The spindle assembly checkpoint arrests cells in mitosis when defects in mitotic spindle assembly or partitioning of the replicated genome are detected. This checkpoint blocks exit from mitosis until the defect is rectified or the cell initiates apoptosis. In this study we have used caffeine to identify components of the mechanism that signals apoptosis in mitotic checkpoint-arrested cells. Addition of caffeine to spindle checkpoint-arrested cells induced >40% apoptosis within 5 h. It also caused proteasome-mediated destruction of cyclin B1, a corresponding reduction in cyclin B1/cdk1 activity, and reduction in MPM-2 reactivity. However, cells retained MAD2 staining at the kinetochores, an indication of continued spindle checkpoint function. Blocking proteasome activity did not block apoptosis, but continued spindle checkpoint function was essential for apoptosis. After systematically eliminating all known targets, we have identified p21-activated kinase PAK1, which has an anti-apoptotic function in spindle checkpoint-arrested cells, as a target for caffeine inhibition. Knockdown of PAK1 also increased apoptosis in spindle checkpoint-arrested cells. This study demonstrates that the spindle checkpoint not only regulates mitotic exit but apoptosis in mitosis through the activity of PAK1.

Cell cycle checkpoints are activated in response to various forms of cellular stress to block cell cycle progression until the stress, e.g. DNA damage, chromatin abnormalities, is correctly resolved. DNA damage triggers cell cycle arrest in the G2 phase through the ATM4/ATR-chk1/2-dependent pathway, which blocks cdc25-dependent activation of cyclin B/cdk1, thereby blocking entry into mitosis (1). Agents such as ionizing radiation and topoisomerase inhibitors that have clinical utility in treatment of a wide spectrum of cancers trigger this checkpoint response in cancer cell lines that are defective for p53, where the checkpoint provides a degree of protection from the cytotoxic actions of these treatments. Inhibitors of either ATM/ATR, the most commonly used being caffeine, or inhibitors of chk1/2, such as UCN01 or more recently Go6976, which abrogate this checkpoint response, can potentiate the cytotoxicity of the therapeutics in cell culture (2–4, 48). Abrogation of this checkpoint results in cells undergoing “mitotic catastrophe.” This term combines the concept of aberrant mitosis and cell death. Cells entering mitosis after bypassing the G2 checkpoint undergo aberrant mitosis with increased cell death, implying that the two are connected (5).

The spindle assembly checkpoint ensures the fidelity of partitioning of the replicated genome by blocking exit from mitosis until bipolar attachment of all chromosomes to the spindle poles and equal tension are achieved. The kinetochores then signal that anaphase can commence. This checkpoint operates by blocking anaphase-promoting complex/cyclosome (APC/C)-dependent degradation of key regulators, cyclin B1 and securin, thereby maintaining mitosis and blocking sister chromatid separation (6). Checkpoint signaling involves a kinetochore-associated protein complex, which includes MAD1, MAD2, Bub3, and BubR1. These proteins associate with unattached kinetochores and regulate the formation of MAD2-CDC20 complexes, which prevents CDC20-mediated ubiquitination of cyclin B1 and its destruction by APC/C (7, 8). Disrupting the spindle checkpoint by expressing dominant negative mutants, siRNA knockdown or knock-out by homologous recombination, or using inhibitors of checkpoint kinases results in cells undergoing aberrant mitosis and exit without correctly partitioning the sister chromatids (9–13). Tumor cell lines treated with anti-microtubule drugs, such as nocodazole or taxol that trigger the spindle assembly checkpoint, arrest in mitosis for an extended period, but eventually cells will adapt and overcome the checkpoint, exiting mitosis without partitioning their genome (13, 14). Normally, the resultant cells either become aneuploid or are blocked in G1 by a p53-dependent mechanism (15–17). Cell death can occur during aberrant mitosis, and it appears to be via an apoptotic mechanism (13, 14, 18).

Cells forced into mitosis with damaged DNA by abrogating the G1 checkpoint arrest are delayed in mitosis for an extended period because of the damage that inhibits normal chromosome partitioning executed by the spindle checkpoint. Cells should remain arrested in mitosis or undergo adaptation, exit-
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Methods—HeLa, HaCaT immortalized human keratinocyte cell line, human normal and A-T lymphoblastoid cell lines C2ABR and AT3ABR, respectively, and GM847/ATRkd containing tetracycline-inducible dominant negative ATR (26) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine donor serum (Serum Supreme, Cambrex, East Rutherford, NJ). HeLa cells stably overexpressing Bcl-2 where as described previously (27). Cells were treated overnight with nocodazole (0.25 μg/ml) or taxol (100 nM), and mitotic cells were isolated by mechanical shake-off, replated, inhibitors added, and cells harvested at later time points. Nocodazole concentration ranging from 0.1 to 0.5 μg/ml gave identical data. Caffeine was used at a final concentration of 5 mM, wortmannin at 5 μM, LY294002, U0126, MG132 at 10 μM, Z-VAD-fmk, G66976 at 100 nM, roscovitine at 50 μM, ZM447439 at 5 μM, ATM inhibitor Ku 005933 at 20 μM, AKT inhibitor SH-5 at 10 μM, and dibutyryl cyclic AMP at 10 μM. In some experiments nocodazole was washed off with three washes of fresh, prewarmed 10% serum-containing media and then cells were replated, inhibitors added, and cells harvested at later time points. Double thymidine block release synchrony of HeLa cells was performed as described previously (25).

HeLa cells were transiently transfected with either GFP or GFP-Bax (kindly provided by Dr. R. Patel, University of Leicester, UK (28)) and then cultured for 48 h. Cells were arrested in mitosis overnight with nocodazole, loaded with 200 nM TMRE, and then treated with caffeine. Cells were followed by time-lapse microscopy capturing images of TMRE and GFP fluorescence and transmitted light for cell morphology.

siRNA knockdown of PAK1 was achieved using synthetic RNA oligonucleotides 5'-CAUCAAAUAUAUCUAGUC-dTdT-3' and 5'-CAGACCCUGUGUAUACAGAdTdT-3' or a scrambled control oligonucleotide. 40 nM siRNA was transfected into HeLa cells using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions, with cultures harvested at 24 and 48 h after transfection for immunoblotting or followed by time-lapse microscopy. ATR shRNA was a kind gift from Y. Shiloh, Tel Aviv University, Israel. HeLa cells were transfected and then after 24 h were selected with 5 μg/ml puromycin for 48 h. Cells were then treated overnight with nocodazole followed by addition of caffeine or the ATM inhibitor Ku-005933 at 20 μM. This concentration was sufficient to block ATM-dependent activity (data not shown).

For time-lapse microscopy experiments, cells were grown on poly-L-lysine-coated multiwell plates; nocodazole or taxol was added for 16 h, and then either inhibitors were added or nocodazole was washed off and inhibitors added. Time-lapse movies were produced using a Zeiss Axiovert 200M Cell Observer with 37 °C incubator hood and 5% CO2 cover. Digital images were taken every 10–15 min with a Zeiss AxioCam and the images processed using AxioVision 4.2 software. Cumulative mitotic and apoptotic cell counts were performed by following cells in three or four random fields over several hours. The time at which cells either entered mitosis or underwent apoptosis was recorded for each cell in the field. The fields were combined and reported as a percentage of the total number of cells in the field at the start of the experiments. The data presented are typical of two to four separate experiments. In some

EXPERIMENTAL PROCEDURES

Materials—Nocodazole, taxol (paclitaxol), caffeine, etoposide, roscovitine, the proteasome inhibitor MG132, forskolin, and dibutyryl cyclic AMP were purchased from Sigma. The pan-caspace inhibitor Z-VAD-fmk was purchased from R&D Systems (Minneapolis, MN); wortmannin, LY294002, G66976, and SH-5 were purchased from Calbiochem. The MEK inhibitor U0126 was purchased from Cell Signaling (Danvers, MA). The Aurora inhibitor ZM447439 was kindly provided by AstraZeneca Pharmaceuticals, UK. ATM inhibitor Ku-005933 was kindly provided by KuDOS Pharmaceuticals, UK (24). Tetramethylrhodamine ethyl ester (TMRE) was purchased from Invitrogen. Antibody to cyclin B1 was produced as described previously (25). Antibodies to phospho-ERK (pERK), ERK1/2, phospho-AKT (pAKT), AKT, Bid, activated caspase 3, and Survivin were purchased from Cell Signaling. PARP antibodies were purchased from Pharmingen and Cell Signaling, and α-tubulin antibody was purchased from Sigma. MMP-2 antibody was purchased from Dako Corp. (Carpinteria, CA); MAD2 antibody was from Covance Research Products (Berkeley, CA), and PAK1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).
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experiments, GFP-H2B-expressing HeLa cells were used to visualize the chromatin in live cells.

FACS analysis of DNA content was performed as described previously (25), and MPM-2 FACS to determine mitotic cell populations was as described (27). Immunoblotting and cyclin B1 immunoprecipitated histone kinase assays were also performed as described previously (25, 29). Briefly, cells were lysed in NETN buffer (20 mM Tris, pH 8, 1 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40) supplemented with 150 mM NaCl, 30 mM NaF, 0.1 mM sodium orthovanadate, protein inhibitor mixture (Sigma), and 0.5 mM phenylmethylsulfonyl fluoride. The lysate supernatant was equalized for protein concentration and then precleared with 5 μg of nonimmune rabbit IgG coupled to 20 μl of protein A-Sepharose for 1 h at 4°C with constant rocking. The cleared supernatants were incubated with 2–5 μg of PAK1 antibody for 4 h at 4°C with constant rocking. Immunoprecipitates were then washed three times with NETN, transferred to a fresh tube, and washed with 20 mM Tris, pH 7.4, 1 mM dithiothreitol. They were then assayed for PAK1 activity by addition of a reaction mixture containing 20 mM Tris, pH 7.4, 10 mM MgCl₂, 1 mM dithiothreitol, 5 μg of myelin basic protein (Sigma), 0.2 mM ATP, and [γ-32P]ATP for 20 min at 30°C. The reactions were stopped by addition of 2× SDS sample buffer and then resolved using 10% SDS-PAGE. The level of phosphorylation of myelin basic protein was quantitated using a Storm PhosphorImager (GE Healthcare).

For immunofluorescence staining, cells were grown on glass coverslips coated with poly-L-lysine. After treatment, cells were either fixed with −20°C methanol and stored at −20°C or fixed briefly with −20°C methanol/acetone for MAD2 staining as described previously (30). Methanol-fixed cells were air-dried and then rehydrated in 3% bovine serum albumin, 0.1% Tween 20 in phosphate-buffered saline (PBS) for 1 h, then incubated with primary antibody in rehydration buffer for 1 h at room temperature in a humidifier chamber. Coverslips were washed in 0.1% Blotto (0.1% skimmed milk powder in PBS) three times, followed by incubation with appropriate fluorophore-conjugated secondary antibodies for 30 min with 0.1 μg/ml 4,6-diamidino-2-phenylindole to stain DNA, and finally washed three times in PBS and then mounted onto glass slides. Micrographs were taken using a Zeiss Axioskop2 equipped with an AxioCam HD digital camera controlled with AxioVision software.

RESULTS

Caffeine Induces Apoptosis in Mitosis following Escape from G₂ Arrest—Caffeine addition to etoposide-arrested HeLa cells results in the rapid release from the G₂ arrest and entry into mitosis by inhibiting ATM activity. Once the cells enter mitosis they would be expected to be delayed in mitosis because of DNA damage and inhibition of topoisomerase II by etoposide-blocking sister chromatid disjunction. Using time-lapse microscopy, we have followed entry into mitosis, characterized by the rounded mitotic phenotype, and apoptosis on the readily distinguishable extensive membrane blebbing under transmitted light microscopy (27) (Fig. 2A). Addition of caffeine to etoposide-treated G₂-arrested HeLa cells resulted in rapid entry into mitosis (Fig. 1A). We also observed delayed transit through mitosis.

FIGURE 1. Caffeine accelerates cell death in G₂ checkpoint-arrested cells. A, etoposide-arrested HeLa cells were treated with either caffeine (+caff) or G6976 (+Go) and then followed by time-lapse microscopy. Cells were scored for mitosis (top panel) and apoptosis (bottom panel). 100–200 cells were followed for each condition. The data are representative of two independent experiments. B, a similar experiment to A, and in this case caffeine was washed off after 2 h (caff w/o), and the mitotic cells at the time of wash off were followed and scored for apoptosis.

Normal HeLa mitosis is generally 60 min in duration. In etoposide- and caffeine-treated HeLa cells, this was extended to an average of 190 min, after which they died with the phenotypic appearance of apoptosis, without exiting mitosis. Addition of the checkpoint kinase chk1/chk2 inhibitor G6976 addition (31) also induced rapid entry into mito-
Caffeine Initiates Apoptosis in Mitotic Checkpoint-arrested Cells—
The mitotic arrest induced in etoposide and checkpoint inhibitor-treated cells was caused by the spindle assembly checkpoint blocking mitotic exit because of the block in topoisomerase II-dependent sister chromatid separation. To investigate the effect of caffeine on mitotic checkpoint-arrested cells, HeLa cells were treated overnight with nocodazole, and the mitotic cells collected by mechanical shake off had either caffeine added or were washed to remove the nocodazole and release them from the mitotic arrest. Removal of nocodazole resulted in the cells exiting mitosis and assuming a flattened interphase phenotype (Fig. 2A) within 2–3 h, with up to 60% of cells exiting mitosis by 5 h. Addition of caffeine to the nocodazole-arrested cells increased the proportion of cells displaying an apoptotic phenotype with membrane blebbing (27) (Fig. 2A). Using flow cytometry, the proportion of cells with subdiploid DNA content increased from 20% in the nocodazole-arrested controls to 50% with 5 h of caffeine treatment, with a corresponding decrease in G2/M phase cells (Fig. 2B).

The presence of a population of cells with subdiploid DNA and phenotypic appearance suggested that the cells were dying by apoptosis, a form of cell death in which DNA is fragmented following activation of caspase proteases. Consistent with this, addition of the caspase inhibitor Z-VAD-fmk blocked the appearance of cells with <2n DNA content (Fig. 2C). Caffeine also induced caspase-dependent cleavage of PARP, an event that is typical of apoptosis that also was blocked by addition of Z-VAD-fmk (Fig. 2D). Cell death was blocked by overexpression of Bcl-2 (Fig. 2E). We also observed a loss of mitochondrial onset delayed to the same extent as treatment with G6976 (Fig. 1B). This observation suggested that, in addition to overcoming the G2 checkpoint arrest, caffeine was causing cells arrested in mitosis to undergo apoptosis. In the absence of either caffeine or G6976, <5% of cell entered mitosis or underwent apoptosis during the time course of the experiment (data not shown).

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sis, but the cells delayed longer in mitosis, averaging 250 min, before 30% of cells exited mitosis to form multinuclear cells. A lower level of apoptosis and a later onset were observed with G6976 compared with caffeine (Fig. 1A). When caffeine was washed out after 2 h and the fate of the cells driven into mitosis was followed, the level of apoptosis was found to be reduced and

FIGURE 2. Caffeine induces apoptosis in mitotic checkpoint-arrested cells. A, HeLa cells were treated with nocodazole for 16 h, and the mitotic population was then harvested by mechanical shake off and then either replated in the continued presence of nocodazole (noco), with or without addition of 5 mM caffeine (+caff), or washed to remove nocodazole and replated in fresh media (wash off). Micrographs were taken at 5 h after either nocodazole wash off or caffeine addition. Apoptotic cells are clearly discernible by the membrane blebbing. B, flow cytometric analysis of nocodazole-arrested HeLa cells treated with caffeine and harvested immediately (open bars) and after 2 h (black bars) and 5 h (gray bars). Percentages of cells in each indicated population from four separate experiments are shown. C, percentage of cells with <2n DNA content in nocodazole-arrested HeLa cultures (noco), treated with caffeine (+caff) or caffeine and the caspase inhibitor Z-VAD-fmk (+zVAD). All cultures were harvested at 5 h after caffeine addition. The data are from three separate experiments. D, nocodazole-arrested HeLa cells were either untreated (noco) or treated with caffeine (+caff) or caffeine and Z-VAD-fmk (+caff+zVAD) and harvested after 5 h. Lysates were immunoblotted for PARP and α-tubulin (α-tub). E, HeLa cells overexpressing Bcl-2 were arrested in mitosis with nocodazole. Cells were treated with caffeine, harvested immediately (open bars) at 2 h (black bars) and 5 h (gray bars) after caffeine treatment, and analyzed by flow cytometry for DNA content. The percentage of cells in each phase is presented. These data are from three independent experiments. F, HeLa cells transiently expressing GFP-Bax were arrested in mitosis with nocodazole, loaded with the intact mitochondria fluorophore TMRE, then treated with caffeine, and followed by time-lapse microscopy. The sequence shown is representative of the changes in GFP-Bax localization and timing of loss of TMRE fluorescence observed in caffeine-treated cultures.
membrane potential, indicated by loss of fluorescence of TMRE, a marker of intact mitochondria. This coincided with the relocation of cytoplasmic GFP-Bax, a pro-apoptotic Bcl-2 family protein, to a region that has previously been TMRE bright, presumably the mitochondria (Fig. 2F). These data demonstrate the involvement of the intrinsic apoptotic pathway in caffeine-induced cell death.

The effect of caffeine was dependent on cells being arrested in mitosis as caffeine did not induce apoptosis in asynchronously growing cells (supplemental Fig. S1), and this was confirmed by time-lapse microscopy. Interestingly, there was no evidence of Bid cleavage, which we have observed previously associated with apoptosis in HeLa cells (29), or destruction of Survivin which has been implicated in a mitotic death mechanism (32, 33) (supplemental Fig. S1).

Apoptosis Is Induced by Novel Targets of Caffeine—To determine whether the caffeine-induced apoptosis was because of the inhibition of known targets, inhibitors of ATM/ATR and PI 3-kinase wortmannin and LY294002, an inhibitor of the checkpoint kinases downstream of ATM/ATR Go6976, and agents that elevated intracellular cAMP levels, dibutyryl cAMP and forskolin, were added individually to nocodazole-arrested cells, and the fate of individual cells was followed by time-lapse microscopy. These experiments revealed that caffeine induced >40% of the mitotic checkpoint arrest cells to undergo apoptosis within 6 h of addition compared with 10% apoptosis in the nocodazole only controls or addition of the checkpoint inhibitor Go6976 (Fig. 3A). Wortmannin used at a concentration that inhibits ATM/ATR and PI 3-kinases (34) induced <20% apoptosis at 6 h after treatment. The more specific PI 3-kinase inhibitor LY294002 had a similar effect to wortmannin, whereas addition of dibutyryl cyclic AMP or forskolin, which activated cAMP-dependent protein kinase in this system, had no effect over nocodazole only treatment (data not shown).

The loss of either ATM by mutation or deletion in lymphoblastoid cell lines derived from ataxia telangiectasia patients or induced expression of dominant negative ATR in GM847 fibroblasts did not affect the level of apoptosis observed following nocodazole treatment when compared with the appropriated wild type or uninduced (data not shown). Specific knockdown of ATR in HeLa cells using an shRNA or inhibition of ATM using a specific small molecule inhibitor did not affect the ability of nocodazole to arrest cells in mitosis or to induce apoptosis (Fig. 3B). Combined ATR knockdown and ATM inhibition also failed to affect either mitotic arrest or induce apoptosis, whereas addition of caffeine initiated apoptosis even in the absence of signaling from these known caffeine targets. Together these data indicate that the known targets of caffeine inhibition do not contribute to the caffeine-induced apoptosis in mitotic checkpoint-arrested cells.

Caffeine Causes Reduction in Mitotic Markers—To define the mechanism by which caffeine induced apoptosis, we examined whether it caused cells to exit from mitosis. Caffeine reduced cyclin B1 levels by 50% within 5 h of addition, similar to the cyclin B1 levels in cells 5 h after release from nocodazole arrest (Fig. 4, A and B). This paralleled the reduction in cyclin B1/cdk1 histone kinase activity (Fig. 4C) and reduction of staining with the mitosis-specific marker MPM-2 (Fig. 4D). The reduction in cyclin B1 level was not a consequence of apoptotic destruction of cyclin B1, as addition of Z-VAD-fmk which inhibited PARP cleavage had no effect on cyclin B1 levels, whereas inhibition of the proteasome using MG132 effectively blocked cyclin B1 destruction (Fig. 4E). The ability of caffeine to induce PARP cleavage and cyclin B1 destruction was also observed in nocodazole-arrested HaCat cells (data not shown), and caffeine induced similar cyclin B1 destruction and PARP cleavage in taxol-arrested HeLa cells (Fig. 4F).

Premature Exit from Mitosis Does Not Trigger Apoptosis—The reduction in cyclin B1 and associated cdk1 kinase activity and the loss of mitosis-specific phosphorylations suggested that premature exit from mitosis may trigger apoptosis. To test this hypothesis, nocodazole-arrested cells were treated with the Aurora inhibitor ZM447439, which also causes cyclin B1 destruction and mitotic exit in microtubule poison-treated cells (12). Treatment with ZM447439 caused similar cyclin B1 destruction to caffeine but little PARP cleavage or activation of caspase 3 over the nocodazole control (Fig. 5A). Time-lapse microscopy revealed the cells exited mitosis and resumed a flattened, interphase morphology. Inhibiting caffeine-induced cyclin B1 destruction also failed to block apoptosis. Addition of the proteasome inhibitor MG132 to nocodazole-arrested caffeine-treated cells inhibited cyclin B1 destruction and maintained elevated levels of cyclin B1 kinase activity, but this did not affect PARP cleavage or caspase 3 activation by caffeine.
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FIGURE 4. Caffeine causes a loss of mitotic markers. A, nocodazole-arrested HeLa cells were treated with caffeine and harvested at the indicated times. The nocodazole control was washed to remove nocodazole, replated in fresh media, and harvested at the indicated times. Lysates were immunoblotted for PARP, cyclin B1 (Cyc B1), and α-tubulin (α-tub) levels. The cleaved PARP fragment is indicated. B, nocodazole-arrested HeLa cells were washed to remove nocodazole, replated in fresh media, and harvested at the indicated times. Lysates were immunoblotted for PARP, cyclin B1 (Cyc B1), and α-tubulin (α-tub) levels. C, cyclin B1/cdk1 was immunoprecipitated from nocodazole-arrested HeLa cells or from cells 5 h after washout (w/o) of nocodazole (noco) or caffeine addition (caff) and assayed for kinase activity. The data represent four separate determinations. D, cells from experiment similar to C were assayed for MPM-2 reactivity by flow cytometry. The data represent three separate experiments. E, nocodazole-arrested HeLa cells were treated with either caffeine (+caff), caffeine and Z-VAD-fmk (+caff+ZVAD), caffeine and the proteasome inhibitor MG132 (+caff+MG), or no addition (noco) and harvested at 5 h after drug addition. Lysates were immunoblotted for cyclin B1 and α-tubulin (α-tub). F, HeLa cells were arrested overnight in taxol and then treated without (taxol) or with caffeine (+caff). Cells were harvested after 5 h, and lysates were immunoblotted for the indicated proteins.

MG132 itself caused some PARP cleavage and caspase 3 activation, but the combined effect of MG132 and caffeine was greater than either drug alone. Addition of the cdk inhibitor roscovitine caused 15% of mitotic cells to resume an interphase phenotype but did not increase the level of apoptosis over nocodazole alone (Fig. 3). These experiments demonstrated that premature destruction of cyclin B1 and inactivation of cyclin B1 kinase activity was not required for caffeine-induced apoptosis.

Spindle Checkpoint Function Is Essential for Caffeine-induced Apoptosis—Caffeine-induced destruction of cyclin B1 was sensitive to inhibition by the proteasome inhibitor MG132 indicating that caffeine was possibly inactivating the spindle assembly checkpoint that blocks APC/C-mediated cyclin B1 destruction. MAD2 is loaded onto unattached kinetochores and is a marker of spindle assembly checkpoint activity (35). Staining for MAD2 revealed robust staining of paired MAD2 foci representing kinetochores on the sister chromatids in all cells with condensed chromatin in nocodazole-arrested cells and cells treated with caffeine, and with caffeine and Z-VAD-fmk to block apoptosis (Fig. 6). Nocodazole-arrested cells treated with the Aurora inhibitor ZM447439 displayed reduced MAD2 staining in cells with apparently condensed chromatin, consistent with the ability of the drug to overcome the spindle checkpoint (12), but this was never detected in caffeine-treated cells. Thus caffeine did not appear to inactivate the spindle checkpoint.

The role of the spindle checkpoint in caffeine-induced apoptosis was investigated. Cells were released from the nocodazole arrest with or without caffeine and followed for 5 h by time-lapse microscopy. Caffeine induced 50% apoptosis (Fig. 7A), and all of these cells died with a rounded mitotic morphology. Of the 30% of caffeine-treated cells that exited mitosis, 90% of these remained viable for the duration of the experiment, a similar percentage survival as control released cells, suggesting that only mitotic checkpoint-arrested cells were sensitive to caffeine-induced apoptosis. The Aurora inhibitor ZM447439 rapidly inactivates the spindle checkpoint in taxol-arrested cells (12) and was used to determine whether continued mitotic checkpoint signaling was required for caffeine-induced apoptosis. ZM447439-induced mitotic exit blocked caffeine-induced apoptosis. Time-lapse microscopy showed that addition of ZM447439 blocked caffeine-induced apoptosis (Fig. 7B). Biochemical analysis also revealed that mitotic exit induced by the Aurora inhibitor reduced PARP cleavage and caspase 3 activation induced by caffeine (Fig. 7C). These data indicate that the caffeine-induced apoptosis required continued signaling from an active spindle checkpoint.
Caffeine-induced Apoptosis Is Independent of Early G₁ Phase AKT Activity—In attempting to identify a mechanism by which caffeine was exerting its apoptotic effect on mitotic checkpoint-arrested cells, a number of anti-apoptotic signaling pathways potentially regulated by caffeine were examined. A previous report from this laboratory has demonstrated a small peak of Ras activity on exit from mitosis and in early G₁ phase (36), and the anti-apoptotic kinase AKT was reported to be activated during G₂/M (37). Caffeine is an inhibitor of PI 3-kinase (22), an upstream activator of AKT, suggesting that PI 3-kinase-AKT was potentially a target for caffeine. Investigation of synchronized HeLa cells revealed the appearance of the phosphorylated and activated AKT (pAKT) soon after exit from mitosis in early G₁ phase (Fig. 8, A and B). Little pAKT was detected in nocodazole-arrested cells, but removal of nocodazole and exit from the mitotic arrest also resulted in elevated levels of pAKT in early G₁ phase. Caffeine had no effect on the low levels of pAKT in nocodazole-arrested cells and only minimally reduced pAKT levels in nocodazole wash off cells experiments (data not shown). Complete inhibition of AKT activation with PI 3-kinase inhibitors LY294002 and wortmannin or the AKT-selective inhibitor SH5 only marginally increased apoptosis in cells released from nocodazole arrest (Fig. 8C and data not shown), demonstrating that inhibition of AKT activation did not contribute to the apoptosis observed with caffeine addition.

Caffeine Is an Inhibitor of PAK1 Activity—Another anti-apoptotic signal activated in mitotic checkpoint-arrested cells is the p21-activated kinase PAK1 (28). Caffeine addition resulted in a small reduction in PAK1 activity immunoprecipitated from caffeine-treated mitotic arrested cells (Fig. 9A). However, caffeine did inhibit PAK1 activity in vitro, with 40% inhibition observed at 1 mM and 80% inhibition at 5 mM caffeine (Fig. 9B). The inhibitory effect of caffeine appeared to be specific as 5 mM caffeine had little effect on cyclin B1/cdk1 activity in vitro (Fig. 9C), or when cyclin B1 destruction was blocked (Fig. 5B), and caffeine addition actually caused the rapid activation of ERK1/2 detected by increased phosphorylated ERK levels (supplemental Fig. S2). ERK activation did not contribute to caffeine-induced apoptosis as inhibition of ERK activation using the MEK
inhibitor U0126 did not reduce the level of apoptosis. The lack of effect of caffeine on PAK1 immunoprecipitated from caffeine-treated cells is because of caffeine being a competitive inhibitor that is removed during the washes of the immunoprecipitated protein.

To directly demonstrate a role for PAK1 in apoptosis in mitotic checkpoint-arrested cells, PAK1 was depleted using two separate siRNA (Fig. 10A). The knockdown was stable until at least 48 h after treatment (data not shown). Knockdown of PAK1 with either siRNA produced a small increase in apoptosis without further treatment. When cells depleted for PAK1 for 24 h were treated for a further 24 h with nocodazole, both PAK1 siRNAs significantly increase apoptosis over either lipofection or scrambled siRNA control, detected by reduction in the full-length PARP (Fig. 10A). To determine whether this apoptosis was specifically occurring in mitotic checkpoint-arrested cells, cells treated as above were followed by time-lapse microscopy. Control and PAK1-depleted cells passed through mitosis without a significant degree of apoptosis; however, when cells were treated with nocodazole, PAK1 depletion resulted in an increased rate of apoptosis that was higher in the siRNA number 1-treated cells, which produced greater knockdown of PAK1 levels.

**DISCUSSION**

Caffeine has been used as an inhibitor of a range of biological activities, including phosphodiesterases and PI 3-kinase related kinases, especially ATM and ATR. In this role it has been used to demonstrate that overcoming a G2 phase checkpoint response to DNA damage augments the cytotoxicity of the genotoxin (38). Using another inhibitor of checkpoint signalling, the chk1/2 inhibitor Go6976, we have found that although caffeine and Go6976 overcame an etoposide-imposed G2 checkpoint arrest with equal efficiency, caffeine treatment caused the rapid apoptosis of cells blocked in mitosis. Go6976-treated cells delayed in mitosis longer than caffeine-treated cultures, and up to 30% of these exited mitosis, suggesting that caffeine had another activity that initiated apoptosis in mitotic checkpoint-arrested cells. An alternative explanation is that
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A

PK1

+nocontro

PAK1

Lipon

PAK1#1

PAK1#2

B

% apoptotic

50

40

30

20

10

0

Time in mitosis (min)

0

100

200

300

400

500

FIGURE 10. Depletion of PAK1 increases apoptosis in mitotic checkpoint-arrested cells. A, HeLa cells were transfected with either lipofection reagent only (Lipo), scrambled siRNA (NS), or either PAK1 siRNA number 1 or 2 (PAK1#1 or PAK1#2). Cells were harvested after 24 h and immunoblotted for PAK1 (indicated by filled arrowhead). The strong band below the PAK1 doublet is a nonspecific band. Similarly treated HeLa cells were treated 24 h after transfection with or without nocodazole for a further 24 h, harvested, and immunoblotted for PARP as a marker of apoptosis. The full-length protein is indicated (open arrowhead). con, control; noco, untreated. B, HeLa cells transfected with either scrambled (N) or the PAK1-directed siRNAs (#1, #2) were treated with nocodazole 24 h after transfection and followed by time-lapse microscopy. The proportion of mitotic cells that undergo apoptosis was scored. Few transfected cells undergo apoptosis in the absence of nocodazole. More than 100 mitotic cells were followed for each condition.

Gö6976 can inactivate the spindle checkpoint permitting cells to exit mitosis and escape apoptosis. Although Gö6976 has been reported to inactivate the spindle checkpoint (39), we found no evidence that it could overcome the nocodazole-induced spindle checkpoint in HeLa cells.

Caffeine has two effects on mitotic checkpoint-arrested cells as follows. It can induce proteasome-mediated destruction of cyclin B1 and loss of MPM-2 reactivity, and it initiates apoptosis. The known targets of caffeine have been examined, and there is no evidence that caffeine inhibition of ATM, ATR, PI 3-kinase, or phosphodiesterase in the mitotic checkpoint-arrested cells produces apoptosis. Other potential targets such as Aurora B can be eliminated as caffeine has no effect on histone H3 Ser-10 phosphorylation, and although both caffeine and the Aurora inhibitor caused destruction of cyclin B1 and decreased MPM-2 reactivity, only the Aurora inhibitor caused loss of kinetochore localized MAD2 leading to mitotic exit and resumption of an interphase morphology (12, 40). The inactivation of the spindle checkpoint by inhibiting Aurora kinase activity or in cells after extended mitotic delay with anti-microtubule drugs, known as adaptation or “mitotic slippage,” normally results in aberrant cell division (reviewed in Ref. 13). However, caffeine does not promote mitotic exit, instead inducing apoptosis. Even when apoptosis is blocked by either caspase inhibitors or overexpression of Bcl-2, caffeine-treated cells maintain a rounded mitotic morphology and condensed chromatin despite signaling proteasome-mediated cyclin B1 destruction.5

It was surprising that inactivation of cyclin B1/cdk1 was not required for caffeine-induced apoptosis. Inhibiting mitotic CDK activity has been reported to promote apoptosis that was dependent on Survivin destruction, although this effect was only observed in taxol-arrested cells, and apoptosis was not observed until 12–24 h after treatment (32). Survivin destruction was also implicated in the caffeine-induced apoptosis after melphalan treatment observed only with nocodazole but not taxol co-treatment (33). The lack of reduction in Survivin levels during the time course of the experiments performed here and the ability of caffeine to induce rapid apoptosis in both taxol- and nocodazole-arrested cells clearly differentiate those mechanisms from the caffeine-induced apoptosis reported here.

The requirement for an intact spindle checkpoint in triggering apoptosis is clearly demonstrated by this study. We have confirmed that inhibiting the spindle checkpoint using Aurora inhibitors causes rapid exit from spindle checkpoint arrest but little apoptosis (12, 40), and inactivation of kinetochore components of the spindle checkpoint does not cause apoptosis in spindle checkpoint-arrested cells (6, 41–43). We have demonstrated that spindle checkpoint signaling is necessary for caffeine-induced apoptosis, as inactivating the checkpoint using either Aurora inhibitor or wash out of nocodazole inhibited apoptosis in cells that exited mitosis. There have been a number of reports demonstrating that an intact mitotic checkpoint is essential for apoptosis of cells that escape a G2 phase DNA damage checkpoint (39, 44) and in taxol-arrested cells (45). Thus the spindle checkpoint not only blocks cell cycle progression in response to mitotic defects, but it also regulates apoptotic signaling during mitotic arrest to ensure that cells that cannot fulfill the requirements for normal partitioning of the replicated genome are destroyed.

In this study, we have demonstrated that caffeine can initiate rapid apoptosis in spindle checkpoint-arrested cells in response to either microtubule poisons or DNA damage. The potential contribution of pathways known to be inhibited by caffeine have been systematically eliminated using other more specific inhibitors and using cell lines containing defective pathways. We have also demonstrated that AKT is activated soon after exit from mitosis. AKT has anti-apoptotic cell growth and cell cycle effects (Ref. 37 and references therein), but it does not contribute to the mitotic apoptosis induced by caffeine. However, its role in triggering apoptosis in early G1 phase cells that

5 B. Gabrielli, Y. Q. Chau, N. Giles, A. Harding, F. Stevens, and H. Beamish, unpublished observations.
have prematurely exited an aberrant mitosis warrants further investigation.

We have identified PAK1 as a novel target for caffeine inhibition that induces apoptosis. PAK1 is activated in mitotic checkpoint-arrested cells using either nocodazole or taxol, and inhibiting PAK1 activity by overexpressing a dominant negative mutant increased apoptosis, while expressing a constitutively activated mutant of PAK1 protected against apoptosis (28). We have shown that caffeine does inhibit PAK1 activity in vitro, the lack of in vivo effect is likely to reflect the reversible nature of the inhibition, and that PAK1 itself rather than other upstream factors is the target for caffeine inhibition. Specific depletion of PAK1 also increases apoptosis only in mitotic checkpoint-arrested cells. Taken together these observations point to PAK1 being at least a significant contributor to the caffeine-induced apoptosis, if not the sole target of caffeine-promoting spindle checkpoint-dependent apoptosis.

The target of PAK1 that transduces this anti-apoptotic activity was not directly identified in the report. However, it was demonstrated that caffeine triggers the intrinsic apoptotic pathway that targets the mitochondrial membrane. The Bcl-2 family proteins function in this pathway to either block or promote disruption of the mitochondrial membrane making this family of proteins excellent candidates as targets. PAK1 does phosphorylate the pro-apoptotic Bcl-2-related Bad protein, inhibiting its pro-apoptotic activity (46). PAK1 has also been demonstrated recently to phosphorylate BimL and its binding partner dynein light chain 1, which inhibits its pro-apoptotic function (47). The mechanism by which the spindle checkpoint regulates PAK1 activity and its apoptotic targets requires further investigation.

The ability of caffeine to induce apoptosis only in cells arrested by the spindle checkpoint suggests that this checkpoint not only blocks progression out of mitosis but also regulates a strong apoptotic response. The ability to destroy cells that fail to partition their genome correctly is a vital mechanism for ensuring genomic stability in a cell population. Spindle checkpoint failure therefore not only results in aneuploidy but also in failure of apoptotic signaling, thereby permitting the survival of the progeny and potentially the transmission of genomic instability. The ability of caffeine to initiate a very rapid and strong apoptotic response suggests that spindle checkpoint-arrested cells have a strong pro-apoptotic signal held in check by an equally strong anti-apoptotic signal to ensure that should mitotic failure occur cells will be rapidly targeted for destruction. This apoptotic signaling targets mitochondrial membrane integrity suggesting that the pro- and anti-apoptotic pathways involve BH3-only proteins. By defining the mechanism linking the spindle checkpoint with apoptotic signaling, it should be possible to increase the potency of anti-microtubule drugs. Potential target may be components of a signaling pathway such as PAK1.

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