Caffeine Inhibits Cell Proliferation by G0/G1 Phase Arrest in JB6 Cells

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ABSTRACT

Caffeine is a major biologically active constituent in coffee and tea. Because caffeine has been reported to inhibit carcinogenesis in UVB-exposed mice, the cancer-preventing effect of caffeine has attracted considerable attention. In the present study, the effect of caffeine in quiescent (G0 phase) cells was investigated. Pretreatment with caffeine suppressed cell proliferation in a dose-dependent manner 36 h after addition of fetal bovine serum as a cell growth stimulator. Analysis by flow cytometry showed that caffeine suppressed cell cycle progression at the G0/G1 phase, i.e., 18 h after addition of fetal bovine serum, the percentages of cells in G0/G1 phase in 1 mM caffeine-treated cells and in caffeine-un-treated cells were 61.7 and 29.0, respectively. The percentage of cells in G0/G1 phase at 0 h was 75.5. Caffeine inhibited phosphorylation of retinoblastoma protein at Ser780 and Ser807/Ser811, the sites where retinoblastoma protein has been reported to be phosphorylated by cyclin-dependent kinase 4 (cdk4). Furthermore, caffeine inhibited the activation of the cyclin D1-cdk4 complex in a dose-dependent manner. However, this compound did not directly inhibit the activity of this complex. In addition, caffeine did not affect p16INK4 or p27kip1 protein levels, but inhibited the phosphorylation of protein kinase B (Akt) and glycogen synthase kinase 3β. Our results showed that caffeine suppressed the progression of quiescent cells into the cell cycle. The inhibitory mechanism may be due to the inhibition of cell growth signal-induced activation of cdk4, which may be involved in the inhibition of carcinogenesis in vivo.

INTRODUCTION

Because it is present in numerous dietary sources, including coffee, tea, cocoa beverages, and soft drinks, caffeine is the most widely consumed behaviorally active substance in the world (1). Caffeine has been the subject of intensive study, and various effects have been reported, including antagonistic effects on adenosine receptors (2), inhibition of phosphodiesterases (3, 4), stimulation of muscle contraction levels (5), and alterations in glucose metabolism (6–8). Topical application of caffeine (6.2 μmol in 100 μl of acetone, once a day, 5 days a week, for 18 weeks) was reported recently to inhibit formation of UVB-induced skin tumors in mice in vivo, and the induction of apoptosis was suggested as one of the responsible mechanisms (9). However, most of the mechanisms of action are as yet still unknown.

Recent studies showed that various concentrations of caffeine induce apoptosis in several cell lines, including 10 mM caffeine in human neuroblastoma cells (10), 4 mM in human pancreatic adenocarcinoma cells (11), 5 mM in human A549 lung adenocarcinoma cells (12), and 450 μM in mouse epidermal JB6 Cl 41 cells (13). Besides apoptosis, suppression of cell proliferation is one of the most important strategies for anticarcinogenesis. Caffeine (5 mM) has been reported to induce TP53-independent G1 phase arrest in a human lung adenocarcinoma cell line (12). On the other hand, this compound has also been reported to prevent G2 phase arrest induced by exposure to radiation or other DNA-damaging agents (14–16). Thus, caffeine shows various effects on the cell cycle and may cause cell growth arrest, resulting in anticarcinogenic effects.

Cells are believed to be quiescent or in G0 phase in most mammals under normal conditions. After stimulation with growth factors, cells progress through the cell cycle phase from G0 to G1 and then G1 to S. The D-type cyclin and cyclin-dependent kinase (cdk)4/6 complex plays an important role in G0 to G1 and G1 to S transitions (17, 18). These complexes phosphorylate protein retinoblastoma (pRb), which binds with transcriptional factors, including E2Fs, and the phosphorylation promotes the expression of target proteins such as cyclin A (17, 18). pRb is a key regulator in the G0 to G1 transition, and its activity is modulated by cyclin-cdk complexes. In addition, the consensus motif for phosphorylation by the cyclin D1-cdk4 complex is different from that for phosphorylation by the cyclin E-cdk2 complex, and the Ser780 site in pRb is specifically phosphorylated by the cyclin D1-cdk4 complex in vivo and in vitro (19).

In this study, to elucidate the mechanism for the anticarcinogenic activity of caffeine, the effect of caffeine in mouse epidermal JB6 cells synchronized in G0 phase was investigated after stimulation with fetal bovine serum (FBS) as a growth factor. Caffeine inhibited FBS-stimulated cell proliferation without the induction of apoptosis, apparently resulting from the suppression of the activation of the cyclin D1-cdk4 complex and the subsequent inhibition of pRb phosphorylation. This inhibitory effect of caffeine on cell proliferation may be one of the mechanisms explaining the anticarcinogenic activity of this compound in vivo.

MATERIALS AND METHODS

Reagents. Caffeine was purchased from Sigma-Aldrich (St. Louis, MO), MEM was from Mediatech (Herndon, VA), FBS was from Gemini Bio-Products (Woodland, CA), and the antibiotics (penicillin and streptomycin) were from Invitrogen (Carlsbad, CA). For cell cycle analysis, propidium iodide and RNase A were purchased from Sigma-Aldrich.

Cell Culture. The mouse epidermal cell line, JB6 Cl 41, was maintained with MEM, containing 5% FBS, 50 IU/ml penicillin G, and 50 μg/ml streptomycin, in an atmosphere of 95% air-5% CO2 at 37°C. Fifty thousand cells were seeded in a 60 mm dish and cultured for 24 h. The cells were serum-starved in 0.1% FBS in MEM for 36 h to synchronize the cell cycle into G0 phase (20). The cells were treated with various concentrations of caffeine or distilled water as a control for 1 h followed by addition of FBS (final 5% concentration) as a cell growth stimulator.

Cell Proliferation Assay [3-(4,3-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium Assay]. Cell proliferation was analyzed using the CellTiter 96 AQ699, One Solution Cell Proliferation Assay (Promega, Madison, WI) following the instructions provided. In brief, cells synchronized into G0 phase in a 96-well multiplate were treated with caffeine and then FBS was added. After an additional 36 h in culture, cells were incubated for 2 h at 37°C with 3-(4,3-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium reagent in a humidified, 5% CO2 atmosphere. The absorbance of a colored formazan product catalyzed by dehydrogenase enzymes in metabolically active cells was recorded at 492 nm and with 690 nm as a background. Data were expressed as the percentage of absorbance versus untreated control cells.

Measurement of Apoptosis by Flow Cytometry. The apoptotic action of caffeine was determined using the Annexin V-FITC Apoptosis Detection kit.
following the instruction protocol provided (MBL International, Woburn, MA). Briefly, 36 h after addition of FBS, cells were trypsinized, washed twice with ice-cold Dulbecco’s phosphate buffered saline (DPBS), and incubated at room temperature for 15 min in the dark with Annexin V-conjugated FITC and propidium iodide in the binding buffer provided. Stained cells were analyzed by flow cytometry using the FACSCalibur (BD Biosciences, San Jose, CA).

**Cell Cycle Analysis.** Cell cycle was analyzed by a published method (20) with slight modifications. At different time points, various hours after stimulation with FBS, cells were trypsinized, washed with ice-cold DPBS, and fixed with ice-cold 70% ethanol at −20°C overnight. Cells were then washed twice with DPBS, incubated with 20 µg/ml RNase A and 200 µg/ml propidium iodide in DPBS at room temperature for 30 min in the dark, and subjected to flow cytometry using the FACSCalibur flow cytometer. Data were analyzed using ModFit LT (Verity Software House, Inc., Topsham, ME).

**Western Blot and Antibodies.** Cells synchronized into G0 phase were pretreated with caffeine for 1 h, and then FBS was added. Cells were washed twice with ice-cold DPBS and harvested with 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium Pp, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin, and 1 µM phenylmethylsulfonfonyl fluoride (PMSF). Immunoblotting was performed by following the protocol provided by Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), with slight modifications. The sources of antibodies were as follows: phospho-retinoblastoma (Rb; Ser780), phosphos-Rb (Ser807/Ser811), phospho-glycogen synthase kinase (GSK)-3α/β (Ser21/9), GSK-3β, phospho-Akt (Thr308), Akt, and p27, Cell Signaling Technology, and p16 and β-actin, Santa Cruz Biotechnology.

**Immunoprecipitation Kinase Assay.** The cyclin D1-cdk4 kinase assay was performed as described (21, 22) with modifications. Briefly, cells synchronized into G0 phase were pretreated for 1 h with the indicated concentration of caffeine, and then FBS was added. Six h after the addition of FBS, cells were washed twice with ice-cold DPBS and harvested with a kinase buffer [25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4, 10 mM MgCl2, and 1 mM PMSF]. Protein content was determined using the modified Lowry method (23). Cyclin D1 was immunoprecipitated from 400 µg of the cell extract using an anticyclin D1 monoclonal antibody (Cell Signaling Technology) diluted at 1:100 overnight. Then 20 µl of protein A/G PLUS-Agarose (Santa Cruz Biotechnology) was added, incubated at 4°C, and then washed at 4°C four times with ice-cold kinase buffer. Immunoprecipitated cyclin D1 was incubated with 1 µg Rb-C fusion protein (Cell Signaling Technology) and 200 µM ATP in a kinase buffer at 30°C for 30 min. Phosphorylation of Rb-C fusion protein was detected by Western blotting with an antiphospho-Rb (Ser780) and (Ser807/Ser811) polyclonal antibodies.

**In Vitro Kinase Assay for the Cyclin D1-cdk4 Complex.** Cells synchronized into G0 phase were treated with FBS for 6 h to allow the activation of the cyclin D1-cdk4 complex. Immunoprecipitated cyclin D1, which was prepared as described in the section “Immunoprecipitation Kinase Assay,” was incubated with 1 µg Rb-C fusion protein, and 200 µM ATP in a kinase buffer at 30°C for 30 min. Phosphorylation of the Rb-C fusion protein was detected by Western blotting with an antiphospho-Rb (Ser780) polyclonal antibody.

**RESULTS**

Caffeine Inhibits Cell Proliferation in Quiescent JB6 Cells Stimulated with Serum. Treatment with caffeine for 24 h was demonstrated recently to induce apoptosis in JB6 cells after 72 h of serum starvation (13). In the present study, the effect of caffeine on cell proliferation in response to cell growth stimulation by FBS in quiescent JB6 cells synchronized into G0 phase by 36-h serum starvation (0.1% FBS) was investigated. As shown in Fig. 1, stimulation with 5% FBS for 36 h increased cell proliferation to 80% above control (100%), which was cultured without addition of 5% FBS for 36 h. Pretreatment with caffeine for 1 h before stimulation with FBS significantly inhibited cell proliferation in a dose-dependent manner (Fig. 1, solid circles). Caffeine at 1 mM inhibited cell proliferation ~75% in response to FBS. The IC50 was ~0.7 mM. On the other hand, caffeine only slightly decreased proliferation in control cells (Fig. 1, open circles).

Flow cytometry was used to determine, under the same conditions as for Fig. 1, whether the inhibition of proliferation is due to apoptosis (Fig. 2). Pretreatment with 1 mM caffeine under 0.1% FBS medium conditions slightly increased the number of apoptotic cells. The percentage of total apoptotic cells stained with Annexin V-FITC was 6.0 ± 2.5 (sum of 4.21 ± 2.25 and 1.87 ± 0.28) and 10.1 ± 0.5 (sum
INDUCTION OF CELL CYCLE ARREST BY CAFFEINE

Fig. 3. Caffeine causes G0/G1 phase arrest in quiescent JB6 cells. A–C, JB6 cells with cell cycle synchronized into the G0 phase were treated for 1 h with 1 mM caffeine (●) or distilled water as a control (○), and a final concentration of 5% fetal bovine serum (FBS) was added to stimulate cell growth. Cell cycle analysis was performed by flow cytometry as described in “Materials and Methods.” Data represent the mean (n = 3); bars, ±SD. *P < 0.001, control JB6 cells untreated with caffeine versus caffeine-treated JB6 cells as determined by Student’s t test. D, JB6 cells in G0 phase were treated with the indicated concentration of caffeine for 1 h. FBS was then added as a growth stimulant, and 16 h after addition of FBS, cell cycle analysis was performed. Data represent the mean (n = 3); bars, ±SD. *P < 0.001, versus JB6 cells added 5% FBS and untreated with caffeine as determined by Student’s t test.

Caffeine Causes G0/G1 Phase Arrest in Quiescent JB6 Cells. To reveal the inhibitory mechanism of caffeine on cell proliferation, the effect of this compound on cell cycle was investigated. In control quiescent cells shown as open circles in Fig. 3, A and B, 14 h after addition of 5% FBS, the percentage of cells in G0/G1 phase started decreasing (Fig. 3A), and the percentage of cells in S phase began increasing (Fig. 3B), meaning that the G0/G1 to S progression was executed in response to FBS stimulation. In quiescent cells treated with 1 mM caffeine shown as closed circles in Fig. 3, A and B, the progression was inhibited significantly. Sequentially in control quiescent cells, 18 h after addition of 5% FBS, the percentage of cells in S phase started decreasing (Fig. 3B), and the percentage of cells in G2/M phase began increasing (Fig. 3C), meaning that the S to G2/M progression was subsequently executed. Caffeine also significantly inhibited this progression as shown as closed circles in Fig. 3, B and C. As shown in Fig. 3D, the inhibitory effects were dose-dependent, and the IC50 was ~0.7 mM.

To investigate whether the inhibitory effect of caffeine is specific to quiescent cells, JB6 cells without serum starvation were cultured for 24 h. Caffeine (≤1 mM) did not affect the phase distribution of the cell cycle, although a high concentration (5 mM) of caffeine caused G0/G1 phase arrest (Fig. 4). Caffeine at high concentrations seems to cause G0/G1 phase arrest through the inhibition of activation of cdk2, a key kinase in the G1 to S transition. A concentration of 5 mM caffeine has already been reported to inhibit the activation of cdk2 in a lung adenocarcinoma cell line (12). Thus, we hypothesized that G0/G1 phase arrest caused by caffeine at low concentrations (≤1 mM) was specific to quiescent cells, and that these concentrations of caffeine might affect the G0 to G1 transition but not the G1 to S transition.

Caffeine Inhibits the Activation of the Cyclin D1-ckd4 Complex. Phosphorylation of pRb plays an important role in the G0 to G1 transition (24). To investigate the effects of caffeine on the G0 to G1 transition, phosphorylation of pRb was examined at Ser780 and Ser807/Ser811, the sites at which pRb has been reported to be phosphorylated by cdk4, a key kinase in the G0 to G1 transition (Fig. 5). In control cells, these sites in pRb were phosphorylated 3–6 h after stimulation by 5% FBS, and the phosphorylation was continued for at least 9 h (Fig. 5A). Pretreatment of cells with 1 mM caffeine before the addition of FBS significantly inhibited the phosphorylation at these sites.

Fig. 4. Caffeine at a 1-mM dose does not cause cell cycle arrest in active JB6 cells. Active JB6 Cl 41 cells without serum starvation were cultured for 24 h with 0.5, 1.0, or 5.0 mM caffeine. Cell cycle analysis was performed by flow cytometry as described in “Materials and Methods.” Data represent the mean (n = 3); bars, ±SD. *P < 0.001, versus control group (caffeine = 0 mM) as determined by Student’s t test.
cells with cell cycle phase synchronized into G0 were treated for 1 h with 1 mM caffeine or distilled water as a control. Both sets of cells were then treated with 5% fetal bovine serum (FBS) for the indicated time. B, synchronized cells were treated with 0.25, 0.5, or 1 mM caffeine for 1 h and then cultured with 0.1% or 5% FBS in MEM for 6 h. The phosphorylation level of pRB was analyzed by Western blotting as described in “Materials and Methods.” Equal loading of protein was confirmed by β-actin, and data shown are representative of at least three independent experiments.

The effect of caffeine on activation of cyclin D1-cdk4 was next investigated because cdk4 is required to form a complex with cyclin D1 for activation (25). Pretreatment with caffeine significantly inhibited the activation of cyclin D1-cdk4 in a dose-dependent manner (Fig. 6A). These results indicate that caffeine inhibited the G0 to G1 transition through the suppression of activation of cyclin D1-cdk4.

Caffeine Inhibits the Akt-GSK-3β Cell Growth Signal. Because cyclin D1-cdk4 is regulated by GSK-3β (26) and also the cdk inhibitors, p16INK4a (27) and p27Kip1 (28), the effect of caffeine on these proteins was investigated. Caffeine had no effect on the protein levels of p16INK4a and p27Kip1, and over time the protein levels tended to decrease in a time-dependent manner after stimulation with FBS (Fig. 6B). In addition, caffeine did not appear to directly affect cyclin D1-cdk4 activity (Fig. 6C). On the other hand, caffeine markedly inhibited the time-dependent phosphorylation of protein kinase B (Akt) Thr308 and its substrate, GSK-3β (Ser9; Fig. 7A). In addition, the inhibitory effect on the phosphorylation was also observed in JB6 cells pretreated with 0.25 and 0.5 mM caffeine 30 min after FBS stimulation (Fig. 7B).

Finally, epidermal growth factor or platelet-derived growth factor was used as a cell growth stimulator instead of FBS. Pretreatment with caffeine (1 mM) for 1 h before addition of epidermal growth factor (10 ng/ml) or platelet-derived growth factor (5 ng/ml) inhibited the phosphorylation of Akt and GSK-3β 30 min after stimulation by these compounds (Fig. 8A). In addition, caffeine suppressed the change in phase distribution in the cell cycle 18 h after stimulation with epidermal growth factor (10 ng/ml; Fig. 8B). Thus, caffeine may inhibit cell proliferation in response to several growth factors through an inhibition of the Akt-GSK-3β cell growth signal.

**DISCUSSION**

This study demonstrated that caffeine caused G0/G1 phase arrest and inhibited proliferation in mouse epidermal JB6 Cl 41 cells synchronized into G0 (quiescent) phase after stimulation with FBS as a cell growth signal (Figs. 1 and 3). Phosphorylation of pRb plays a critical role in the G0 to G1 and G1 to S transitions (24). The phosphorylation leads to the disruption of the pRb-E2F transcription factor complex, and the release of active E2F additionally triggers the activation of a number of genes (18). Cdk4/6 and cdk2 are key kinases, which phosphorylate pRb in the G0 to G1 and G1 to S transitions (29). Ser780 in pRb is a specific site for phosphorylation by cdk4 (19). Ser807 and Ser811 in pRb are also phosphorylated by cdk4 in vivo and in vitro (30). As shown in Fig. 5, caffeine inhibited the phosphorylation of Ser780 and Ser807/Ser811 after stimulation with FBS. Cdk4/6 forms a complex with D-type cyclins in response to growth factor stimulation, and the active complex phosphorylates pRb (25). Thus, the cyclin D1-cdk4 complex is a key factor in regulation of the G0 to G1 transition (31). Pretreatment with caffeine resulted in an inhibition of cyclin D1-cdk4 complex activation in a dose-dependent manner (Fig. 6A). Taken together, these data suggest that caffeine causes G0/G1 phase arrest accompanied by inhibition of cyclin D1-cdk4 complex activation and cell proliferation in response to cell growth stimulation. Some effects of caffeine on the cell cycle have already been reported, including abrogation of the G2 phase arrest that was observed in cells exposed to radiation or other DNA-damaging agents (14–16) and suppression of the activation of cdk2 in a TP53-independent mechanism (12). The present study shows a new effect of caffeine on cell cycle, the suppression of cyclin D1-cdk4 activation. Direct inhibition by caffeine of several kinases, ataxia-telangiectasia mutated kinase (IC50, 0.2 mM), ATM- and Rad3-related kinase
PI3K p100 activity of cdk4 (Fig. 6C). In addition, caffeine (8 mM) had no effect on the activity of cdk2 by a TP53-independent mechanism in a lung adenocarcinoma cell line (12). Also in the present study, 5 mM, but not 1 mM caffeine, caused G0/G1 phase arrest. JB6 cells with cell cycle synchronized into the G0 phase were treated with 0.25, 0.5, or 1 mM caffeine for 1 h and then cultured in 0.1% or 5% FBS in MEM for 30 min. The phosphorylation of Akt at Thr308 (p-Akt) and GSK-3β at Ser9 (p-GSK-3β) was analyzed by Western blotting using the corresponding antibody as described in “Materials and Methods.” Equal loading of protein was monitored by total Akt and GSK-3β, and data shown are representative of at least three independent experiments.

Fig. 7. Caffeine suppresses the Akt-glycogen synthase kinase (GSK)-3β cell growth signal pathway. A. JB6 cells with cell cycle phase synchronized into G0 were treated with 1 mM caffeine or distilled water (control) for 1 h and then treated with 5% fetal bovine serum (FBS) for the indicated time. B. synchronized cells were treated with 0.25, 0.5, or 1 mM caffeine for 1 h and then cultured in 0.1% or 5% FBS in MEM for 30 min. The phosphorylation of Akt at Thr308 (p-Akt) and GSK-3β at Ser9 (p-GSK-3β) was analyzed by Western blotting using the corresponding antibody as described in “Materials and Methods.” Data shown are representative of at least three independent experiments.

PI3K p100 activity of cdk4 (Fig. 6C). In addition, caffeine (8 mM) had no effect on the activity of cdk2 by a TP53-independent mechanism in a lung adenocarcinoma cell line (12). Also in the present study, 5 mM, but not 1 mM caffeine, caused G0/G1 phase arrest. JB6 cells with cell cycle synchronized into the G0 phase were treated with 0.25, 0.5, or 1 mM caffeine for 1 h and then cultured in 0.1% or 5% FBS in MEM for 30 min. The phosphorylation of Akt at Thr308 (p-Akt) and GSK-3β at Ser9 (p-GSK-3β) was analyzed by Western blotting using the corresponding antibody as described in “Materials and Methods.” Data shown are representative of at least three independent experiments.

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(CIC50, 1.1 mM), mammalian target of rapamycin (IC50, 0.4 mM), DNA-dependent protein kinase (IC50, <10 mM), hChk1 (IC50, <5 mM), phosphatidylinositol 3’-kinase (PI3K) p100α (IC50, 0.4 mM), PI3K p100β (IC50, 4.4 mM), PI3K p100γ (IC50, 1.0 mM), and PI3K p100δ (IC50, 75 μM; Refs. 32, 33) has been reported. The inhibitory effect may be due to an antagonistic effect on ATP because caffeine is a purine derivative. However, caffeine did not directly inhibit the activity of cdk4 (Fig. 6C). In addition, caffeine (8 mM) had no effect on the activity of active cdk2/cyclin E (Upstate, Lake Placid, NY; data not shown), although 5 mM caffeine has been reported to inhibit the activity of cdk2 by a TP53-independent mechanism in a lung adenocarcinoma cell line (12). Also in the present study, 5 mM, but not 1 mM, caffeine caused G0/G1 phase arrest in JB6 Cl 41 cells without serum starvation (Fig. 4), and the effect might be due to the inhibition of cdk4 as was reported previously (12). Furthermore, caffeine has been reported to enhance the activity of cdk1 in cells exposed to DNA-damaging agents (34, 35). Thus, caffeine is unlikely to directly inhibit cdk5 specifically.

Cdk5 are regulated by cdk inhibitors, including the INK4 family and Cip/Kip family (36). In particular, the cyclin D1-cdk4 complex is regulated by p16INK4a, a member of the INK4 family (25). In the current study, caffeine did not affect the protein level of p16INK4a (Fig. 6B). A member of the Kip family, p27Kip1 is also known to regulate the cyclin D1-cdk4 complex (28), and to be exported from the nucleus and degraded by the ubiquitin-proteasome pathway (37). In this study, caffeine did not affect protein levels of p27Kip1, although the levels were time-dependently decreased after stimulation with FBS as a growth signal (Fig. 6B). Therefore, the inhibitory effect of caffeine on the activation of the cyclin D1-cdk4 complex is not due to the regulation of p16INK4a and p27Kip1 levels.

Cell growth signals sequentially mediate Ras, PI3K, and Akt, resulting in the phosphorylation of GSK-3β. GSK-3β phosphorylates Thr286 in cyclin D1, thereby triggering cyclin D1 turnover and decreasing the activity of cdk4 (26). Pretreatment with caffeine suppressed the phosphorylation of both Akt and GSK-3β as shown in Fig. 7. In addition, caffeine has already been reported to inhibit PI3K directly (33). Therefore, the inhibitory effects of caffeine on the cell growth signaling of Akt-GSK-3β may result from the direct inhibition of PI3K, which is upstream of Akt. In fact, the inhibition of PI3K by caffeine is believed to have several effects on the physiological functions of cells, including the inhibition of translocation of glucose transporter to the cell membrane in response to insulin stimulation (33). In this study, caffeine suppressed the Akt-GSK-3β signal stimulated by not only FBS but also by epidermal growth factor and platelet-derived growth factor (Fig. 8A), and caused G0/G1 phase arrest (Fig. 8B). On the other hand, a cell growth signal might bifurcate to a signal leading to the ubiquitination of p27Kip1 and a signal leading to Akt-GSK-3β upstream from PI3K, because caffeine did not affect p27Kip1 in response to FBS stimulation (Fig. 6B). However, inhibition of the PI3K pathway has been reported to induce an increase in p27Kip1 in a senescence-like growth arrest, which is induced in mouse primary embryo fibroblasts by inhibitors of PI3K (38). This contradiction may be due to the existence of a signal pathway for p27Kip1 synthesis downstream from PI3K. Therefore, the rate of disruption of p27Kip1 may be faster than the synthesis. The disruption of p27Kip1 occurs by an ubiquitin-proteasome pathway (37), and the phosphorylation of Ser10 in p27Kip1 is required for the disruption (39). However, the kinase responsible is still unknown.

The ED50 at which caffeine causes G0/G1 phase arrest, was ~0.7 mM as estimated from Figs. 1 and 2. He et al. (13) showed that 0.45 mM caffeine suppressed the phosphorylation of Akt and glycogen synthase kinase (GSK)-3β, and caused G0/G1 phase arrest (Fig. 6A). Therefore, the inhibitory effect of caffeine on the activation of the cyclin D1-cdk4 complex is not due to the regulation of p16INK4a and p27Kip1 levels.

Fig. 8. Caffeine inhibits epidermal growth factor (EGF)- and platelet-derived growth factor (PDGF)-induced phosphorylation of Akt and glycogen synthase kinase (GSK)-3β and causes G0/G1 phase arrest. JB6 cells with cell cycle synchronized into the G0 phase were treated with 1 mM caffeine or distilled water for 1 h, and then a final concentration of 10 ng/ml EGF or 5 ng/ml PDGF in MEM (0.1% fetal bovine serum; FBS) was added to stimulate cell growth. A. cells were harvested 30 min after the addition of EGF or PDGF, and the phosphorylation of Akt at Thr308 (p-Akt) and GSK-3β at Ser9 (p-GSK-3β) was analyzed by Western blotting using the corresponding antibody as described in “Materials and Methods.” Data shown are representative of at least three independent experiments. B. cells were synchronized by addition of EGF and cell cycle analysis was performed by flow cytometry as described in “Materials and Methods.” Data represent the mean (n = 5); bars, ±SE. *, P < 0.01, versus JB6 cells added EGF and untreated with caffeine as determined by Student’s t test.
mm caffeine induced apoptosis in JB6 cells after 24 h culture in 0.1% FBS-containing MEM. These cells were serum-starved in advance for 72 h, and the LD₅₀ was 2.7 mm. Therefore, cell culture studies indicate that millimolar concentrations of caffeine induce not only inhibition of cell proliferation but also induction of apoptosis in starved or quiescent JB6 cells. On the other hand, animal studies suggested that 6.2 µmol caffeine in 100 µl aceton (62 mm) in a topical application inhibited UV-induced carcinogenesis; induction of apoptosis was suggested as one of the anticarcinogenic mechanisms (9). The current study suggests that not only induction of apoptosis, but also inhibition of proliferation in G₀ (quiescent) cells may be an important mechanism explaining the anticarcinogenic effect of caffeine.

ACKNOWLEDGMENTS

We thank Andria Hansen for secretarial assistance.

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*Cancer Res* 2004;64:3344-3349.

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