Caffeine Inhibits Checkpoint Responses without Inhibiting the Ataxia-Telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) Protein Kinases*

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The ataxia-telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) kinases regulate cell cycle checkpoints by phosphorylating multiple substrates including the CHK1 and -2 protein kinases and p53. Caffeine has been widely used to study ATM and ATR signaling because it inhibits these kinases in vitro and overcomes cell cycle checkpoint responses in vivo. Thus, caffeine has been thought to overcome the checkpoint through its ability to prevent phosphorylation of ATM and ATR substrates. Surprisingly, I have found that multiple ATM-ATR substrates including CHK1 and -2 are hyperphosphorylated in cells treated with caffeine and genotoxic agents such as hydroxyurea or ionizing radiation. ATM autophosphorylation in cells is also increased when caffeine is used in combination with inhibitors of replication suggesting that ATM activity is not inhibited in vivo by caffeine. Furthermore, CHK1 hyperphosphorylation induced by caffeine in combination with hydroxyurea is ATR-dependent suggesting that ATR activity is stimulated by caffeine. Finally, the G2/M checkpoint in response to ionizing radiation or hydroxyurea is abrogated by caffeine treatment without a corresponding decrease in ATM-ATR-dependent signaling. This data suggests that although caffeine is an inhibitor of ATM-ATR kinase activity in vitro, it can block checkpoints without inhibiting ATM-ATR activation in vivo.

DNA damage-initiated cell cycle checkpoints are principally regulated by the ataxia-telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) protein kinases that belong to a family of phosphatidylinositol 3-kinase (PIK)-related kinases (1, 2). ATM and ATR share sequence homology and many of the same substrates. They differ with respect to which types of genotoxic stresses promote their activation. ATM primarily responds to agents such as ionizing radiation (IR) that cause DNA double strand breaks, whereas ATR signals in response to IR and many agents that cause bulky adducts on DNA or otherwise cause stalling of replication forks and generation of single-stranded DNA.

ATM and ATR are also activated by different methods. ATM activation requires autophosphorylation that results in the disruption of an ATM dimer (3). How autophosphorylation is triggered is still unknown. ATR is also capable of autophosphorylation, but it is unclear whether it forms inactive dimers and active monomers in cells. Activation of ATR signaling appears to be dependent on localization of ATR to regions of single-stranded DNA, which is accomplished through the function of the ATRIP regulatory subunit (4, 5).

ATM and ATR signal to induce cell cycle arrest in part by activating a signaling cascade involving two other protein kinases CHK2 and -1. CHK2 is phosphorylated by ATM on Thr-68, and this phosphorylation promotes CHK2 activation (6–8). CHK1 is activated by ATR-dependent phosphorylation of Ser-345 and Ser-317 (9–12). Active CHK1 and -2 phosphorylate the Cdc25C phosphatase on Ser-216 (7, 8, 13, 14). This phosphorylation decreases Cdc25C activity and sequesters it in the cytoplasm by creating a binding site for 14-3-3 proteins. Inactivation of Cdc25C prevents dephosphorylation and activation of cyclin B-cdc2, thereby inhibiting mitosis. Thus, ATM and ATR signal in response to DNA damage to activate the G2/M checkpoint through phosphorylation of CHK1-CHK2, which prevent activation of mitotic cyclin-dependent kinases. Other ATM and ATR substrates, such as p53, BRCA1, and SMC1, participate in multiple checkpoint responses (15–20).

Mutations in ATM cause the inherited cancer-predisposition syndrome ataxia-telangiectasia (21). Ataxia-telangiectasia cells are hypersensitive to IR and exhibit defects in G1, S, and G2/M checkpoints in response to IR. ATR is essential for cell viability and mouse development, and transient loss of ATR function causes extreme chromosomal instability (4, 22–24). Recently, a hypomorphic mutation in the ATR gene was linked to Seckel syndrome, which is characterized by growth retardation, microcephaly, and mental retardation (25).

Caffeine is a methylxanthine that has been used extensively to study ATM-ATR signaling and checkpoint responses. Caffeine is a relatively non-selective agent and causes many effects in cells. For example, caffeine inhibits the nucleotide exchange activity of RCC1 (26), alkaline phosphatase activity (27), and phosphodiesterase activity (28, 29).

One of the most studied actions of caffeine is its ability to cause radiosensitization and to inhibit cell cycle checkpoints. p53-deficient cells appear to be preferentially sensitized to IR by caffeine (30, 31). Caffeine has also been shown to inhibit multiple ATM-ATR-dependent cell cycle checkpoint responses including the G2/M- and S-phase checkpoints (32–34).

The ability of caffeine to produce phenotypes that are similar to ATM-ATR-deficient cellular phenotypes suggested that it might act to inhibit these kinases. Several reports have shown
that addition of caffeine to immunopurified ATM and ATR kinases prevents phosphorylation of substrates in vitro (35–38). ATM is inhibited by caffeine with an IC$_{50}$ of 0.2 mM, whereas ATR is inhibited with an IC$_{50}$ of 1.1 mM. The related PI3 kinase mTOR is also inhibited with an IC$_{50}$ of 0.4 mM, but DNA-PK and CHK1 are relatively resistant to caffeine with an IC$_{50}$ of greater than 10 mM (38). These concentrations for ATM and ATR inhibition in vitro are consistent with the concentrations of caffeine needed to cause radiosensitization and inhibition of checkpoint activation (10). Based on these findings, it was proposed that caffeine causes checkpoint inhibition in cells by inhibiting ATM and ATR. Consistent with this conclusion, the phosphorylation of CHK1, CHK2, and p53 can be reduced by caffeine treatment in some circumstances (11, 36, 38, 39).

Based on these results caffeine has been used extensively as an inhibitor of ATM and ATR to study loss of function phenotypes. Furthermore, the ability of caffeine to cause radiosensitization of cells has supported the idea that inhibitors of checkpoints generally and ATM-ATR specifically may be useful clinically as radio- and chemosensitizing agents (40).

In the course of studying ATR-dependent signaling events, I observed that some ATM-ATR substrates such as p53 and CHK2 were hyperphosphorylated in the absence of ATR. I suspected this was due to a feedback mechanism involving activation of ATM in these cells. To confirm this hypothesis I attempted to use caffeine to inhibit both ATM and ATR. Surprisingly, I found that treatment of cells with caffeine does not prevent ATM-ATR-dependent phosphorylation of CHK1 and -2 yet still inhibits checkpoints initiated by IR and hydroxyurea. Thus, at least in some cellular contexts caffeine prevents check-point responses without directly inhibiting the ATM and ATR kinases.

EXPERIMENTAL PROCEDURES

Cell Culture—HCT116 cells were maintained in McCoy’s medium supplemented with 10% fetal bovine serum. hTERT-RPE1 cells were obtained from Clontech and maintained in Dulbecco’s modified Eagle’s medium/F12 medium supplemented with 0.348% sodium bicarbonate and 10% fetal bovine serum. ATR$^{+/−}$ cells were maintained in McCoy’s medium supplemented with 10% fetal bovine serum. M059J and M059K cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Deletion of the ATR gene from the ATR$^{+/−}$ cells was performed as described previously (4).

Drug Treatment and DNA Damage—Caffeine was purchased from Sigma (no. C8960). It was either dissolved in growth medium at a stock concentration of 80 mM just prior to use or dissolved in water at a concentration of 200 mM. Cells were pretreated with caffeine for 30 min prior to exposer to any genotoxic agents. Hydroxyurea was purchased from Sigma, dissolved in water at 1 mM, and stored frozen. Cells were treated with ionizing radiation from a 137Cs source at a dose rate of ~1.8 grays/min. UV was administered with a Stratalinker (Stratagene) after cells were washed one time with PBS. Aprotinin was purchased from Sigma, dissolved in MeSO at 100 mg/ml, and stored at −80 degrees.

Western Blotting—Cells were lysed in 50 mM Tris (pH 8.0), 200 mM NaCl, 1% Igepal CA-630 supplemented with 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1 mM NaF, 20 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Lysates were cleared by centrifugation prior to protein concentration (Bio-Rad). Anti-CHK1 P-S345, p53 P-S15, and anti-CHK2 P-T68 antibodies were purchased from Cell Signaling. Anti-ATM P-S1861 antibodies were purchased from Rockland. Anti-ATM antibodies were purchased from Novus. Anti-CHK1 antibody was purchased from Santa Cruz. Anti-P-HAX antibody was purchased from Cell Signaling Solutions.

Kinase Assay—ATM and ATR kinase assays were performed using recombinant FLAG-ATM or -ATR produced in HEK 293T cells. The FLAG-ATM and -ATR kinases were purified and assayed using a fragment of BRCA1 protein as described previously (17).

Checkpoint Assay—Untreated or treated cells were harvested and fixed in 70% ethanol. The fixed cells were washed twice with PBS and permeabilized with 0.25% Triton X-100 in PBS on ice for 15 min.

RESULTS

Loss of ATR Promotes Double Strand Breaks and Hyperphosphorylation of p53 and CHK2 in Response to Stalled Replication Forks—Hydroxyurea-treated cells arrest with stalled replication forks in S-phase due to the inhibition of ribonucleotide reductase. Stalling of replication forks promotes activation of an ATR-dependent checkpoint response that slows DNA replication, stabilizes replication forks, and prevents entry into mitosis (41).

To examine this checkpoint in more detail I analyzed the phosphorylation of ATR substrates such as CHK1 and p53 in response to hydroxyurea treatment in cells that had been engineered to contain a conditional allele of ATR (4). These ATR$^{+/−}$ cells can be induced to lose the ATR gene by introducing the CRE recombinase to delete ATR or encoding GFP as a control were treated with (A) 1 mM hydroxyurea (HU) for the indicated times, (B) 0 or 1 mM hydroxyurea for 6 h, or (C) IR (5 grays) or UV (50 J/m²) and incubated for the indicated times or treated with 1 mM hydroxyurea for the indicated times. Cell lysates were prepared, separated by SDS-PAGE, and blotted with the indicated antibodies. Permeabilized cells were rinsed in PBS-containing 1% bovine serum albumin and then stained with anti-phospho-S10 histone H3 antibody for 1.5 h at room temperature. Cells were washed twice with PBS-containing 1% bovine serum albumin and then stained with fluorescein isothiocyanate-conjugated donkey anti-rabbit secondary antibody (Jackson Immunologicals). Cells were washed three times in PBS and then stained with propidium iodide (25 µg/ml) in the presence of 0.1 mg/ml RNase. Flow cytometry was performed on a BD Biosciences FACSCalibur flow cytometer, and the percentage of mitotic cells was determined as the percentage of those cells that were fluorescein isothiocyanate-positive and contained 4N DNA content.

FIG. 1. Loss of ATR promotes activation of ATM and hyperphosphorylation of p53, CHK2, and H2AX. ATR$^{+/−}$ or ATR$^{+/−}$/H11011 cells infected with adenovirus either encoding the CRE recombinase to delete ATR or encoding GFP as a control were treated with (A) 1 mM hydroxyurea (HU) for the indicated times, (B) 0 or 1 mM hydroxyurea for 6 h, or (C) IR (5 grays) or UV (50 J/m²) and incubated for the indicated times or treated with 1 mM hydroxyurea for the indicated times. Cell lysates were prepared, separated by SDS-PAGE, and blotted with the indicated antibodies.

CHK2 is phosphorylated on Thr-68 in an ATM-dependent manner in response to DNA damage such as double strand
Breaks (6). Loss of ATR has been reported to induce chromosomal abnormalities (22, 42). Therefore I suspected that the increased CHK2 and p53 phosphorylation in ATR-deficient cells was due to an increase in double strand breaks that might form at stalled replication forks when ATR is absent. Consistent with this hypothesis, H2AX phosphorylation was increased in ATR-deficient cells both before and after treatment with hydroxyurea (Fig. 1B). Furthermore, ATM phosphorylation on Ser-1981 is also increased both before and after treatment of ATR-deficient cells with DNA-damaging agents or replication inhibitors (Fig. 1C). Ser-1981 is an autophosphorylation site on ATM that promotes its activity by disrupting the inactive ATM dimer (3). Therefore, it is likely that the increase in CHK2 and p53 phosphorylation that I observed in the hydroxyurea-treated ATR-deficient cells was due to the formation of double strand breaks and activation of the ATM pathway. Loss of ATR increases the amount of double strand breaks and activates ATM in cells both before and after treatment with genotoxic agents.

Caffeine Promotes Hyperphosphorylation of ATM-ATR Substrates—To confirm that ATM was indeed required for the p53 and CHK2 phosphorylation in the absence of ATR, I attempted to use caffeine to inhibit both kinases. Caffeine has been reported to inhibit the ATM kinase at an IC50 of 0.2 mM and the ATR kinase at an IC50 of 1.1 mM (35–38). Furthermore, the intracellular concentration of caffeine quickly equilibrates with the extracellular concentration (43). Therefore treatment of ATR−/− cells with concentrations of caffeine between 2 and 16 mM should inhibit both ATM and ATR kinases. I pretreated HCT116 cells with caffeine, added hydroxyurea, and then analyzed the phosphorylation of multiple ATM and ATR substrates by immunoblotting with antiphosphopeptide-specific antibodies or by analyzing gel mobility shifts. I expected a dose-dependent decrease in substrate phosphorylation, but I observed a dose-dependent increase of CHK1, replication protein A, CHK2, and p53 phosphorylation (Fig. 2A).

Hyperphosphorylation of these ATM-ATR substrates might be expected if caffeine transiently inhibited ATM and ATR but then was degraded and no longer present. To test this I first checked whether caffeine treatment inhibited CHK1 phosphorylation at earlier times after hydroxyurea exposure. There was a similar dose-dependent increase in CHK1 phosphorylation at 2 h after hydroxyurea exposure (Fig. 2B). Addition of fresh caffeine and growth medium every hour during the hydroxyurea treatment also failed to inhibit CHK1 phosphorylation (data not shown).

The ATM and ATR kinases belong to the PIK class of kinases that can be inhibited by wortmannin. I confirmed that the caffeine-induced hyperphosphorylation of CHK1 and replication protein A was at least partially sensitive to wortmannin suggesting the involvement of a PIK kinase (Fig. 2C). The dose of wortmannin (20 μM) used is expected to efficiently inhibit ATM and DNA-PK while partially inhibiting ATR (44). DNA-PK has been reported to phosphorylate some ATR and ATM substrates and is wortmannin-sensitive, so I examined whether the caffeine-stimulated hyperphosphorylation of CHK1 was dependent on DNA-PK. I saw no difference between the ability of caffeine to affect CHK1 phosphorylation after hydroxyurea treatment in DNA-PK-proficient or -deficient cells (Fig. 3A) suggesting that DNA-PK is not involved. These cells also express reduced levels of ATM and harbor an ATM mutation (45) suggesting that ATM is also not involved in the CHK1 phosphorylation.

The caffeine-induced hyperphosphorylation of ATM-ATR substrates is not limited to cells treated with hydroxyurea. I used aphidicolin as another agent that can block replication fork progression and found that CHK1 phosphorylation at 6 h after aphidicolin addition is also stimulated by caffeine (Fig. 3B). Furthermore, I directly examined ATM autophosphorylation using the phospho-S1981 ATM antibody and found that
caffeine promoted phosphorylation on this site in the presence of aphidicolin (Fig. 3B).

**ATM and ATR Signaling Is Not Inhibited in Vivo by Caffeine**—HCT116 cells are a colon carcinoma cell line that harbors many mutations. To ensure that caffeine’s effect on ATM-ATR-dependent phosphorylation events was not limited to this tumor cell line I analyzed its effects in telomerase-immortalized human retinal pigment epithelial cells (hTERT-RPE1). Both ATM autophosphorylation as well as phosphorylation of the ATM substrate CHK2 are increased when caffeine is added to hTERT-RPE1 cells in addition to hydroxyurea (Fig. 4A). The increase in phosphorylation of Ser-1981 on ATM caused by adding caffeine and inhibitors of replication suggests that caffeine may promote the activation of ATM in these cells. As ATM activation is normally a response to double strand breaks I tested whether caffeine had an effect on IR-induced ATM activation. I found that ATM autophosphorylation was already maximal with 5 grays of IR and that caffeine did not decrease this phosphorylation. Furthermore, caffeine consistently caused a slight increase in CHK2 phosphorylation on Thr-68 and also increased CHK1 phosphorylation in a dose-dependent manner after ionizing radiation treatment (Fig. 4A). This data is inconsistent with an inhibitory function of caffeine on ATM in vitro and suggests that caffeine may promote the activation of ATM in cells. It is interesting to note that the level of ATM autophosphorylation did not strictly correlate with the amount of CHK2 Thr-68 phosphorylation. For example, hydroxyurea treatment in the presence of 4 or 8 mM caffeine caused nearly the same amount of ATM autophosphorylation on Ser-1981 as did IR treatment, but the level of CHK2 Thr-68 phosphorylation was much less. This observation suggests that there must be additional mechanisms regulating CHK2 phosphorylation besides the disruption of the ATM dimer by Ser-1981 autophosphorylation.

I have observed a requirement for ATR for CHK1 phosphorylation in response to UV, hydroxyurea, and IR in HCT116 cells using the ATR<sup>fl</sup>/Cre<sup>+/−</sup> system (see Fig. 1) (data not shown), yet caffeine treatment stimulates CHK1 phosphorylation in response to hydroxyurea and IR suggesting it may function to activate ATR signaling. If caffeine does function as an activator of ATR then CHK1 hyperphosphorylation in response to caffeine treatment should be dependent on ATR. I tested this by treating ATR<sup>fl</sup>/Cre<sup>+/−</sup> cells with hydroxyurea in the presence or absence of caffeine. The control AdGFP-infected cells containing ATR showed increased CHK1 phosphorylation at the 6-h time point after hydroxyurea treatment when caffeine was present and showed no evidence of caffeine inhibition at earlier time points. However, this caffeine-stimulated CHK1 phosphorylation was not observed in the AdCRE-infected cells, which have lost the ATR gene even though ATM was activated based on the presence of Ser-1981 phosphorylation (Fig. 4B). Thus, the caffeine-induced hyperphosphorylation of CHK1 is ATR-dependent. This data also shows that Ser-345 of CHK1 is not capable of being phosphorylated by active ATM in the absence of ATR.

**Caffeine Is an Inhibitor of ATM-ATR in Vitro and an Inhibitor of the G2/M Checkpoint in Vivo**—Previous publications have documented that caffeine is an inhibitor of the ATM and ATR kinases in vitro and an inhibitor of checkpoint responses in cells. However, my data indicated that at least in the cell types and conditions that I examined, caffeine did not inhibit ATM or ATR. This could be explained if the caffeine was inactivated, metabolized, or unable to enter the cells in the growth conditions that I employed. Therefore, I tested whether the caffeine would inhibit ATM and ATR in vitro and whether it would inhibit the checkpoint in the same cells in which a hyperactivation of ATM-ATR signaling is observed. Consistent with previous publications, ATM kinase activity was inhibited at low (1 mM) concentrations of caffeine in vitro. Both autophosphorylation and trans-phosphorylation of the BRCA1 substrate were inhibited (Fig. 5A) in kinase assays. I also observed inhibition of the ATR kinase at slightly higher concentrations of caffeine consistent with previously reported results (data not shown).

To test whether caffeine could inhibit the G2/M checkpoint I incubated HCT116 or hTERT-RPE1 cells with caffeine, exposed them to IR or hydroxyurea, and then analyzed the percentage of mitotic cells. I found that caffeine efficiently prevented the G2 arrest normally observed in cells treated with either IR or hydroxyurea consistent with previous observations of the inhibitory effect of caffeine on checkpoints (Fig. 5, B and C). Caffeine efficiently overcame the IR- and hydroxyurea-induced G2/M checkpoint at all time points examined. Furthermore, it inhibited the checkpoint at concentrations and time
points at which there is hyperphosphorylation of ATM-ATR substrates in these cells.

DISCUSSION

The ATM and ATR kinases are master regulators of the DNA damage-induced cell cycle checkpoints. They function in largely parallel pathways to induce the phosphorylation and activation of multiple proteins including CHK1-CHK2 and p53. I have now shown that loss of ATR from cells causes activation of ATM probably as a result of increased double strand breaks that form during DNA replication. Therefore, loss of ATR actually increases phosphorylation of some ATM-ATR substrates such as p53 in response to agents such as hydroxyurea and ultraviolet light that are normally thought to promote checkpoint responses primarily through ATR activation. CHK1 phosphorylation, however, remains ATR-dependent suggesting that ATM can substitute for ATR to phosphorylate only a subset of ATR substrates.

Caffeine has been used extensively as an inhibitor of checkpoint responses. Addition of caffeine to cells will overcome S-phase and G2/M checkpoint responses to multiple genotoxic stresses including ionizing radiation and hydroxyurea. Although its mechanism of action in cells is not clear, caffeine does inhibit the kinase activity of ATM and ATR in vitro (35–38). My results using in vitro kinase assays to measure ATM and ATR sensitivity to caffeine are consistent with the previously reported inhibitory effect of caffeine on these kinases (35–38). ATM is inhibited at lower concentrations than ATR, and both kinases are inhibited in the low millimolar concentrations in vitro. Furthermore, I have confirmed previous observations that low millimolar concentrations of caffeine override cell cycle checkpoint responses to both IR and hydroxyurea. ATM and ATR signaling is essential for these checkpoint responses in cells. Thus, it seems logical to expect that the mode of action of caffeine in regard to checkpoint inhibition in cells is through its ability to inhibit ATM and ATR kinase activity.

Paradoxically, I have found that multiple ATM and ATR substrates are actually hyperphosphorylated when cells are treated with caffeine in the presence of either replication inhibitors or IR. CHK2 and -1 are the proximal downstream substrates of ATM and ATR, respectively, that mediate the G2/M checkpoint response. Phosphorylation of these proteins promotes their activation (6–12). If caffeine acts to inhibit cell cycle checkpoints by inhibiting ATM and ATR then I would expect the phosphorylation of CHK2 and -1 on their ATM-ATR-dependent phosphorylation sites to be decreased by caffeine treatment. I observed the opposite effect. Phosphorylation of CHK1 and -2 was increased in a dose-dependent manner by caffeine treatment in cells that were also treated with hydroxyurea, IR or hydroxyurea for the indicated times. The percentage of mitotic cells is determined by phosphohistone staining.

There is some evidence in the literature that supports the idea that caffeine does not inhibit ATM-ATR in vivo. Pretreatment of cells with caffeine failed to inhibit ATM activation by...
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IR when measured in immunoprecipitation-kinase reactions (38). This data was interpreted to mean that during the immunoprecipitation step the caffeine was washed away from ATM and that the mechanism of ATM activation in vitro is insensitive to caffeine. It is now known that ATM activation requires ATM autophosphorylation on Ser-1981. Furthermore, ATM is activated in vitro by autophosphorylation since preincubation of ATM with ATP stimulates its activity (46). Caffeine is unable to inhibit this activation step, and pretreatment of ATM with caffeine actually slightly increases its activity (46). These data are consistent with my conclusions that ATM autophosphorylation and activity are increased in cells treated with caffeine.

It is possible for caffeine to act as an inhibitor of ATM and ATR kinase activities in cells and still yield the same experimental results. Deletion of the ATR gene stimulates hyperphosphorylation of some ATM-ATR substrates such as p53 in response to replication inhibitors or other DNA-damaging agents (Fig. 1). This is likely a result of ATM activation due to increased double strand breaks forming at sites of stalled replication forks because of the lack of an ATR-dependent signal to stabilize these stalled forks. I could hypothesize that the caffeine-dependent hyperphosphorylation of checkpoint-signaling proteins in response to hydroxyurea treatment is acting through a similar mechanism of ATR inhibition leading to increased double strand breaks and activation of another PIK kinase. I have observed increases in H2AX phosphorylation after caffeine and hydroxyurea treatment, which would be consistent with this model (data not shown).

However, there are several arguments against this model. First, since ATM is inhibited at lower concentrations than ATR in vitro, the caffeine + hydroxyurea-activated PIK kinase should not be ATM, and I have shown it is not DNA-PK. An unidentified, caffeine-insensitive, wortmannin-sensitive kinase capable of phosphorylating ATM-ATR substrates would need to be invoked. Second, CHK1 phosphorylation on Ser-345 is increased by combined caffeine and hydroxyurea treatment but is completely absent by the same treatment in cells that have lost the ATR gene. Thus, if ATR is inhibited and not responsible for the hyperphosphorylation of CHK1 in response to hydroxyurea combined with caffeine, then the responsible kinase must depend on the presence of the inhibited ATR kinase to recognize CHK1. Third, Ser-1981 of ATM has only been shown to be phosphorylated by ATM itself (3). Phosphorylation of this site activates ATM by disrupting the inactive ATM dimer. Since I observe an increase in Ser-1981 phosphorylation in the presence of caffeine, it seems highly unlikely that it is not reflective of increased ATM kinase activity.

A second model that might explain the results is if caffeine transiently inhibits ATM-ATR in such a manner as to induce irreparable DNA damage that is then sensed by ATM and ATR proteins that are not inhibited. This situation could arise in two ways. First, if only a portion of ATR or ATM molecules that are required to prevent this DNA damage are accessible to caffeine then the remaining, uninhibited molecules could become superactivated. Such a scenario seems unlikely as there is no reason to believe caffeine would be selective for only a portion of ATM-ATR molecules in cells. Second, caffeine could transiently inhibit most of ATM-ATR molecules but then be degraded/metabolized so that it is no longer present or otherwise has its inhibitory effect inactivated. This scenario also seems unlikely as increasing the dosage of caffeine actually increases apparent ATM-ATR activation, and re-addition of fresh caffeine at 1-h intervals does not prevent the hyperphosphorylation of substrates (data not shown).

Even if one of these mechanisms is functioning they do not explain why the checkpoint is still inactivated by caffeine even when multiple ATM-ATR substrates including CHK1 and -2 are hyperphosphorylated. This observation argues strongly that caffeine is inhibiting the G2/M checkpoint somewhere downstream of the essential ATM-ATR-dependent phosphorylation of CHK1 and -2. Phosphorylation of CHK1 and -2 is required for their activation and is often used as an indicator of activity. However, it is possible that phosphorylation is not sufficient for activation and that they remain inhibited in the presence of caffeine. CHK2 has been reported to be inactive in caffeine-treated cells (37), and caffeine abrogation of the checkpoint appears to be upstream of the essential CHK1- and -2-dependent inactivation of Cdc25C (30, 47). Direct inactivation of CHK1 and -2 by caffeine would be expected to yield similar phenotypic consequences as ATM and ATR inactivation. However, CHK1 and -2 are not inhibited by caffeine at low millimolar concentrations in vitro (37, 38). Therefore, the mechanism of action of caffeine to overcome DNA damage-induced checkpoints in the HCT116 and hTERT-RPE1 cells remains unresolved.

These results raise a number of important issues. First, the use of caffeine to study ATM- and ATR-dependent signaling in cells is likely to be problematic without a clearer understanding of its effects. Second, caffeine does inhibit cell cycle arrest in response to many types of genotoxic stress including DNA damage or replication inhibition. If it is not inhibiting cell cycle arrest by inhibiting ATM-ATR signaling, then what are the relevant targets of caffeine in this response? Third, caffeine augments the ATM-ATR-dependent signaling response to replication inhibitors. Is this due to an indirect or direct effect of caffeine on the ATM and ATR kinases? Caffeine can intercalate into DNA and interfere with DNA repair activities by preventing the binding of repair enzymes (48–50). Therefore, caffeine may be preventing the repair of hydroxyurea-, aphidicolin-, and IR-induced DNA damage and thereby promoting hyperactivation of ATM and ATR. Caffeine has multiple known targets, and it is not clear how many other unknown targets are being affected. Thus, a caffeine-insensitive ATM or ATR mutant would be an essential reagent to validate the specificity of the effects of caffeine on these kinases in cells.

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