc2} kinase activity and arrests the cell cycle at G2. *FASEB J.* 13, 1359–1370 (1999)

Key Words: SKF 104976 · cholesterol synthesis · cell proliferation · low density lipoprotein · caffeine

As an essential component of mammalian cell membranes, proliferating cells require cholesterol, which is either obtained from plasma lipoproteins, mainly through the low density lipoprotein (LDL)[2] receptor pathway, or synthesized intracellularly from acetyl-coenzyme A. The main rate-limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) (1) and several other enzymes participating in cholesterol synthesis—HMG-CoA synthase (2), farnesyl diphosphate synthase (3–4), squalene synthase (5)—as well as the LDL receptor are down-regulated at the transcriptional level by the intracellular cholesterol levels (6, 7) through a mechanism that involves the transcription factors SREBP (SRE binding proteins) and the participation of SCAP, a protein that senses the changes of cholesterol levels in the endoplasmic reticulum (8).

As originally pointed out by Chen et al. (9) and Brown and Goldstein (10), cholesterol synthesis is tightly related to cell proliferation. Phytohemagglutinin-stimulated mouse lymphocytes undergo a sixfold increase in cholesterol synthesis just before the cells enter the S phase of the cell cycle (11). In synchronized BHK-21 cells, it was also observed that cholesterol synthesis reached a maximum during the G1 phase, concurrent with the activation of HMG-CoA reductase (12). In general, thus, proliferating cells display both a

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[2] Abbreviations: cdk, cyclin-dependent kinases; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HPLC, high-performance liquid chromatography; LDL, low density lipoproteins; LPDS, lipoprotein-deficient serum; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SRE, sterol regulatory elements; SREBP, SRE binding proteins.

high rate of cholesterol synthesis and an elevated LDL receptor activity (13), which warrant the provision of this lipid. On the other hand, inhibition of HMG-CoA reductase with vastatins, which depletes the cells of both cholesterol and other mevalonate derivatives, results in a dramatic reduction of DNA synthesis, inducing the arrest of the cell cycle at G1 (14–16). This block of the cell cycle is specific because supplementing the medium with mevalonic acid can readily reverse it (14, 17). Recent studies indicate that in addition to cholesterol, several other mevalonate derivatives are necessary for DNA synthesis, such as the phosphorylated forms of isopentane (18), farnesol (19, 20), geranylgeraniol (21, 22), and dolichol (23, 24).

Aside from vastatins, other cholesterol synthesis inhibitors also block cell proliferation. Thus, ketoconazole, which is an inhibitor of P_{450} enzymes and lanosterol conversion into cholesterol, has been shown to produce an arrest of a minor portion of cells in G2 (25). Fluoromevalonate [an inhibitor of pyrophosphatemevalonate decarboxylase (26, 27)], azole derivatives, and oxysterols (14, 28) also reduce DNA synthesis but their action on the cell cycle has not been elucidated.

Eukaryotic cell cycle is governed by cyclin-dependent kinases (cdk) coupled to specific cyclins. The protein–kinase complex $p34^{cdc2}$-cyclin B controls the traversal of both the G2 and the M phases (29, 30). Expression levels of cyclin B change along the cell cycle, increasing during S and G2 phases and abruptly decreasing at the metaphase/anaphase transition (31). Moreover, the activity of $p34^{cdc2}$ is regulated by phosphorylation/dephosphorylation reactions mediated by different kinases (wee1, mik1, myt1) and phosphatase cdc25, respectively (32). Finally, entry into mitosis is caused by degradation of cyclin B and $p34^{cdc2}$ inactivation (33).

It has been reported recently that lovastatin increases the levels of cyclin-dependent kinase inhibitors p21 and p27, which results in a decrease of cdk2 kinase activity (34–36). This finding agrees with the G1 block this drug produces (15, 16). However, the potential role of cholesterol in the cell cycle could not be established from these studies since vastatins compromise the provision of both cholesterol and nonsterol mevalonate derivatives.

Here we document the effect of cholesterol starvation and readdition on the cell cycle progression and the activity of $p34^{cdc2}$ in both MOLT-4 and HL-60 cells. The results indicate that cholesterol is essential for the transition from G2 to mitosis and that cholesterol availability affects $p34^{cdc2}$ activity.

MATERIALS AND METHODS

Materials

MOLT-4 (ATCC CRL 1582) and HL-60 (ATCC CCL 240) cell lines were obtained from the American Type Culture Collection (Rockville, Md.). RPMI 1640 and antibiotics were purchased from Gibco BRL (Barcelona, Spain) and fetal calf serum from PAA Laboratories (Linz, Austria). Multiscreen-HV culture plates were acquired from Millipore (Milford, Mass.). SKF 104976 was kindly provided by Dr. T. A. Berkhaut (SmithKline Beecham, Betchworth, Surrey, U.K.). [^{3}H-methyl]-thymidine (5 Ci/mmol) was from Amersham (Little Chalfont, U.K.). Mevalonate, farnesol, and fluorodeoxyuridine were provided by Sigma Chemical Co. (St. Louis, Mo.), and geranylgeraniol by American Radiolabeled Chemicals, Inc. (St. Louis); all other chemicals were of analytical grade. LDL was isolated from normolipidemic human sera by a single vertical spin ultracentrifugation (VTi 50 rotor, Beckman) (37).

Cell culture

MOLT-4 and HL-60 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 100 U penicillin/ml, 100 μg streptomycin/ml, and 10 μg gentamicin/ml at 37°C in a humidified atmosphere of 5% CO_2. Before each experiment, the cells were washed three times with RPMI 1640 to remove fetal calf serum and grown in RPMI 1640 supplemented with 10% lipoprotein-deficient serum (LPDS) (final cholesterol concentration in medium <0.2 μg/ml). The cell viability was greater than 90–95% in all experiments, as determined by the trypan blue exclusion test.

Metabolic labeling and high-performance liquid chromatography (HPLC) analysis of isoprene lipids

MOLT-4 (15×10^6 cells) were preincubated in 2 ml RPMI 1640, 10% LPDS with or without SKF 104976 for 2 h and supplemented with 40 μCi of [2-^{14}C]-acetate (53 mCi/mmol) (Amersham) for a further 8 h. The cells were agitated on a rocking platform at 37°C in a 5% CO_2 atmosphere. At the end of the incubation, the cells were washed with ice-cold phosphate-buffered saline (PBS) and resuspended in 0.5 ml of 10% KOH. The samples were sequentially treated with chloroform:methanol (2:1) and distilled water to obtain the lipidic and aqueous-soluble fractions (38). The lipid extract was further subfractionated into the saponifiable and the nonsaponifiable fractions, as reported (39). The nonsaponifiable lipids were analyzed by reverse phase HPLC with a Nova Pack-C_{18} column (150 × 3.9 mm, 4 μM, Waters Chromatography Division, Millipore Corp.). Lipids were eluted with methanol-acetonitrile-water (65:25:10) during the first 9 min and then with methanol-acetonitrile (10:90) at a flow rate of 1.2 ml/min. The column effluent was monitored simultaneously by UV (Beckman 168 variable wavelength detector; Beckman Instruments, Palo Alto, Calif.) and on-line radioactivity detection (LB-506 C-1, Berthold, Bad Widbad, Germany). The aqueous-soluble fractions were analyzed by HPLC by using an ion exclusion column Aminex HPX-87H (300 mm × 7.8 mm, Bio-Rad Lab, Richmond, Calif.) with a cation-H refill cartridge (30 mm × 4.6 mm). The organic acids were eluted with 5 mM sulfuric acid at a flow rate of 0.6 ml/min at 42°C and radioactivity was monitored on-line, as before.

Measurement of cell proliferation

Incubations were carried out in sterile 96-well plates (Multiscreen-HV), each well containing 36×10^3 cells in a final volume of 225 μl. The culture medium (RPMI 1640 with 10% LPDS) was supplemented with either the cholesterol synthesis inhibitor SKF 104976 dissolved in dimethyl sulfoxide (final concentration in the wells, 0.05%), LDL, mevalonate, or free

cholesterol dissolved in ethanol (final concentration, 0.05%), as indicated. DNA synthesis was assessed by measuring the incorporation of [^{3}H-methyl]-thymidine into DNA as reported elsewhere (40). Cell number and viability were determined by trypan blue dye exclusion using a hemocytometer.

Cell cycle analysis

Cells sampled for flow cytometry were harvested by centrifugation and washed three times with PBS. Cells were then fixed in 70% cold ethanol for 30 min at −20°C, washed three times with PBS, and incubated for 1 h at 37°C in PBS containing 100 μg/ml ribonuclease A and 50 μg/ml propidium iodide. Cells were analyzed with a FACScan flow cytometer (Becton-Dickinson, San Jose, Calif.) using a 488 nm argon ion laser. Data were acquired using selective gating excluding doublet cells and analyzed with Cell FIT software (Becton-Dickinson). The fraction of cells in each cell cycle compartment was calculated according to the SOBR analysis model.

To ascertain whether cells in S phase were actively synthesizing DNA, 2–4 × 10^6 cells were incubated with 100 μM bromodeoxyuridine (BrdU) for 1 h in the CO_2 incubator at 37°C, fixed in 70% cold ethanol at −20°C, and washed three times with PBS. After a 20 min incubation at room temperature with 2 N HCl, cells were washed twice with PBS and incubated for 15 min at room temperature in PBS, 0.5% Tween 20, 1% normal goat serum (Vector Laboratories, Inc., Burlingame, Calif.). Subsequently, cells were centrifuged, resuspended in 0.5% Tween 20, 1% normal goat serum containing 20 μl of Anti-BrdU FITC (Becton Dickinson) for 1 h at room temperature, stained with propidium iodide, and analyzed by flow cytometry.

Analysis of apoptosis

For morphological analysis, cells were stained with Hoechst 33258 and examined with a fluorescence microscope (Olympus IX70, Hamburg, Germany) as reported (40). Apoptosis was also assessed by a TUNEL assay with the Apoptosis Detection System kit (Promega Corporation, Madison, Wis.), following the manufacturer's instructions, and analyzed by flow cytometry.

Gel electrophoresis and immunoblots

Cells extracts were obtained by using lysis buffer containing 50 mM Tris-HCl pH 8, 120 mM NaCl, 0.5% Nonidet P40, 100 mM NaF, 40 μg/ml PMSF, 40 μg/ml aprotinin, and 40 μg/ml leupeptin. Equal amounts of protein (41) were run in each lane of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (12% acrylamide) and transferred to nitrocellulose paper. The $p34^{cdc2}$ protein was detected by using an anti-cdc2 polyclonal antibody (Ab-1, Calbiochem, Cambridge, Mass.) and then probed with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G. Immunoreactive bands were detected after addition of ECL reagent (ECL kit; Amersham), followed by exposure to photographic film.

Assessment of $p34^{cdc2}$ kinase activity

$p34^{cdc2}$ kinase activity was assayed as described by Poon et al. (34). Briefly, equal protein amounts of cell extracts were resuspended in immunoprecipitation buffer (50 mM Tris-HCl pH 8, 120 mM NaCl, 30 mM NaF, 30 mM pNPP, 1% Nonidet P40, 1% Triton X-100, 1 mM benzamide, 0.1 mM PMSF) containing 1 μg of anti-cdc2 polyclonal antibody Ab-1. The samples were precipitated with 25 μl of protein A-Sepharose. The immunoprecipitates were resuspended in 25 μl of kinase buffer (80 mM Na-β-glycerophosphate, 20 mM EGTA, 15 mM $Mg(OAc)_2$, 1 mM DTT, 32 μM ATP, 6.4 μCi[γ-^{32}P] ATP, and 5 μg histone H1) and incubated for 30 min at 30°C. The reaction was terminated by the addition of 12.5 μl of SDS sample buffer. The samples were subjected to 12% SDS-PAGE gel electrophoresis, transference to nitrocellulose membrane, and autoradiography. Phosphorylation was quantified by scanning the film and image analysis (Diversity One Software Package, PDI, New York).

RESULTS

Effect of SKF 104976 on cholesterol synthesis

To determine the extent of the cholesterol synthesis inhibition produced by SKF 104976, MOLT-4 cells were incubated with the drug and [2-^{14}C]-acetate to measure the incorporation of ^{14}C-radioactivity into lipids and organic acids by HPLC. As shown in **Fig. 1**, in the control condition most of the radioactivity appearing in nonsaponifiable lipids corresponded to cholesterol. By contrast, in cells treated with SKF 104976, the predominant ^{14}C-labeled sterols were lanosterol and dihydrolanosterol, with practically undetectable radioactivity in cholesterol. Since it was reported that secondary to the inhibition of lanosterol 14-α demethylase, SKF 104976 also reduced HMG-CoA reductase (42), we measured the incorporation of [^{14}C]-acetate into organic acids (**Fig. 2**) and found that [^{14}C]-mevalonate levels in cells treated with SKF 104976 were similar to those in the control (49,460±4938 and 52,352±3045 dpm/well, respectively; n=5, not significant).

Effect of SKF 104976 on MOLT-4 and HL-60 cell proliferation

To study cell proliferation, the incorporation of [^{3}H]-thymidine into DNA was first determined. SKF 104976 inhibited the proliferation of MOLT-4 and HL-60 cell lines in a dose-dependent manner when incubated in a cholesterol-free medium with an IC_{50} of ~0.1 μM (**Fig. 3**). Time course studies revealed that DNA synthesis was significantly reduced at 24 h of treatment and practically undetectable at 48 h or longer (**Fig. 4**).

To determine whether this effect on cell proliferation was specific to the inhibition of lanosterol 14-α demethylase simultaneous with the addition of the inhibitor, the cultures were supplemented with LDL, free cholesterol, and mevalonate in different combinations. As shown in **Fig. 5** for HL-60 cells, both LDL and free cholesterol restored cell proliferation in a dose-dependent manner, LDL being more efficient than free cholesterol. Similar results were observed with MOLT-4 cells (data not shown). In contrast,

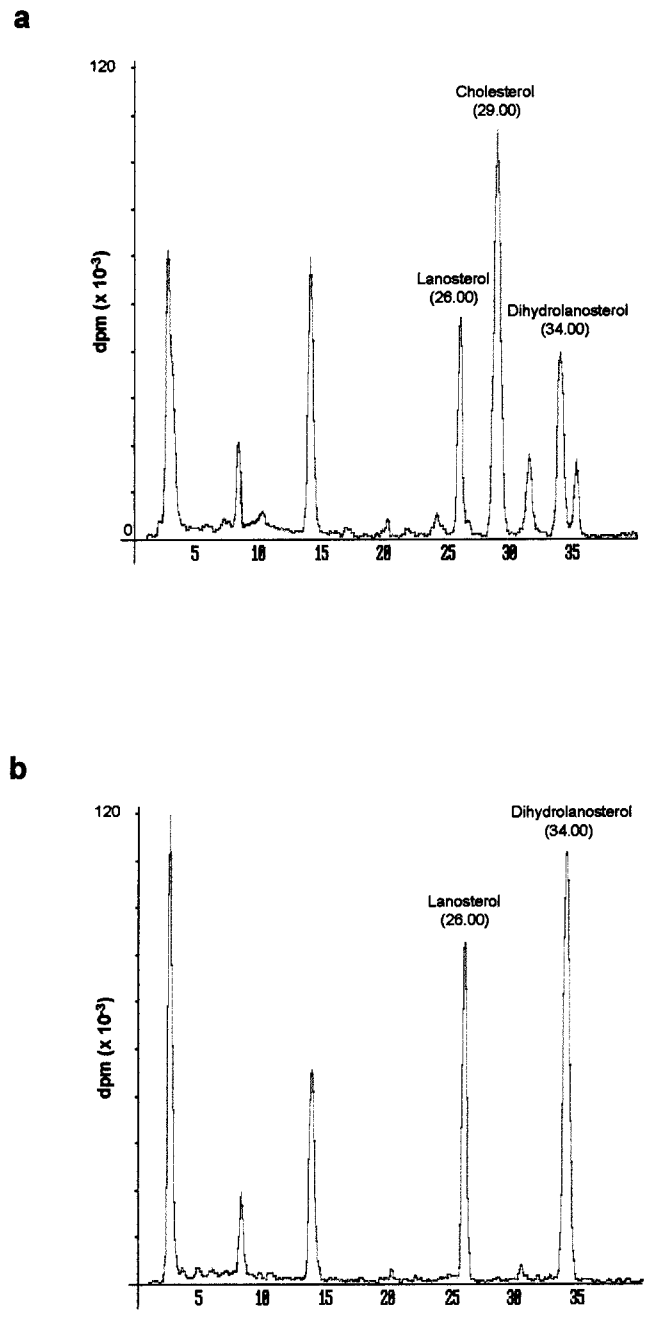

Figure 1. Effect of SKF 104976 on [^{14}C]-acetate incorporation into nonsaponifiable lipids. MOLT-4 cells were preincubated in RPMI 1640, 10% LPDS in the absence (*a*) or presence (*b*) of 1.5 μM SKF 104976 for 2 h, and supplemented with 40 μCi of [2-^{14}C]-acetate for a further 8 h. The cell extracts containing the nonsaponifiable lipid fraction were analyzed by reverse phase HPLC and on-line radioactivity counting.

neither mevalonate, all-*trans* farnesol, nor all-*trans* geranylgeraniol prevented the inhibitory effect of SKF 104976 or affected the reversal due to LDL or free cholesterol (data not shown). Viable cells were counted at different incubation times (**Fig. 6**). At 24 h, cell counts in cultures containing SKF 104976 did not differ from the controls. Whereas the controls displayed an ascending pattern, in the presence of the drug the number of viable cells declined to practically zero at 72 h (Fig. 6). LDL or free cholesterol supplementation abolished the effect of SKF 104976 on cell growth, whereas mevalonate was ineffective. On the other hand, mevalonate did not produce any benefit over cholesterol supplementation (Fig. 6). Altogether, these results demonstrate

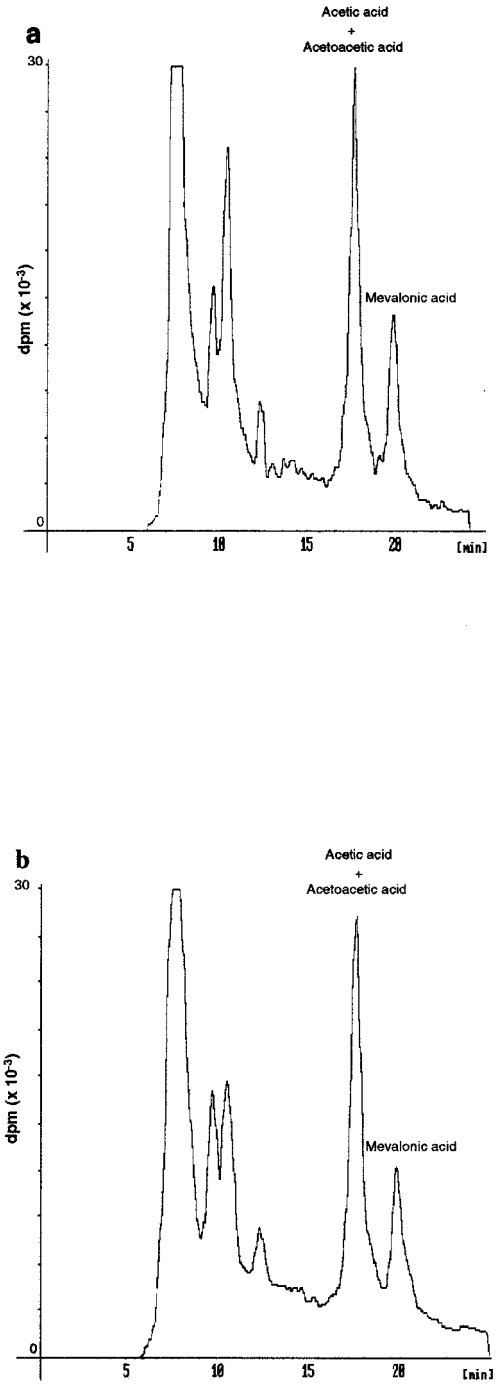

Figure 2. Effect of SKF 104976 on [^{14}C]-acetate incorporation into organic acids. MOLT-4 cells were preincubated in RPMI 1640, 10% LPDS in the absence (*a*) or presence (*b*) of 1.5 μM SKF 104976 for 2 h, then supplemented with 40 μCi of [2-^{14}C]-acetate for a further 8 h. The cells were processed and the aqueous-soluble fractions were analyzed by ion exclusion HPLC and on-line radioactivity counting.

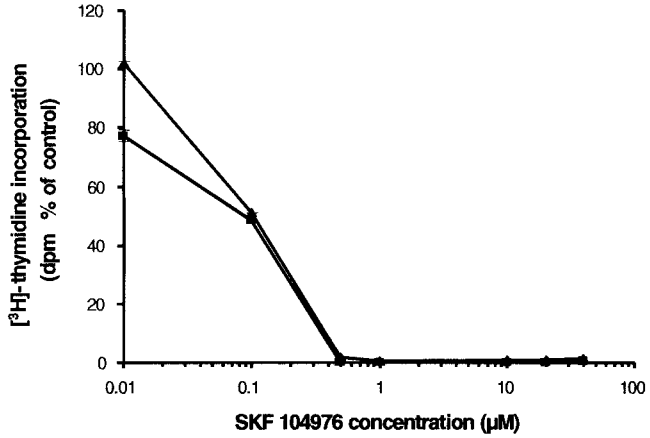

Figure 3. Dose effect of SKF 104976 on DNA synthesis. MOLT-4 (▲) and HL-60 (■) cells were incubated in RPMI 1640, 10% LPDS in the presence of increasing concentrations of SKF 104976 for 72 h and then received an 18 h pulse of [^{3}H]-thymidine. Cells were harvested and radioactivity incorporated into DNA was counted. Results are expressed as % of the control (cells incubated in the absence of the inhibitor): MOLT-4, 170378 ± 6034 dpm/well; HL-60, 152918 ± 885 dpm/well. Data correspond to the means ± SE of four replicates from a representative experiment.

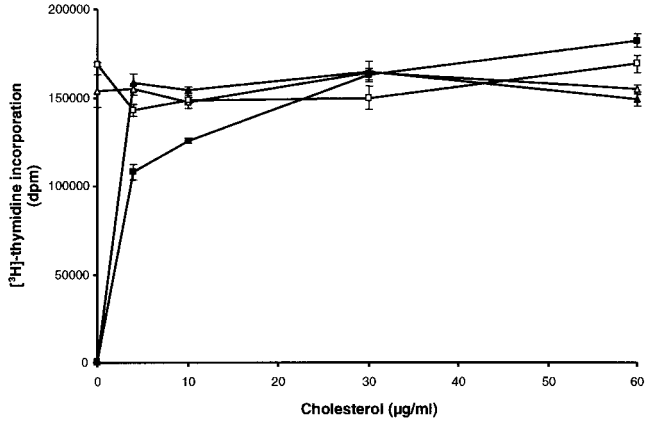

Figure 5. Reversal of SKF 104976-induced DNA synthesis inhibition by LDL and free cholesterol supplementation. HL-60 cells were incubated in RPMI 1640, 10% LPDS for 72 h in the absence (Δ, □) or presence of 1.5 μM SKF 104976 (▲, ■) and increasing concentrations of LDL (Δ, ▲) or free cholesterol (□, ■). Then the cells received a 18 h pulse with [^{3}H]-thymidine and radioactivity incorporated into DNA was determined. Data correspond to the means ± SE of four replicates from a representative experiment.

that the inhibition of cell growth by SKF 104976 was attributable to the deficiency in cholesterol but not in mevalonic acid and/or its nonsterol derivatives.

Effects of SKF 104976 on cell cycle and rescue by LDL or free cholesterol

The effect of SKF 104976 on cell cycle distribution and its prevention by LDL, free cholesterol, and mevalonate was then studied with flow cytometry after propidium iodide DNA staining. As observed in **Fig. 7**, SKF 104976 treatment resulted in a progressive increase in the proportion of cells in G2/M phase and a decrease in G1. The simultaneous addition of either LDL or free cholesterol prevented the accumulation of cells in G2 and allowed the cell cycle to progress normally despite the continuous presence of the inhibitor (Fig. 7). Mevalonate supplementation did not alter the effect of SKF 104976 on cell cycle nor its reversion by LDL or free cholesterol (Fig. 7). Similar results were found with MOLT-4 cells (data not shown). In the absence of any exogenous source of cholesterol, prolonging

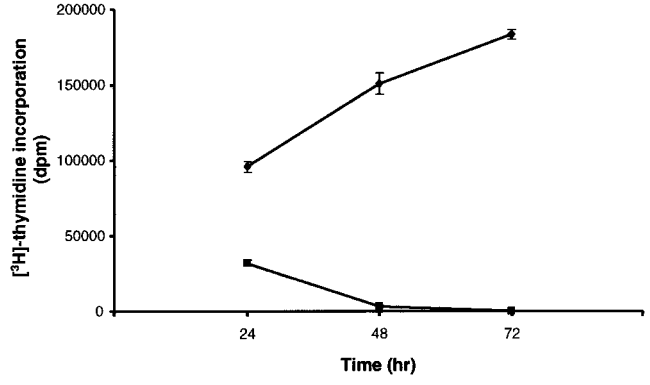

Figure 4. Time course effect of SKF 104976 on DNA synthesis. HL-60 cells were incubated in RPMI 1640, 10% LPDS in the absence (control) (♦) or presence of 1.5 μM SKF 104976 (■). At the indicated times, the cells received a 18 h pulse of [^{3}H]-thymidine and radioactivity incorporated into DNA was determined. Data correspond to the means ± SE of four replicates from a representative experiment.

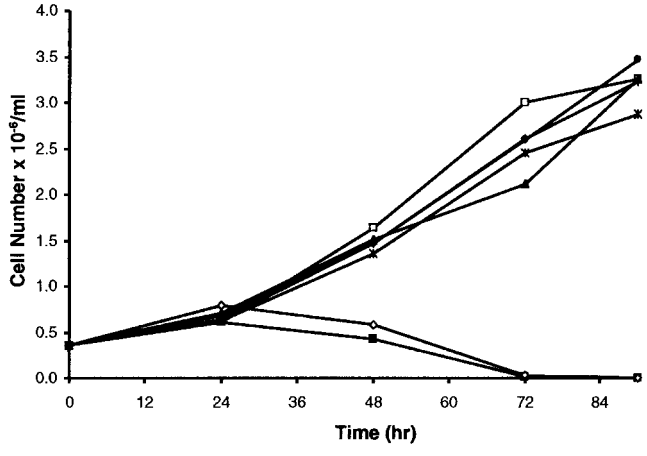

Figure 6. Effect of LDL, free cholesterol and mevalonate on the on the growth of SKF 104976-treated cells. HL-60 cells were incubated in RPMI 1640, 10% LPDS in the absence (□) or presence of 1.5 μM SKF 104976 alone (■) or in combination with 30 μg/ml LDL (+), 30 μg/ml free cholesterol (T), 1 mM mevalonate (◇), 30 μg/ml LDL plus 1 mM mevalonate (●), or 30 μg/ml free cholesterol plus 1 mM mevalonate (▲). At the indicated times, the viable cells were counted. Data correspond to the mean of duplicates from a representative experiment out of several with similar results.

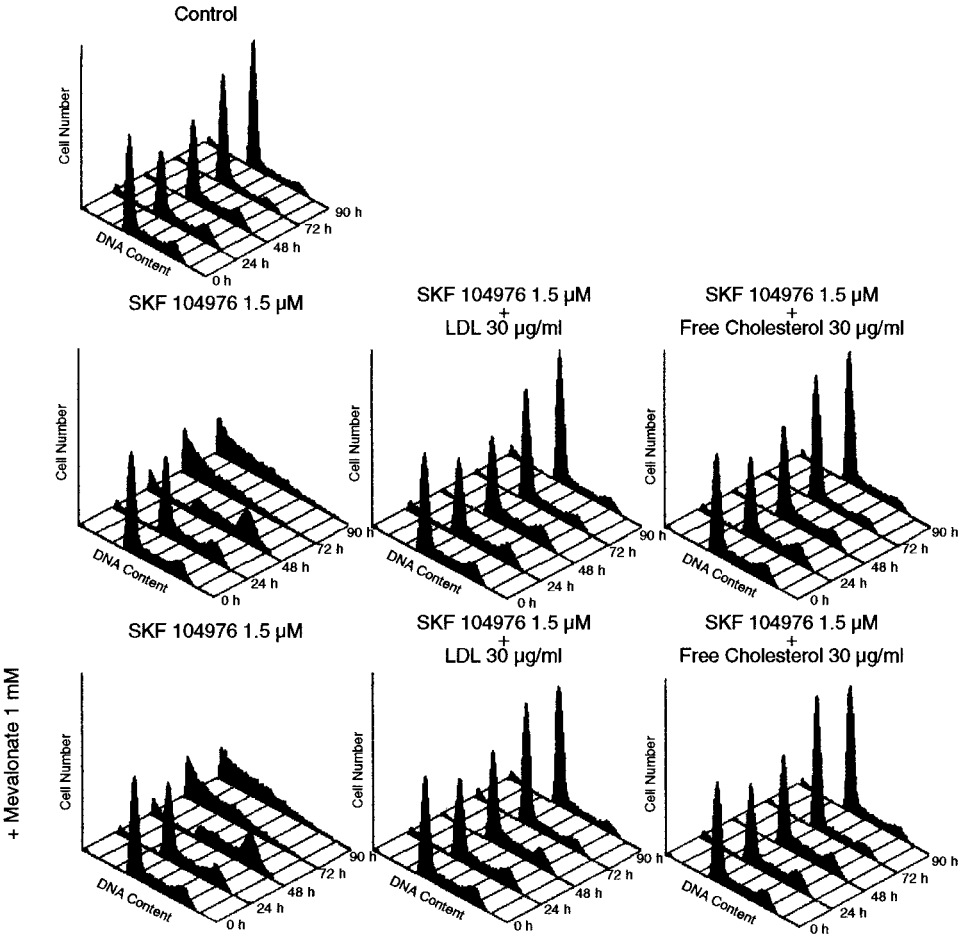

Figure 7. Inhibition of the cell cycle progression by SKF 104976 and effect of LDL, free cholesterol, and mevalonate. HL-60 cells were incubated in RPMI 1640, 10% LPDS in the absence (control) or presence of 1.5 µM SKF 104976 alone or in combination with 30 µg/ml LDL or 30 µg/ml free cholesterol and in the absence or the presence of 1 mM mevalonate. At the indicated times, the cells were harvested, stained with propidium iodide, and analyzed by flow cytometry. Data were acquired using selective gating that excluded doublet cells and were analyzed with Cell FIT software.

incubation with the inhibitor for 72 h or longer resulted in generalized cell death, as indicated by the prominent Sub G1 (Fig. 7). To confirm that cell death corresponded to apoptosis, a TUNEL assay coupled to flow cytometry was performed. As observed in **Fig. 8**, cells treated with the drug for 72 h had much more fluorescence than controls, which demonstrates apoptosis-induced DNA fragmentation, whereas supplementing the medium with LDL totally prevented this effect. In keeping with this, many condensed and fragmented nuclei were visualized by fluorescence microscopy in cultures exposed to SKF 104976 after staining with Hoechst 33258 (data not shown). On the other hand, this technique also revealed that the intact, not fragmented nuclei remaining after 48 h of treatment with SKF 104976 were larger than the average in the control condition and showed the complete absence of mitotic figures (separating chromosomes) (data not shown). The latter results, in combination with those derived from flow cytometry, indicated that SKF 104976 arrested the cell cycle at G2 rather than at M.

To determine whether the G2 arrest could be released by cholesterol, HL-60 cells were preincubated with SKF 104976 for 48 h and then the medium was supplemented with placebo, LDL, or free cholesterol. As shown in **Fig. 9**, LDL produced a rapid increase of the number of cells in G1 and complete restoration of the cell cycle distribution 24 h after its addition. Free cholesterol also allowed the traversal of G2/M phase but less efficiently than LDL, since the cell cycle required almost 48 h to be

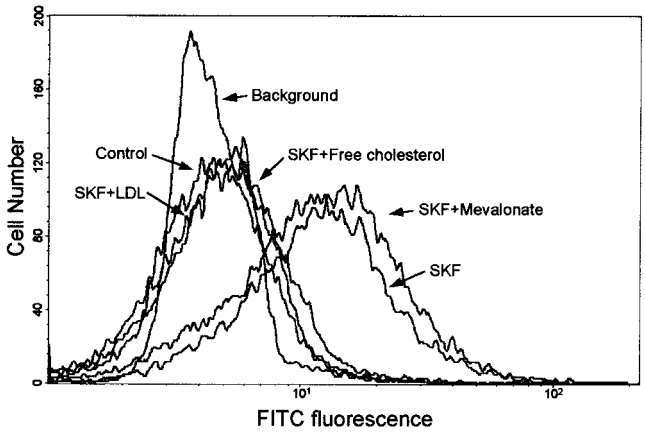

Figure 8. Flow cytometry analysis of DNA end labeling of apoptotic cells. HL-60 cells were incubated in RPMI 1640, 10% LPDS for 72 h in the absence (control) or presence of 1.5 µM SKF 104976 supplemented or not with LDL (60 µg cholesterol/ml) and/or mevalonate (1 mM). At the end of the incubation, DNA was labeled with FITC-dUTP by the action of terminal transferase and processed for flow cytometry.

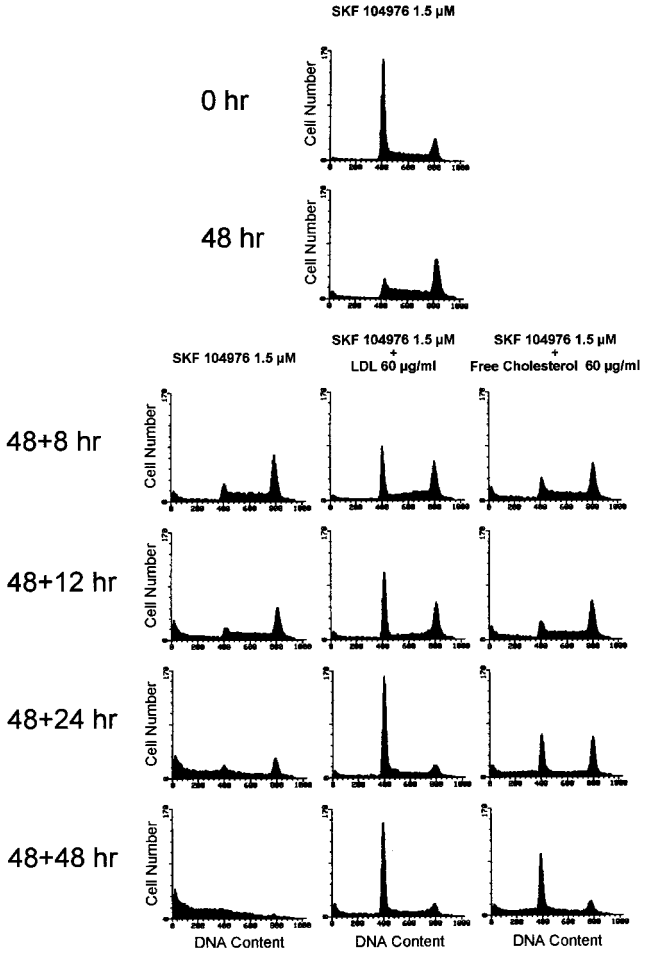

Figure 9. Release of SKF 104976-induced cell cycle arrest by LDL and free cholesterol supplementation. HL-60 cells were incubated in RPMI 1640, 10% LPDS in the presence of 1.5 µM SKF 104976 for 48 h and the medium was supplemented with 60 µg/ml LDL, 60 µg/ml free cholesterol, or not supplemented. At the indicated times, the cells were harvested, stained with propidium iodide, and analyzed by flow cytometry.

entirely reestablished (Fig. 9). In addition, the number of cells increased after supplementing the medium with cholesterol, which confirmed release of the cell cycle arrest (data not shown). In contrast, supplementing the medium with mevalonate either alone or in combination with LDL or free cholesterol exerted no effect on the release of the G2 block (data not shown). Altogether, these results demonstrate that cholesterol enables the cells to surpass the G2 block induced by SKF 104976, allowing the cell cycle to be resumed.

Changes in $p34^{cdc2}$ activity associated to the G2 arrest induced by SKF 104976

In eukaryotic cells, the G2 to M transition is known to be controlled by the protein kinase complex $p34^{cdc2}$-cyclin B. To determine whether the SKF 104976-induced G2 arrest was associated with changes in this kinase activity, we analyzed $p34^{cdc2}$ protein levels by Western blotting and H1 kinase activity in $p34^{cdc2}$ immunoprecipitates. As depicted in **Fig. 10**, $p34^{cdc2}$ protein levels increased during treatment in parallel to the increase in the proportion of cells in G2 phase (Fig. 8), which is in accordance with previous results by others using other G2-arrest inductors (43). Notably, the relative proportion of the slow-migrating band, which corresponds to the hyperphosphorylated, inactive form of the enzyme (44–45), increased with time. Consistent with this, $p34^{cdc2}$ kinase activity showed a biphasic pattern, with a transient increase at 36 h of treatment and a marked decrease thereafter (Fig. 10). At 60 h of treatment, despite the fact that 72% of live cells were in G2 phase in this experiment, the $p34^{cdc2}$ kinase activity was even lower than in the control (Fig. 10). Thus, apparently, the G2 arrest induced by SKF 104976 was associated with diminished $p34^{cdc2}$ kinase activity.

We next studied whether cholesterol provision could restore $p34^{cdc2}$ activity in previously arrested cells. We found that $p34^{cdc2}$ activity increased upon supplementing the medium with either cholesterol source (**Fig. 11**). This recovery was faster with LDL (Fig. 11*A*) than with free cholesterol (Fig. 11*B*), which is in keeping with their effects in releasing the G2 arrest as determined by flow cytometry (Fig. 10).

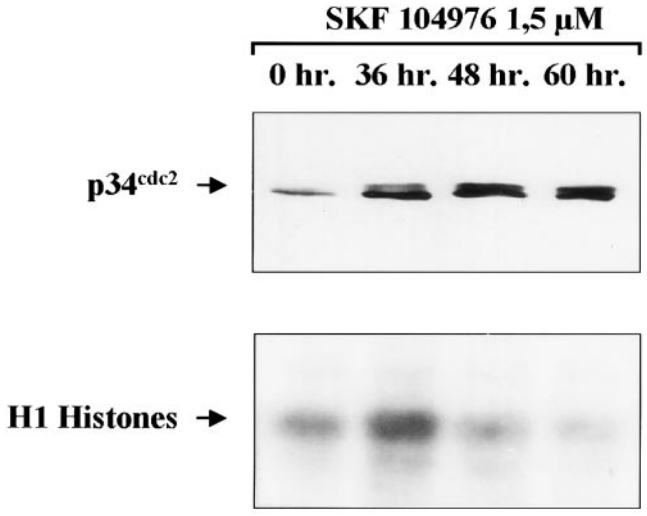

Figure 10. Expression of $p34^{cdc2}$ and its kinase activity in HL-60 cells treated with SKF 104976. HL-60 cells were incubated in RPMI 1640, 10% LPDS in the presence of 1.5 µM SKF 104976 and aliquots were extracted at the times indicated. Western blotting of $p34^{cdc2}$ was performed by 12% SDS-PAGE, transference to nitrocellulose membrane, and reaction with an anti-cdc2 polyclonal antibody. $p34^{cdc2}$ H1 kinase assay: cell extracts were incubated with an anti-cdc2 polyclonal antibody and the resultant immunoprecipitates were used for histone H1 phosphorylation assay. Phosphorylation was quantified by scanning the film with densitometer.

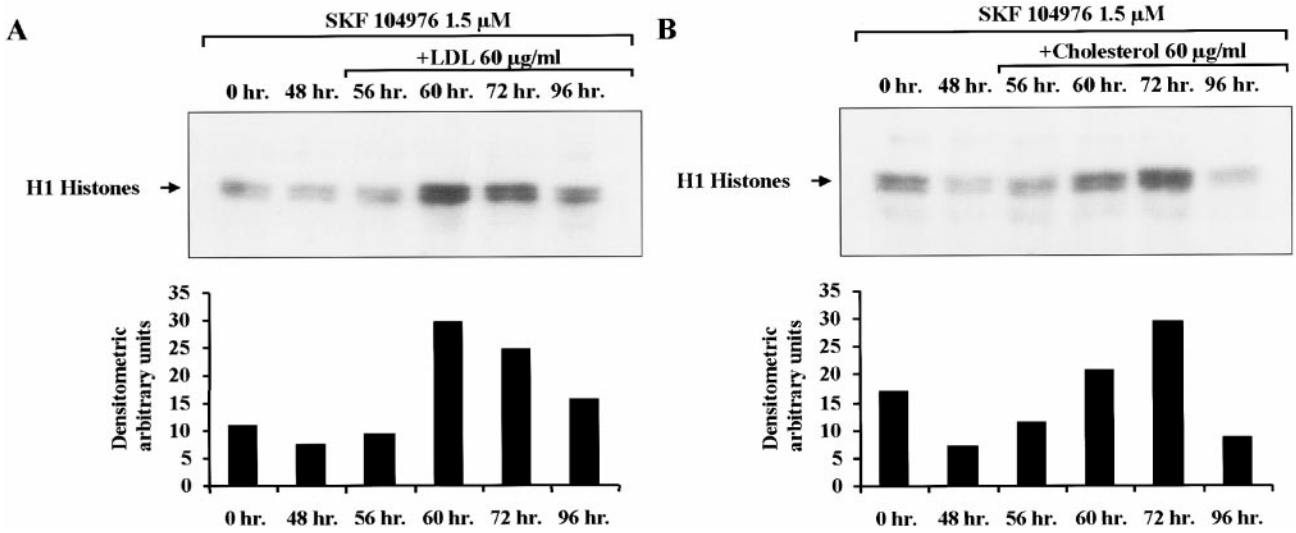

Figure 11. Effect of LDL and free cholesterol on $p34^{cdc2}$ kinase activity of HL-60 cells treated with SKF 104976. HL-60 cells were incubated in RPMI 1640, 10% LPDS in the presence of 1.5 µM SKF 104976 for 48 h; the medium then was supplemented with 60 µg/ml LDL (*A*) or 60 µg/ml free cholesterol (*B*). At the indicated times, cell extracts were incubated with an anti-cdc2 polyclonal antibody and the resultant immunoprecipitates were used for histone H1 phosphorylation assay. Phosphorylation was quantified by scanning the film with densitometer.

Caffeine release of the SKF 104976-induced G2 arrest

To further analyze the level at which cholesterol availability was acting in the regulation of the cell cycle, we took advantage of the well-known effect of caffeine in promoting the unscheduled entry into mitosis by preventing the phosphorylation-mediated $p34^{cdc2}$ inactivation (46, 47). HL-60 cells were incubated in the presence of SKF 104976 for 36 h, a time when the S phase was very active as determined by BrdUr incorporation (data not shown), and 60 h, when most of the cells were arrested at G2 (Fig. 9); then the medium was supplemented with 5 mM caffeine or placebo. As shown in **Fig. 12**, caffeine reduced the proportion of cells in G2 and raised the

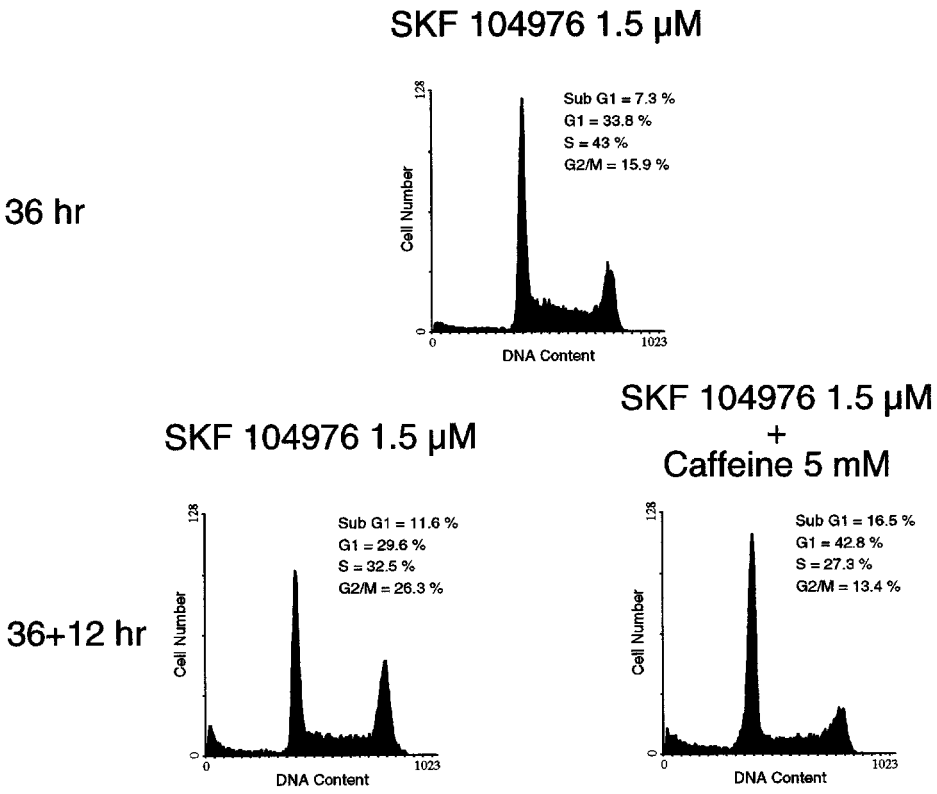

Figure 12. Effect of caffeine on cell cycle progression in SKF 104976-treated cells. HL-60 cells were incubated in RPMI 1640, 10% LPDS in the presence of 1.5 µM SKF 104976 for 36 h, the medium was supplemented with 5 mM caffeine or placebo, and cultured for additional 12 h. At the indicated times, the cells were harvested, stained with propidium iodide, and analyzed by flow cytometry.

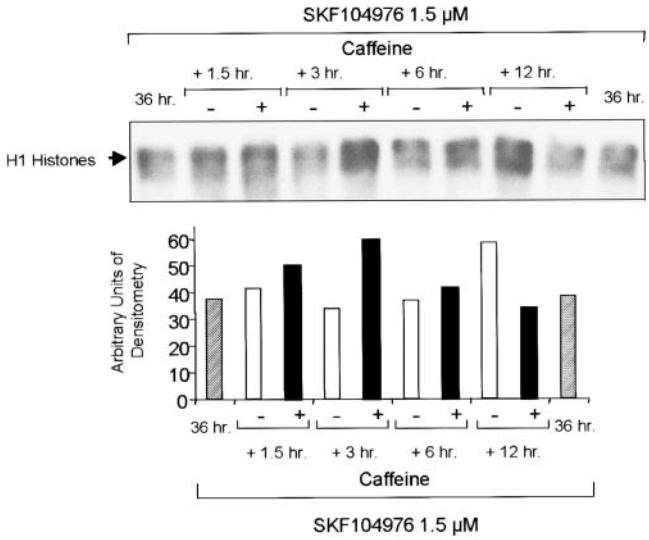

Figure 13. Effect of caffeine on $p34^{cdc2}$ kinase activity in cells treated with SKF 104976. HL-60 cells were incubated in RPMI 1640, 10% LPDS in the presence of 1.5 μM SKF 104976 for 36 h, then the medium was supplemented with 5 mM caffeine (+) or placebo (−) and cultured for an additional 12 h. At the indicated times, cell extracts were incubated with an anti-cdc2 polyclonal antibody and the resultant immunoprecipitates were used for histone H1 phosphorylation assay. Phosphorylation was quantified by scanning the film with densitometer.

proportion in G1. This effect of caffeine was preceded by a rapid and transient stimulation of $p34^{cdc2}$ activity (**Fig. 13**), which is in accordance with previous results by others in other systems (43, 48, 49). The effect of caffeine on cell cycle progression, however, depended on the cell cycle distribution at the time of application. For example, in a culture treated with SKF 104976 for 60 h (when the cell cycle distribution was 20.3% in G1, 17.0% in S, and 62.7% in G2), the addition of caffeine did not produce any significant change after 12 h (21.0% in G1, 18.1% in S, and 60.9% in G2 for 12 h). Thus, the different effects probably relied on the proportion of cells in S phase in transit to G2, caffeine being ineffective on cells previously arrested at G2 by cholesterol deprivation.

DISCUSSION

It is well recognized that cholesterol is essential for cell growth. In the present work, we show that cholesterol starvation induces cell cycle arrest specifically at G2 phase in a process mediated by the $p34^{cdc2}$ cyclin-dependent kinase. This arrest is reversed on provision of exogenous cholesterol, which results in the stimulation of $p34^{cdc2}$ activity and cell cycle restoration. Hence, it is proposed that the G2 checkpoint is sensitive to cholesterol availability.

To explore the role of cholesterol on cell cycle, MOLT-4 and HL-60 cell lines were cultured in a cholesterol-deficient medium and simultaneously exposed to SKF 104976, which is a specific inhibitor of lanosterol 14-α demethylase (42). We demonstrated that SKF 104976 efficiently blocks the incorporation of [^{14}C]-acetate into cholesterol, resulting in the accumulation of both lanosterol and dihydrolanosterol, without affecting significantly the synthesis of mevalonic acid.

The strong inhibitory effect of SKF 104976 on cell growth, which is described here for the first time, is in keeping with previous observations indicating that cells deficient in lanosterol 14-α demethylase require cholesterol for normal cell growth (50). The effect of SKF 104976 was fully restored by either LDL or free cholesterol, whereas mevalonate was ineffective. These results demonstrate that SKF 104976-treated cells were not deprived of the nonsterol mevalonate derivatives essential for cell proliferation (17–24), but just of cholesterol.

Flow cytometry and morphological examination clearly showed that SKF 104976 treatment arrested the cells specifically at the G2 phase, whereas both G1 traversal and the transition through S were unaffected. A similar block in G2/M was observed in lanosterol 14-α demethylase-deficient cells when incubated in a cholesterol-poor medium (51). It has been reported that other cholesterol synthesis inhibitors also disturb cell cycle progression. Ketoconazole, which is an inhibitor of cytochrome P_{450}-dependent enzymes, including lanosterol 14-α demethylase, blocks the cell cycle primarily at G1, although a small accumulation of cells in G2 is also observed when used at high concentration (25). Both vastatins and dehydroepiandrosterone, which deplete endogenous mevalonate pools, have been shown to arrest the cell cycle predominantly at G1, but they also delayed the cells in G2/M transitorily (15, 52, 53). These observations and our results suggest that the accumulation of cells in G2/M is due to the cholesterol deficiency these drugs produce. On other hand, the block in G1 is probably the result of either reduced protein prenylation (53, 54) or depletion of certain nonsterol isoprene derivatives (i.e., dolichol, isopentenyladenine) (18, 23, 24).

One of the main findings of this study is the requirement of cholesterol for G2 traversal. In fact, cells arrested at G2 entered into mitosis and the cell cycle resumed on provision of cholesterol, either in the form of LDL or free cholesterol dissolved in ethanol, despite the continuous presence of the inhibitor. This reinforces the concept that cholesterol is essential for G2 traversal. Given that SKF 104976-treated cells are temporarily viable and the cell cycle can easily be resumed by cholesterol addition, this model offers a valuable tool for studying the mechanisms governing the traversal of G2 phase.

The G2 arrest that occurs in response to DNA

damage or other insults is imposed by transient inactivation of $p34^{cdc2}$ kinase, the universal determinant of entry into mitosis (29–32). The observation that $p34^{cdc2}$ in cells arrested at G2 by prolonged cholesterol starvation is predominantly in its hyperphosphorylated form and inactive (Fig. 10), and that cholesterol provision to these cells increases $p34^{cdc2}$ activity (Fig. 11) and releases the block (Fig. 9), suggests that the cell cycle arrest was mediated by the reduced $p34^{cdc2}$ activity. We found that caffeine activated $p34^{cdc2}$ and partially reduced the accumulation of cells in G2 provided it was added when the culture contained a substantial number of cells in S phase, on transit to G2. By contrast, cells arrested at G2, not responding to caffeine, still responded to cholesterol by $p34^{cdc2}$ activation and proliferation. Therefore, the results suggest that cholesterol acts downstream of caffeine on the regulation of the cell cycle. This hypothesis and the extrapolation of present results to other cell types deserve further verification.

The finding that cholesterol is required specifically for the traversal of the G2 phase extends previous studies that demonstrated the necessity of this sterol for cell growth (9, 10). Whether $p34^{cdc2}$ activity responds to other sterols as it does to cholesterol was not directly addressed here. However, the fact that cells treated with SKF 104976 accumulated lanosterol and dihydrolanosterol suggests that the regulatory response herein described is stringent for cholesterol. Previous studies in *Mycoplasma capricolum* (55, 56) and *Saccharomyces cerevisiae* (57, 58) have led to the notion that sterols serve a dual role in cells: one structural, ensuring the integrity of membranes; another of regulatory nature (59). The structural role is accomplished by the amount of the cellular sterol. Although the preferred sterol is the one synthesized by each organism (i.e., cholesterol in mammals, ergosterol in yeast, sitosterol and others in plants), this role has a relatively broad specificity; in fact, other exogenously supplied sterols can be used for membrane formation provided there is some compensatory change in membrane fatty acids (60–62). In contrast, the regulatory role is restricted to the sterol that is characteristic for the organism (55, 56). These two actions explain the synergistic effects of different sterols on cell growth as observed in several species (57, 58). It was reported that extremely low concentrations of ergosterol (< 1 nM) in the budding yeast activated a pp60v-src-related protein kinase involved in the cell cycle control division (59). This finding allowed the suggestion that this kinase activity might be part of the signal transduction process that senses the availability of sterol needed for cell growth and commits the cell to a new mitotic division (59).

Similar to that reported for ergosterol in yeast, we observed that cholesterol provision in cholesterol-deprived mammalian cells leads to the rapid stimulation of $p34^{cdc2}$, a cell cycle-related protein kinase. How the cell senses cholesterol availability and how this signal is transduced are still unknown. Cholesterol is an important regulator of the expression of several lipid metabolism-related genes, which contain the so-called sterol regulatory elements (SRE) in their promoter regions (8). When the intracellular cholesterol levels decrease, the endoplasmic reticulum-bound SCAP activates the proteolytic processing of SRE binding proteins to form transcription factors (SREBP-1a, -1c, -2), which migrate to the nucleus and activate gene transcription by binding to the SRE (8). Whether an analogous mechanism is involved in the modulation of $p34^{cdc2}$ activity by cholesterol deprivation remains to be established. FJ

This study was supported by grants from the Fondo de Investigación Sanitaria (FIS 94/0484) and Comisión Interministerial de Ciencia y Tecnología (SAF96/0011), Spain, to M.A.L., and FIS 97/0389 to D.G.-C. We thank Miguel Martin for excellent technical assistance, Drs. M. A. Vega, F. Domínguez and R. F. Martínez-Botas for revising the manuscript, and SmithKline Beecham for providing SKF 104976.

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Received for publication April 9, 1998.
Revised for publication February 22, 1999.