Caffeine Inhibits Checkpoint Responses without Inhibiting ATM and ATR

David Cortez

Vanderbilt University

Department of Biochemistry

Nashville, TN 37232

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Corresponding author:

David Cortez

613 Light Hall

Vanderbilt University

Nashville, TN 37232

Phone: (615) 322-8547

Fax: (615) 322-8547

Email: david.cortez@vanderbilt.edu
Summary

The ATM and ATR kinases regulate cell cycle checkpoints by phosphorylating multiple substrates including the Chk1/2 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, we have found that multiple ATM/ATR substrates including Chk1 and Chk2 are hyper-phosphorylated in cells treated with caffeine and genotoxic agents such as hydroxyurea or ionizing radiation. ATM auto-phosphorylation 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 hyper-phosphorylation 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 while caffeine is an inhibitor of ATM/ATR kinase activity in vitro, it can block checkpoints without inhibiting ATM/ATR activation in vivo.

Introduction

DNA damage-initiated cell cycle checkpoints are principally regulated by the ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3 related) protein kinases that belong
to a family of phosphatidylinositol 3-kinase related kinases (PIK) (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 that cause DNA double strand breaks such as ionizing radiation (IR) while 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 Chk1. Chk2 is phosphorylated by ATM on T68 and this phosphorylation promotes Chk2 activation (6-8). Chk1 is activated by ATR-dependent phosphorylation of S345 and S317 (9-12). Active Chk1 and Chk2 phosphorylate the Cdc25C phosphatase on S216 (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 cyclinB-cdc2, thereby inhibiting mitosis. Thus, ATM and ATR signal in response to DNA damage to activate the G2/M checkpoint through phosphorylation of Chk1/2 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 (A-T) (21). A-T 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\textsubscript{50} of 0.2mM while ATR is inhibited with an IC\textsubscript{50} of 1.1mM. The related PIK kinase mTor is also inhibited with IC\textsubscript{50} of 0.4 mM but DNA-PK and Chk1 are relatively resistant to caffeine with an IC\textsubscript{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 checkpoints in vivo. 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 chemo-sensitizing agents (40).

In the course of studying ATR-dependent signaling events, we observed that some ATM/ATR-substrates such as p53 and Chk2 were hyper-phosphorylated in the absence of ATR. We suspected this was due to a feedback mechanism involving activation of ATM in these cells. To confirm this hypothesis we attempted to use caffeine to inhibit both ATM and ATR. Surprisingly, we found that treatment of cells with caffeine does not prevent ATM/ATR-dependent phosphorylation of Chk1 and Chk2 yet still inhibits checkpoints initiated by IR and HU. Thus, at least in some cellular contexts caffeine prevents checkpoint responses without directly inhibiting the ATM and ATR kinases.

**Experimental Procedures**

**Cell culture**

HCT116 cells were maintained in McCoy’s media supplemented with 10% fetal bovine serum (FBS). hTERT-RPE cells were obtained from Clontech and maintained in DMEM/F12 media supplemented with 0.348% sodium bicarbonate and 10%FBS. ATR\textsuperscript{floX/-} cells were maintained in McCoy’s media supplemented with 10%FBS. M059J and M059K cells were
maintained in DMEM supplemented with 10%FBS. Deletion of the ATR gene from the ATR\textsuperscript{flox/-} cells was performed as described previously (4).

Drug treatment and DNA damage

Caffeine was purchased from Sigma (#C8960). It was dissolved in either growth media at a stock concentration of 80mM just prior to use or dissolved in water at a concentration of 200mM. Cells were pre-treated with caffeine for 30 minutes prior to exposing them to any genotoxic agents. Hydroxyurea was purchased from Sigma dissolved in water at 1M and stored frozen. Cells were treated with ionizing radiation from a \textsuperscript{137}Cs source at a dose rate of approximately 1.8 Gy/min. UV was administered with a Stratalinker (Stratagene) after cells were washed one time with PBS. Aphidicolin was purchased from Sigma, dissolved in DMSO at 5mg/ml and stored at –80 degrees.

Western blotting

Cells were lysed in 50mM Tris pH=8.0, 200mM NaCl, 1% Igepal CA-630 supplemented with 5μg/ml aprotinin, 5μg/ml leupeptin, 1mM NaF, 20mM \textsuperscript{2}-glycerophosphate, 1mM sodium vanadate, 1 mM DTT, and 1mM PMSF. Lysates were cleared by centrifugation prior to protein concentration determination (BioRad). Anti-Chk1 P-S345, p53 P-S15 and anti-Chk2 P-T68 antibodies were purchased from Cell Signaling. Anti-ATM P-S1981 antibodies were purchased from Rockland. Anti-ATM antibodies were purchased from Novus. Anti-Chk1 antibody was purchased from Santa Cruz. Anti-P-H2AX antibody was purchased from UBI.
**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 previously described (17).

**Checkpoint assay**

Untreated or treated cells were harvested and fixed in 70% ethanol. The fixed cells were washed twice with PBS and permeabalized with 0.25% Triton X-100 in PBS on ice for 15 minutes. Permeabalized cells were rinsed in 1%BSA/PBS then stained with anti-phospho-S10 histone H3 antibody for 1.5 hours at room temperature. Cells were washed twice with 1%BSA/PBS then stained with FITC-conjugated donkey anti-rabbit secondary antibody (Jackson Immunologicals). Cells were washed three times in PBS then stained with propidium iodide (25ug/ml) in the presence of 0.1mg/ml RNAse. Flow cytometry was performed on a Becton Dickinson FACSCalibur flow cytometer and the percentage of mitotic cells was determined as those cells that were FITC-positive and contained 4N DNA content.

**Results**

*Loss of ATR promotes double strand breaks and hyper-phosphorylation of p53 and Chk2 in response to stalled replication forks.*

Cells treated with hydroxyurea 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 we analyzed the phosphorylation of ATR substrates such as Chk1 and p53 in response to HU treatment in cells that had been engineered to contain a conditional allele of ATR (4). These ATR\textsuperscript{flx/c} cells can be induced to lose the ATR gene by introducing the Cre recombinase by adenoviral infection (AdCRE). Loss of ATR prevented the HU-induced phosphorylation of Chk1 on S345 which is required for Chk1 activation (Fig 1A). This data is consistent with a requirement for ATR for Chk1 phosphorylation and activation. However, another putative ATR substrate p53 was actually hyper-phosphorylated in response to HU treatment in cells lacking ATR (Fig. 1A). p53 phosphorylation was also slightly elevated in untreated ATR-deficient cells compared to either wild-type cells treated with AdCRE or ATR\textsuperscript{flx/c} cells treated with AdGFP. This hyper-phosphorylation correlated with an increase in phosphorylation of Chk2 as measured by a gel mobility shift.

Chk2 is phosphorylated on T68 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 we 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 HU (Fig 1B). Furthermore, ATM phosphorylation on S1981 is also increased both before and after treatment of ATR-deficient cells with DNA damaging agents or replication inhibitors (Fig. 1C). S1981 is an autophosphorylation site on ATM which promotes its activity by
disrupting the inactive ATM dimer (3). Therefore, it is likely that the increase in Chk2 and p53 phosphorylation that we observed in the HU-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 hyper-phosphorylation of ATM/ATR substrates

To confirm that ATM was indeed required for the p53 and Chk2 phosphorylation in the absence of ATR, we attempted to use caffeine to inhibit both kinases. Caffeine has been reported to inhibit the ATM kinase at an IC$_{50}$ of 0.2mM and the ATR kinase at an IC$_{50}$ of 1.1mM (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 16mM should inhibit both ATM and ATR kinases. We pre-treated HCT116 cells with caffeine, added hydroxyurea, then analyzed the phosphorylation of multiple ATM and ATR substrates by immunoblotting with anti-phospho-peptide specific antibodies or by analyzing gel mobility shifts. We expected a dose-dependent decrease in substrate phosphorylation but we observed a dose-dependent increase of Chk1, RPA, Chk2, and p53 phosphorylation (Fig. 2A).

Hyper-phosphorylation 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 we first checked whether caffeine treatment inhibited Chk1 phosphorylation at earlier times after HU exposure. There was a similar dose-dependent increase in Chk1 phosphorylation at 2
hours after HU exposure (Fig 2B). Addition of fresh caffeine and growth media every hour during the HU 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. We confirmed that the caffeine-induced hyper-phosphorylation of Chk1 and RPA 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 we examined whether the caffeine-stimulated hyper-phosphorylation of Chk1 was dependent on DNA-PK. We saw no difference between the ability of caffeine to affect Chk1 phosphorylation after HU 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 hyper-phosphorylation of ATM/ATR substrates is not limited to cells treated with hydroxyurea. We used aphidicolin as another agent that can block replication fork progression and found that Chk1 phosphorylation at 6h after aphidicolin addition is also stimulated by caffeine (Fig 3B). Furthermore, we 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 are not inhibited in vivo by caffeine.**

HCT116 cells are a colon carcinoma cell line that harbor many mutations. To ensure that caffeine’s effect on ATM/ATR-dependent phosphorylation events was not limited to this
tumor cell line we analyzed its effects in telomerase immortalized human retinal pigment epithelial cells (hTERT-RPE1) cells. 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 S1981 on ATM caused by adding caffeine and inhibitors of replication suggests that caffeine may promote the activation of ATM in these cells. Since ATM activation is normally a response to double strand breaks we tested whether caffeine had an effect on IR-induced ATM activation. We found that ATM auto-phosphorylation was already maximal with 5Gy of IR and caffeine did not decrease this phosphorylation. Furthermore, caffeine consistently caused a slight increase in Chk2 phosphorylation on T68 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 vivo 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 T68 phosphorylation. For example, HU treatment in the presence of 4 or 8mM caffeine caused nearly the same amount of ATM autophosphorylation on S1981 as did IR treatment, but the level of Chk2 T68 phosphorylation was much less. This observation suggests that there must be additional mechanisms regulating Chk2 phosphorylation besides the disruption of the ATM dimer by S1981 auto-phosphorylation.

We have observed a requirement for ATR for Chk1 phosphorylation in response to UV, HU, and IR in HCT116 cells using the ATR<sup>flox/−</sup> system (see figure 1 and data not shown). Yet caffeine treatment stimulates Chk1 phosphorylation in response to HU and IR suggesting it may function to activate ATR signaling. If caffeine does function as an activator of ATR then
Chk1 hyper-phosphorylation in response to caffeine treatment should be dependent on ATR. We tested this by treating ATR$^{\text{lox/}}$ cells with hydroxyurea in the presence or absence of caffeine. The control AdGFP infected cells containing ATR showed increased Chk1 phosphorylation at the 6-hour time point after HU treatment when caffeine was present and 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 S1981 phosphorylation (Fig. 4B). Thus, the caffeine-induced hyper-phosphorylation of Chk1 is ATR dependent. This data also shows that S345 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, our data indicated that at least in the cell types and conditions that we examined, caffeine did not inhibit ATM or ATR. This could be explained if our caffeine was inactivated, metabolized, or unable to enter the cells in the growth conditions that we employed. Therefore, we tested whether our caffeine would inhibit ATM and ATR *in vitro* and whether it would inhibit the checkpoint in the same cells in which we observe a hyper-activation of ATM/ATR signaling. Consistent with previous publications, ATM kinase activity was inhibited at low (1mM) concentrations of caffeine *in vitro*. Both autophosphorylation and trans-phosphorylation of the BRCA1 substrate was inhibited (Fig. 5A) in kinase assays. We 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 we incubated HCT116 or hTERT-RPE1 cells with caffeine, exposed them to IR or HU, then analyzed the percentage of mitotic cells. We found that caffeine efficiently prevented the G2 arrest normally observed in cells treated with either IR or HU consistent with previous observations of caffeine’s inhibitory effect on checkpoints (Fig. 5B and 5C). Caffeine efficiently overcame the IR and HU-induced G2/M checkpoint at all time points examined. Furthermore, it inhibited the checkpoint at concentrations and time points at which there is hyper-phosphorylation 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 Chk1/2 and p53. We 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. While its mechanism of action in cells is not clear, caffeine does inhibit the kinase activity of ATM and ATR \textit{in vitro} (35-38). Our results using \textit{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 \textit{in vitro}. Furthermore, we have confirmed previous observations that low millimolar concentrations of caffeine overrides cell cycle checkpoint responses to both IR and HU. ATM and ATR signaling is essential for these checkpoint responses in cells. Thus, it seems logical to expect that caffeine’s mode of action in regard to checkpoint inhibition in cells is through its ability to inhibit ATM and ATR kinase activity.

Paradoxically, we have found that multiple ATM and ATR substrates are actually hyper-phosphorylated when cells are treated with caffeine in the presence of either replication inhibitors or IR. Chk2 and Chk1 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 we would expect the phosphorylation of Chk2 and Chk1 on their ATM/ATR-dependent phosphorylation sites to be decreased by caffeine treatment. We observed the opposite effect. Phosphorylation of Chk1 and Chk2 was increased in a dose dependent manner by caffeine treatment in cells that were also treated with HU, IR, or aphidicolin. Furthermore, we found that phosphorylation of the S1981 on ATM itself is increased by caffeine treatment. S1981 is an autophosphorylation site that is required for ATM
activation (3). The simplest explanation of this data is that ATM and ATR are actually super-activated by replication inhibitors or other DNA damaging agents in the presence of caffeine. We did not observe activation of ATM or ATR by caffeine treatment alone although we can’t rule out the possibility that activation occurred at levels below the sensitivity of our detection reagents.

There is some evidence in the literature that supports the idea that caffeine does not inhibit ATM/ATR in vivo. Pre-treatment of cells with caffeine failed to inhibit ATM activation by 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 vivo is insensitive to caffeine. It is now known that ATM activation requires ATM autophosphorylation on S1981. Furthermore, ATM is activated in vitro by autophosphorylation since pre-incubation of ATM with ATP stimulates its activity (46). Caffeine is unable to inhibit this activation step, and pre-treatment of ATM with caffeine actually slightly increases its activity (46). These data are consistent with our conclusions that ATM autophosphorylation and activity is 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 hyper-phosphorylation 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. We could hypothesize that the caffeine-dependent hyper-phosphorylation of checkpoint signaling proteins in response to HU
treatment is acting through a similar mechanism of ATR inhibition leading to increased double strand breaks and activation of another PIK kinase. We 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+HU activated PIK kinase should not be ATM and we have shown it is not DNA-PK. An unidentified, caffeine-insensitive, wortmanin-sensitive kinase capable of phosphorylating ATM/ATR substrates would need to be invoked. Second, Chk1 phosphorylation on S345 is increased by combined caffeine and HU 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 hyper-phosphorylation of Chk1 in response to HU combined with caffeine, then the responsible kinase must depend on the presence of the inhibited ATR kinase to recognize Chk1. Third, S1981 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 we observe an increase in S1981 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 unrepairable 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 super-activated. Such a scenario seems unlikely since 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 have its inhibitory effect inactivated. This scenario also seems unlikely since increasing the dosage of caffeine actually increases apparent ATM/ATM activation and re-addition of fresh caffeine at one-hour intervals does not prevent the hyper-phosphorylation 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 Chk2 are hyper-phosphorylated. This observation argues strongly that caffeine is inhibiting the G2/M checkpoint somewhere downstream of the essential ATM/ATR-dependent phosphorylation of Chk1 and Chk2. Phosphorylation of Chk1 and Chk2 is required for their activation and is often used as indicators 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 Chk-dependent inactivation of Cdc25C (30,47). Direct inactivation of Chk1 and Chk2 by caffeine would be expected to yield similar phenotypic consequences as ATM and ATR inhibition. However, Chk1 and Chk2 are not inhibited by caffeine at low millimolar concentrations in vitro (37,38). Therefore, caffeine’s mechanism of action 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 doing it 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 HU, aphidicolin and IR induced DNA damage and thereby promoting hyper-activation 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 caffeine’s effects on these kinases in cells.

References


Footnotes

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The abbreviations used are: HU, hydroxyurea; IR, ionizing radiation; UV, ultraviolet light; ATM, ataxia telangiectasia mutated; ATR, ATM and Rad3 related; GFP, green fluorescent protein.

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Figure Legends

Figure 1. Loss of ATR promotes activation of ATM and hyper-phosphorylation of p53, Chk2, and H2AX. ATR\(^{+/+}\) or ATR\(^{flox/-}\) cells infected with adenovirus encoding either the Cre recombinase to delete ATR or GFP as a control were treated with (A) 1mM hydroxyurea (HU) for the indicated times (B) 0 or 1mM HU for 6 hours, or (C) IR (5Gy) or UV (50J/m\(^2\)) and incubated for the indicated times or treated with 1mM HU for the indicated times. Cell lysates were prepared, separated by SDS-PAGE and blotted with the indicated antibodies.

Figure 2. Caffeine promotes hyper-phosphorylation of ATM/ATR substrates following HU treatment. HCT116 cells were treated with the indicated concentrations of caffeine and 1mM HU for (A) six hours or (B) two hours. Cell lysates were prepared, separated by SDS-PAGE, and blotted with the indicated antibodies. (C) Cells were treated with 0 or 4mM caffeine, 0 or 20\(\mu\)M wortmanin, and 0 or 1mM HU as indicated for six hours. Cell lysates were separated by SDS-PAGE and blotted with the indicated antibodies.

Figure 3. Phosphorylation of ATM/ATR substrates in response to caffeine treatment is not DNA-PK dependent or limited to hydroxyurea treatment. (A) DNA-PK proficient (M059K) or deficient (M059J) cells were treated with 0, 4 or 8mM caffeine and 1mM HU for 2 or 6 hours. Cell lysates were prepared, separated by SDS-PAGE, and blotted with antibodies to DNA-PK or phospo-S345 Chk1. (B) HCT116 cells were treated with 0 or 8mM caffeine and exposed to 5\(\mu\)g/ml aphidicolin or 1mM HU for 1 or 6 hours. Cell lysates were prepared, separated by SDS-PAGE and blotted with the indicated antibodies.
Figure 4. ATM and ATR signaling is hyper-activated by caffeine treatment. (A) hTERT-RPE1 cells were treated with the indicated concentrations of caffeine and 1mM HU for 4 hours or exposed to 5 Gy of ionizing radiation (IR) and allowed to recover for 1 hour. Cell lysates were prepared, separated by SDS-PAGE, and blotted with the indicated antibodies. (B) ATR\(^{+/+}\) or ATR\(^{flox/-}\) cells infected with adenovirus encoding either the Cre recombinase or GFP were treated with caffeine and 1mM hydroxyurea as indicated for 2, 4 or 6 hours. Cell lysates were prepared, separated by SDS-PAGE, and blotted with the indicated antibodies.

Figure 5. Caffeine inhibits ATM kinase activity in vitro and overcomes the G2/M checkpoint in vivo. (A) Wild-type or kinase-deficient ATM were immunoprecipitated from transfected cells, placed in kinase reaction conditions containing \(\text{[\text{32P}]ATP}\) and incubated with a BRCA1 fragment as a substrate in the presence or absence of caffeine. Reactions were separated by SDS-PAGE, stained with coomassie, and exposed to film. (B) HCT116 cells were treated with 0 or 8mM caffeine and 1mM HU for 6 hours. Cells were harvested, stained with anti-phospho-histone H3 antibody and FITC-conjugated secondary antibody. Following PI staining the cells were examined by flow cytometry. (C) HCT116 or hTERT-RPE1 cells were treated with 0 or 8mM caffeine and IR or HU for the indicated times. The percentage of mitotic cells was determined by PI and phospho-histone H3 staining.
Figure 1

A

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Chk1 P-S345
Chk1
p53 P-S15
Chk2

B

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P-H2AX

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ATM P-S1981
ATM
Figure 2

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HU 6h

Chk1 P-S345
Chk1
RPAp34
Chk2 P-T68
p53-P-S15

B

<table>
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<th>4</th>
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HU 2h

Chk1 P-S345

C

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<tr>
<th>Caf</th>
<th>Caf+ Wort</th>
<th>- HU</th>
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<th>- HU</th>
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Figure 3

A

<table>
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<tr>
<th></th>
<th>M059K</th>
<th>M059J</th>
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<tr>
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<td>4mM 8mM</td>
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<tr>
<td>0 2 6</td>
<td>0 2 6 6</td>
<td>0 2 6 6</td>
</tr>
<tr>
<td>hours</td>
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DNA-PK

- Chk1 P-S345

B

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<th></th>
<th>Aphidicolin</th>
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<td>- - + +</td>
<td>6 6 hours</td>
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<tr>
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<tr>
<td>hours</td>
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Chk1 P-S345

Chk1

ATM P-S1981

ATM
Figure 4

A

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<th>HU 1mM 4h</th>
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- ATM P-S1981
- ATM
- Chk1 P-S345
- Chk1
- Chk2 P-T68

B

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<th>ATR^{flox/-}</th>
<th>Cre</th>
<th>ATR^{flox/-}</th>
<th>GFP</th>
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<tbody>
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<td>HU (h)</td>
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<td>6</td>
</tr>
<tr>
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<td>4</td>
<td>6</td>
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<tr>
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<tr>
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- ATM P-S1981
- ATM
- Chk1 P-S345
- Chk1
- ATR
Figure 5

A

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<td>1</td>
</tr>
<tr>
<td>8</td>
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- ATM
- BRCA1 1351-1552

32p

Coomassie

- BRCA1 1351-1552
- IgG

B

HU

Anti-P-H3 FITC

HU + Caffeine

DNA Content

C

HCT116 Cells

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</tr>
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hTERT-RPE1 Cells

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<td>0.5</td>
</tr>
<tr>
<td>2h IR 5Gy</td>
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