Heat Shock Inhibition of CDK5 Increases NOXA Levels through miR-23a Repression*

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Background: HSP70 inhibits heat-induced apoptosis by preventing NOXA accumulation.

Results: Inhibition of CDK5 in heat-shocked cells reduces levels of miR-23a leading to increased NOXA abundance, which is prevented by HSP70.

Conclusion: CDK5 regulates miR-23a expression and affects NOXA abundance.

Significance: The prosurvival function of HSP70 can be attributed to its ability to prevent the stress-induced inhibition of CDK5.

Hyperthermia is a proteotoxic stress that is lethal when exposure is extreme but also cytoprotective in that sublethal exposure leads to the synthesis of heat shock proteins, including HSP70, which are able to inhibit stress-induced apoptosis. CDK5 is an atypical cyclin-dependent kinase family member that regulates many cellular functions including motility and survival. Here we show that exposure of a human lymphoid cell line to hyperthermia causes CDK5 insolubilization and loss of tyrosine-15 phosphorylation, both of which were prevented in cells overexpressing HSP70. Inhibition of CDK5 activity with roscovitine-sensitized cells to heat induced apoptosis indicating a protective role for CDK5 in inhibiting heat-induced apoptosis. Both roscovitine and heat shock treatment caused increased accumulation of NOXA a pro-apoptotic BH3-only member of the BCL2 family. The increased abundance of NOXA by CDK5 inhibition was not a result of changes in NOXA protein turnover. Instead, CDK5 inhibition increased NOXA mRNA and protein levels by decreasing the expression of miR-23a, whereas overexpressing the CDK5 activator p35 attenuated both of these effects on NOXA and miR-23a expression. Lastly, overexpression of miR-23a prevented apoptosis under conditions in which CDK5 activity was inhibited. These results demonstrate that CDK5 activity provides resistance to heat-induced apoptosis through the expression of miR-23a and subsequent suppression of NOXA synthesis. Additionally, they indicate that hyperthermia induces apoptosis through the insolubilization and inhibition of CDK5 activity.

Cyclin-dependent kinase 5 (CDK5) 3 is a member of the cyclin-dependent kinase family with unique roles unrelated to cell cycle progression. It shares sequence homology with typical CDKs, such as CDK1, and phosphorylates substrates having the same consensus motif ((S/T)P(X(K/H/R)), however its activity is dependent upon the binding of the activators p35 (CDK5R1) or p39 (CDK5R2), which are not cyclins but do contain a cyclin box necessary for CDK5 binding (1, 2). CDK5 is also atypical in that threonine phosphorylation of the activation loop is not required for its activity and its activity is not inhibited by Tyr-15 phosphorylation. Instead, phosphorylation of Tyr-15 by the non-receptor tyrosine kinases c-Abl and Fyn increases CDK5 activity (3, 4). CDK5 was originally believed to be restricted to neuronal cells where it plays fundamental roles in brain development through regulation of cell migration, neurite outgrowth, and synapse formation (1, 2). However, CDK5 also regulates numerous activities in a wide variety of non-neuronal cells including lymphocytes (5–7).

Under normal physiological conditions, CDK5 plays a prosurvival role as its inhibition by treatment with the somewhat selective inhibitor roscovitine, expression of a competitive-interfering loss of function mutant of CDK5 (CDK5-D145N) or siRNA-mediated knockdown results in cell dysfunction and cell death (1). Substrates of CDK5 include transcription factors, kinases, cytoskeletal proteins, and apoptotic regulators. CDK5 directly phosphorylates NOXA (PMAIP1) (8) and HTRA2 (9) inhibiting their pro-apoptotic function and increases the anti-apoptotic activity of BCL2 by direct phosphorylation (10) as well as indirectly increasing its expression (11). Under pathological conditions, such as Alzheimer disease, Parkinson disease, and Huntington disease, p35 can be cleaved by calpain proteases to generate a p25 fragment that has an extended half-life and lacks myristolation that induces hyper-activation and mis-location of CDK5 (2, 12). Deregulation of CDK5 is also associated with cancer where it plays a role in regulating cell motility and metastasis (13). Elevated expression of CDK5 correlates with poor prognosis in non-small-cell lung cancer (14) and the pharmacological inhibition of CDK5 as a strategy for cancer therapy has progressed to phase II clinical trials (5).

Protein damaging stress, including hyperthermia, can lead to apoptosis through BAX activation, mitochondrial release of cytochrome c, and caspase activation, although the molecular mechanisms responsible for BAX activation in heat-stressed
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cells are unclear (15, 16). Sublethal exposure to hyperthermia induces the expression of molecular chaperone proteins, such as HSP70, which can inhibit apoptosis (17, 18). This pro-survival function of HSP70 contributes to the tumorigenic potential of cancer cells. As well, HSP70 plays a cytoprotective role in a number of neurodegenerative disorders by chaperoning the aggregation-prone proteins that are associated with these diseases (19). We have previously shown that BAX activation in heat stressed cells is mediated by a NOXA-dependent depletion of the anti-apoptotic protein MCL1 (20). Since CDK5 has been shown to be a critical mediator of cell survival in stressed cells we investigated the effect of hyperthermia on CDK5 and examined whether its inhibition could increase the sensitivity of cells with elevated levels of HSP70 to hyperthermia.

EXPERIMENTAL PROCEDURES

Cells and Treatments—The effects of HSP70 overexpression were examined using a human acute lymphoblastic T-cell line (PEER) with tetracycline-regulated expression of human HSP70 (PErTA70) (21). HSP70 (HSPlA1A) was induced by incubation with 1.0 μg/ml doxycycline for 24 h prior to each experiment. A stably transfected PEER cell line with tetracycline regulated expression of CDK5-D145N was created by transfection of PErTA cells (PEER cells stably expressing rTA) with the plasmid pTR5-DC/CDK5D145N-GFPq*tk/hygro and selection for hygromycin resistance. This plasmid was produced by subcloning the CDK5-D145N sequence containing a C-terminal HA-tag from pCMVCDK5-D145N (CDK5-DN-HA was a gift from Sander van den Heuvel, Addgene plasmid #1873) (22) into the pTR5-DC/GFPq*tk/hygro plasmid (21). Cells were maintained at 37 °C in a humidified 5% CO2 incubator in RPMI medium with 10% fetal bovine serum (Invitrogen Inc., Burlington, Ontario, Canada). Cells were heat shocked in medium supplemented with 20 mM HEPES buffer (pH 7.2) by immersion of log-phase cells in a circulating water bath. Roscovitine (Cell Signaling Technology, Danvers, MA) was dissolved in DMSO and added to cells for 30 min prior to exposure to hyperthermia. Control cells received an equivalent volume of DMSO, which was no more than 0.1%. NOXA protein turnover was measured by treating cells with cycloheximide (200 μg/ml) at 37 °C for up to 2 h and subsequent Western blotting.

Measurement of NOXA turnover in cells expressing either WT or non-phosphorylatable NOXA-S13A was examined by transient transfection of HEK-293-rTA cells using calcium phosphate co-precipitation. Plasmids to express NOXA were created by amplifying the NOXA cDNA, which was reverse transcribed from PEER cell mRNA using primers corresponding to the NOXA mRNA sequence (NM_021127). PCR was performed using primers that added a single N-terminal c-myc tag and restriction enzyme sites to allow cloning into the pB-L-EYFP plasmid (Clontech). Serine-to-alanine mutation of the codon encoding serine-13 was achieved by PCR mutagenesis. Stably transfected PEER cells expressing either a control microRNA (C-miR) or miR-23a were generated by transfection with pEZX-M04 derived plasmids (GeneCopoeia, Rockville, MD) and have been described previously (23).

HeLa cells were transiently transfected with plasmids to overexpress CDK5-DN-HA (Addgene plasmid 1873) and p35 (pCMV-P35 was a gift from Li-Huei Tsai, Addgene plasmid #1347) to test whether the effect of CDK5-D145N expression on miR-23a levels could be rescued by p35 overexpression. All transfections contained an equivalent total amount of plasmid DNA, which was adjusted by the addition of the pCMV-P35 plasmid that had the p35 insert removed by BamHI digestion and re-ligation.

Cell Viability Assays—Cell viability was determined by measuring the reduction of resazurin (Alamar Blue) as previously described (24). Briefly, cells were seeded at a concentration of 2.5 × 10^6 cells/ml in 12-well microplates and treated with various doses of roscovitine. After a 30 min pre-treatment at 37 °C, the plates were sealed with parafilm and heated at 42 or 43 °C in a circulating waterbath or incubated at 37 °C in an incubator. Following the 1-h heat treatment the cells were returned to 37 °C and incubated in the presence of roscovitine for 20 h. Cell suspensions (100 μl in triplicate) were then seeded into a 96-well plate with 100 μl of medium containing 50 μM resazurin (Sigma-Aldrich, Oakville, Canada) and incubated at 37 °C for 4 h. Fluorescence generated from resazurin reduction was measured in a microplate fluorescence reader (Ex516/20, Em590/35). Viability is expressed as a percentage of the control values. Apoptosis was assessed by Annexin-V staining using a Beckman Coulter FC500 flow cytometer on cells stained with an Annexin-V Alexa Fluor®647 conjugate (Life Technologies).

Cell Lysis and Immunoblotting—To examine protein solubility following exposure to hyperthermia, cells were lysed in Triton X-100 buffer (10 mM HEPES pH 7.4, 100 mM NaCl, 5 mM MgCl2, 1 mM EGTA, 1% Triton X-100, 10 μg/ml each of peptatin A, leupeptin, and aprotinin, 10 mM sodium fluoride, 1 mM sodium vanadate, 20 mM sodium phosphate, 3 mM β-glycerolphosphate, 5 mM sodium pyrophosphate). Lysates were centrifuged at 15,000 × g for 10 min at 4 °C. Protein concentration in the supernatants was determined using the BCA Protein Assay (Pierce/Thermo Scientific, Markham, Ontario, Canada). The supernatants were then mixed with 2× Laemmli buffer (100 mM Tris-Cl pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol) and heated to 95 °C for 5 min. Pellets were resuspended in the same total volume of 1× Laemmli buffer as the supernatant fractions and then sonicated and heated.

Subcellular fractions were prepared by digitonin lysis to monitor the release of cytochrome c and HtrA2 from mitochondria as described previously (25). Cells (5 × 10^6) were lysed for 10 min on ice in digitonin lysis buffer (phosphate buffered saline (pH 7.4) containing 250 mM sucrose, 70 mM KCl, 0.025% digitonin, protease and phosphatase inhibitors). Lysis was monitored by trypan blue exclusion. The lysates were centrifuged at 15,000 × g for 10 min at 4 °C and the supernatants, containing soluble proteins (S), were collected. The pelleted membrane fraction (M), was lysed in a volume of 1× Laemmli buffer equivalent to that of the soluble fraction, sonicated and heated at 95 °C for 5 min. Protein concentration in the soluble fraction was determined and equivalent amounts of protein were loaded for each sample. Efficiency of separation was confirmed by blotting for tubulin and HSP60.
SDS-PAGE and immunoblotting were performed as described previously (25). The following antibodies were used for immunoblotting: Actin (ACTN05: NeoMarkers, Fremont, CA), CDK5 (2506: Cell Signaling Technology, Danvers, MA), cleaved caspase-3 Asp175 (9664: Cell Signaling Technology), cytochrome c (65981A: BD Biosciences PharMingen, Mississauga, Ontario, Canada), c-myc from 9E10 hybridoma supernatant, HSP60 (SPC-105: StressMarq Biosciences, Victoria, British Columbia, Canada), HSP70 (C92F3A-5: Stressgen/Assay Designs, Ann Arbor, MI, USA), HtrA2 (AF1458: R&D Systems/ Cederlane, Burlington, Ontario, Canada), MCL1 (SC-819: Santa Cruz Biotechnology, Santa Cruz, CA), NOXA (ALX-804–408: Enzo Life Sciences), p35/25 (2680: Cell Signaling Technology), phospho-MAPK/CDK substrates (PXS^P or S^PXR/K, 2325: Cell Signaling Technology), phospho-CDK5 Tyr15 (CG1085: Cell Applications, San Diego, CA), tubulin (MABT205: Millipore, Billerica, MA).

RT-PCR and RT-qPCR—Cells were collected by centrifugation, washed with PBS, and RNA was isolated using TRIzol® Reagent (Invitrogen-Life Technologies, Burlington, Ontario, Canada). RNA was quantified by Nanodrop and cDNA was synthesized from 5 μg of RNA using an oligo(dT) primer and Superscript II Reverse Transcriptase kit in a total volume of 19 μl (Invitrogen-Life Technologies). PCR was carried out using GOTAq® Flexi DNA Polymerase (Promega, Madison, WI). Each 25 μl reaction contained 10 μM primers and 1 μl of cDNA in 1× GOTAq® Flexi buffer. All PCR reactions were 30 cycles except for miR-23a, which was 35 cycles. PCR products were mixed with RedSafe dye (FroggaBio, Toronto, Ontario, Canada) and analyzed by agarose gel electrophoresis and imaged using a Bio-Rad ChemiDoc™ XRS+ imaging system (Bio-Rad).

For RT-qPCR, cDNA was synthesized from 0.017 μg purified RNA with random primers using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems-Life Technologies). qPCR was performed using PerfeCTa® FastMix® II from Quanta Biosciences and the Applied Biosystems StepOnePlus real-time PCR instrument at the University of Guelph Advanced Analysis Centre. Primer-specific amplification efficiencies were determined by constructing a standard curve of serially diluted cDNA. For each sample, the relative amount of starting template was determined by calculating the ΔΔCt after correcting the Ct values for expression of RPL4 (ribosomal protein L4). Primers used for RT-PCR and RT-qPCR were: miR-23a-fwd: 5'-ctgggttctggtttgg-3'; miR-23a-rev: 5'-gtctgg-gttggaacctgct-3'; NOXA-fwd: 5'-gtcggagtctagtgtgctca-3'; NOXA-rev: 5'–gagttccccctcatgcaagt-3'; RPL4-fwd: 5'-gtctgg-gccaggtgttttg-3'; RPL4-rev: 5'-atgcccgtatcggtttgggtt-3').

RESULTS

We set out to determine whether hyperthermia affects CDK5 activity, given its role in regulating stress-induced apoptosis, and also to examine whether this might be affected by overexpression of HSP70, which has potent anti-apoptotic activity. For this we used PeRTA70 cells, a human acute lymphoblastic T-cell line with tetracycline-regulated expression of HSP70 (21). Doxycycline induced cells, which overexpress HSP70 (+ HSP70) and non-induced cells (−HSP70) were exposed to 42, 43 or 44 °C for 1 h and then returned to 37 °C for 6 h. Western blotting showed a temperature dependent decrease in the abundance of both total and Tyr-15-phosphorylated CDK5 (pY15-CDK5) in Triton X-100 soluble extracts (Fig. 1A). Loss of pY15-CDK5 was more significant than that of total CDK5 in the heat-shocked cells. Overexpression of HSP70 suppressed the loss of total CDK5 and pY15-CDK5, particularly in cells exposed to 43 °C (Fig. 1B) and as shown previously (21) also inhibited caspase-3 activation (Fig. 1A). Levels of the CDK5 activator p35 were also decreased by hyperthermic treatment although not to the same extent as CDK5 or pY15-CDK5.

We next examined whether HSP70 expressing cells suffered less heat-inactivation of CDK5 and/or were able to recover soluble CDK5 and pY15-CDK5 more effectively when returned to 37 °C following heat shock. Cells were exposed to 42, 43, or 44 °C for 1 h and then either collected immediately or incubated at 37 °C for 2, 4 or 6 h before harvesting and lysis in Triton-X buffer (Fig. 1C). In the non-induced cells, pY15-CDK5 levels demonstrated mild recovery in cells heat-shocked at 42 °C and following 6 h of incubation at 37 °C, though no recovery of pY15-CDK5 was observed in cells exposed to either 43 or 44 °C. However, in HSP70-expressing cells complete recovery of pY15-CDK5 levels were observed by 2 h at 37 °C following exposure to 42 °C and a nearly complete return to basal levels occurred after 6 h of incubation following exposure to 43 °C. CDK5 was found in the Triton X-insoluble fractions of the non-induced cells following exposure to 42, 43 and 44 °C, while insoluble pY15-CDK5 was only present after exposure to 44 °C. In the HSP70-overexpressing cells CDK5 was observed in the insoluble fraction only after exposure to 44 °C and no appreciable level of pY15-CDK5 became insoluble at any temperature in these cells. These data demonstrate that total and pY15-CDK5 levels are sensitive to hyperthermia-induced loss. Furthermore, HSP70 overexpression enhances the recovery of pY15-CDK5 and prevents the insolubilization of total CDK5 following exposure to hyperthermia.

We further explored the effect of hyperthermia on CDK5 solubility by exposing PeRTA70 cells to 43 °C for various lengths of time followed by 6 h incubation at 37 °C (Fig. 2A). Levels of CDK5 and pY15-CDK5 were lost in a time-dependent manner following prolonged heat shock exposure in the non-induced cells though this loss was much less extreme in the HSP70-expressing cells (Fig. 2B). Loss of total CDK5 from the Triton X-soluble fraction was accompanied by its appearance in the insoluble fraction. However, pY15-CDK5 was not found in the insoluble fraction, indicating that hyperthermia leads to a rapid loss of CDK5 phosphorylation combined with the insolubilization of the pool of total CDK5. Probing for phosphorylated MAPK/CDK substrates revealed that hyperthermia caused an accumulation of high MW phosphoproteins in the insoluble fraction in the non-induced cells that did not occur in the HSP70-expressing cells (Fig. 2A). Heat shock also increased the level of phosphorylation of some proteins, notably proteins with a molecular size of ~25 and 60 kDa. To test whether some of these proteins could potentially be targets of CDK5 we exposed cells to hyperthermia in the presence of the CDK5 inhibitor roscovitine (Fig. 2C). Inhibition of CDK5 in cells exposed to hyperthermia further decreased the level of pMAPK/CDK substrates and prevented the increased phos-
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Phosphorylation of the 25 and 60 kDa proteins observed in Fig. 2A. These results demonstrate that hyperthermia globally reduces the solubility and abundance of pMAPK/CDK substrates, including proteins phosphorylated by CDK5. As well, HSP70 has a protective effect in reducing the extent of pMAPK/CDK substrate insolubilization in heat-shocked cells.

Since exposure to mild hyperthermia (42 °C for 1 h) produced only a transient loss of pY15-CDK5 and had minimal effects on total CDK5 (Fig. 1C), we hypothesized that chemical inhibition of CDK5 activity using roscovitine would sensitize these cells to heat-induced apoptosis. To test this we exposed PEtTA70 cells to 42 °C for 1 h with or without roscovitine (5 or 10 μM) and then incubated them for 6 h at 37 °C, either without or with roscovitine, before measuring apoptosis by Annexin V staining (Fig. 3A). Treatment of both non-induced and HSP70-overexpressing cells with roscovitine at 37 °C produced a dose-dependent increase in apoptosis, which was further augmented by exposure to hyperthermia. However, overexpression of HSP70 lessened the effect of roscovitine on heat-induced apoptosis. We also looked at long-term survival following CDK5 inhibition in cells exposed to either 42 or 43 °C in the presence of various concentrations of roscovitine. Cells were heat shocked for 1 h in the presence of roscovitine and then incubated at 37 °C for 48 h in the continuous presence of roscovitine before measuring cell viability using the resazurin reduction assay (Fig. 3B). The dose that reduced relative cell number by 50% (ED50) was similar in both the non-induced and HSP70-expressing cells treated at 37 °C (12 μM). However, when exposed to hyperthermia the non-induced cells were more sensitive to the combined effects of hyperthermia and roscovitine treatment (ED50, 6 μM) as compared with HSP70-expressing cells (ED, 10 μM). Therefore, inhibition of CDK5 activity with roscovitine effectively sensitized both non-induced and HSP70-overexpressing cells to hyperthermia, although HSP70 overexpression provided some protection against roscovitine-dependent heat sensitivity.

We next examined whether the effect of CDK5 inhibition in PEtTA70 cells exposed to mild hyperthermia correlated with an increased release of pro-apoptotic proteins from mitochondria (Fig. 4). In non-induced cells exposure to 42 °C caused the release of both cytochrome c and HtrA2 that was augmented in a dose-dependent manner by treatment with roscovitine, dem-

FIGURE 1. Loss of pY15-CDK5 and insolubilization of total CDK5 in cells exposed to hyperthermia. A, PEtTA70 cells that were either not induced (−HSP70) or induced to express HSP70 (+HSP70) were incubated at 42, 43, or 44 °C for 1 h and then returned to 37 °C for 6 h. Control cells remained at 37 °C. Cells were lysed in buffer containing 1% Triton X-100 and following centrifugation the Triton X-100 soluble fraction was examined by SDS-PAGE and Western blotting. B, quantification of the results shown in A (mean ± S.E., n = 3, * indicates a significant difference between −HSP70 and +HSP70 cells p < 0.05). C, cells were exposed to 42, 43, or 44 °C for 1 h and then either collected immediately or after incubation at 37 °C for 2, 4, or 6 h before collection. Cells were lysed as in A and following centrifugation the Triton X-100 soluble and insoluble (PELLET) fractions were examined by Western blotting.
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Demonstrating that inhibition of CDK5 activity increases sensitivity to heat-induced apoptosis. Treatment of HSP70-overexpressing cells with 10 μM roscovitine that were exposed to 42 °C produced a significant increase in cytochrome c release although HtrA2 release was not affected by this combined treatment. Altogether these results show that inhibition of CDK5 with roscovitine sensitizes both non-induced and HSP70 overexpressing cells to hyperthermia-induced apoptosis.

The proapoptotic activity of NOXA is regulated by CDK5 under conditions of glucose deprivation (8). We were therefore interested in determining whether inhibition of CDK5 by roscovitine treatment would augment the effect of mild hyperthermia on NOXA and MCL1 protein levels. PErTA70 cells were treated with either 5 or 10 μM roscovitine and exposed to 42 °C for 60 or 120 min in the absence or presence of 20 μM roscovitine and collected. Roscovitine-treated cells were pre-incubated with the drug for 6 h at 37 °C before exposure to hyperthermia. Control cells were incubated at 37 °C. Cells were lysed with Triton X-100 lysis buffer, and the Triton X-100 soluble fractions were examined by Western blotting.

FIGURE 2. Effect of hyperthermia on phosphorylation of MAPK/CDK targets. A, non-induced (−HSP70) and induced (+HSP70) PErTA70 were exposed to 43 °C for 30, 60, 90, or 120 min and then incubated at 37 °C for 6 h before being lysed in 1% Triton X-100 buffer. Control cells were incubated at 37 °C (C). Triton X-100 soluble and insoluble (PELLET) fractions were examined by Western blotting. B, quantification of the results shown in A (mean ± S.E., n = 3, * indicates a significant difference between −HSP70 and +HSP70 cells, p < 0.05). C, roscovitine enhances the loss of MAPK/CDK target protein phosphorylation in heat shocked cells and inhibits the hyperthermia-induced increase in phosphorylation of specific MAPK/CDK targets. PErTA70 cells were exposed to 43 °C for 60 or 120 min in the presence of 20 μM roscovitine and collected. Roscovitine-treated cells were pre-incubated with the drug for 6 h at 37 °C before exposure to hyperthermia. Control cells were incubated at 37 °C. Cells were lysed with Triton X-100 lysis buffer, and the Triton X-100 soluble fractions were examined by Western blotting.
ence of roscovitine resulted in an increased abundance of NOXA in the HSP70-overexpressing cells and further increased the levels of NOXA protein in the non-induced cells (Fig. 5B). This increased abundance of NOXA correlated with a significant decrease in the levels of MCL1 in both the absence and presence of HSP70 (Fig. 5C). The pro-apoptotic function of NOXA is mediated in part by its ability to target MCL1 for proteasomal degradation (26).

We next sought to address the mechanism responsible for the increased abundance of NOXA in the roscovitine-treated cells. We considered the possibility that inhibition of CDK5 activity could reduce NOXA phosphorylation, resulting in a reduced rate of protein turnover and a corresponding increase in protein abundance. For these experiments we used PEER cells (27), which were used previously as the parental line to create stable PErTA70 cells. PEER cells were incubated with 10 μM roscovitine for 6 h at 37 °C and subsequently treated with cycloheximide for up to 120 min to prevent further NOXA protein synthesis. Cells were collected at various times follow-

FIGURE 3. The CDK5 inhibitor roscovitine sensitizes cells to heat-induced apoptosis. A, non-induced (−HSP70) and HSP70-expressing (+HSP70) cells were treated with 0, 5, or 10 μM roscovitine and incubated at either 37 °C for 7 h or exposed to 42 °C for 1 h followed by 6 h at 37 °C. Apoptosis was measured by Annexin-V staining (mean ± S.E., n = 3, * indicates significant difference in value compared with treatment in the absence of roscovitine p < 0.05). B, cells were incubated with various concentrations of roscovitine (0.5–20 μM), exposed to either 42 or 43 °C for 1 h and then incubated at 37 °C in the presence of roscovitine for 48 h. Viability was then measured using Alamar blue and plotted relative to cells that were not exposed to roscovitine (mean ± S.E., n = 3).

FIGURE 4. Roscovitine treatment augments the effects of hyperthermia on the release of cytochrome c and HtrA2 from mitochondria. A, cells were treated with 0, 5, or 10 μM roscovitine and incubated at 37 °C for 7 h or exposed to 42 °C for 1 h and then incubated at 37 °C for 6 h. Cells were lysed in 0.025% digitonin buffer and centrifugation to obtain membrane M and soluble S fractions, which were analyzed by Western blotting. Quantification of the results are shown for levels of cytochrome c (B) and HtrA2 (C), (mean ± S.E., n = 3, * indicates a significant difference between non-treated and roscovitine treated cells p < 0.05).
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We lastly examined the effect of miR-23a overexpression on resistance to apoptosis in cells exposed to hyperthermia in the presence and absence of roscovitine. Stably transfected PEER cells overexpressing miR-23a produced lower levels of NOXA mRNA relative to stably transfected cells expressing a control miRNA (Fig. 8A). Roscovitine treatment of the control miRNA-expressing cells resulted in increased NOXA protein levels and caspase-3 cleavage (Fig. 8B). The extent of NOXA protein accumulation and caspase-3 cleavage was higher when these cells were exposed to hyperthermia (42 °C) while in the presence of roscovitine. Remarkably the miR-23a-expressing cells showed no increase in NOXA protein levels or caspase-3 cleavage when treated with roscovitine either alone or in combination with hyperthermia. Additionally, while roscovitine treatment of the control miRNA expressing cells resulted in apoptosis, as indicated by the appearance of cleaved caspase-3 (Fig. 8B) and by an increased percentage of Annexin V positive cells (Fig. 8C), the
miR-23a expressing cells were highly resistant to the combined treatment of roscovitine with hyperthermia (Fig. 8, B and C). Therefore, the effects of CDK5 inhibition on sensitivity to hyperthermia-induced apoptosis can be abrogated by the over-expression of miR-23a and its ability to suppress NOXA expression.

**DISCUSSION**

Proteotoxic stress, including hyperthermic exposure, causes protein misfolding and aggregation leading to loss of function. Signaling pathways controlling cell survival are acutely affected by proteotoxic stress. Hyperthermia can either activate or inhibit signaling pathways by inhibiting the activity of specific kinases or the phosphatases that regulate their activity (17, 28–30). Induction of apoptosis in cells exposed to hyperthermic stress serves as a mechanism to remove irreparably damaged cells. However, prior exposure to a mild proteotoxic stress induces an adaptive response leading to the enhanced expression of the heat-shock family of molecular chaperones, including HSP70, which have anti-apoptotic properties (16). HSP70 inhibits apoptosis by preventing the activation of BAX, a pro-apoptotic member of the BCL2 family of apoptotic regulators (25). The activation of BAX in heat-stressed cells is controlled by a NOXA-dependent loss of the anti-apoptotic BCL2 family member MCL1 (20).

The proapoptotic activity of NOXA is regulated by CDK5 dependent phosphorylation of residue serine 13 (8). Lymphoid cells deprived of glucose undergo apoptosis that is associated with reduced CDK5-mediated NOXA serine 13 phosphorylation, increased NOXA/MCL1 association and MCL1 depletion (8). We were therefore interested in examining the potential effect of hyperthermia on CDK5 activity and its relation to NOXA-induced heat-sensitivity. We found that CDK5 is sensitive to heat-induced insolubilization and that HSP70 overexpression maintains CDK5 solubility in heat-stressed cells. We also observed a dramatic loss of tyrosine-15 phosphorylated CDK5 after exposure to hyperthermia, which was prevented by HSP70 overexpression. Previously, several studies have shown that the kinase activity of CDK5 is stimulated by Tyr-15 phosphorylation and that this is mediated by c-Abl, Fyn, and Src kinases (1). One study investigating c-Abl has shown that hyperthermia reduces c-Abl mRNA expression in vivo in mice testes (31), while another study demonstrated that hyperthermia reduces activity of purified recombinant c-Abl (32). In
addition, Gao et al., (33) have reported heat-induced insolubilization of CDK5 in astrocytes and showed that overexpression of CDK5 protected these cells from the effects of hyperthermia. Therefore, in combination with previous studies, our results suggest that CDK5 activity is impaired during hyperthermia, and that this loss of activity could contribute to hyperthermia-induced apoptosis through decreased substrate phosphorylation, such as NOXA. Lastly, we hypothesize that HSP70 could potentially prevent hyperthermia-induced apoptosis by maintaining CDK5 activity through preservation of CDK5 solubility and/or Tyr-15 phosphorylation via maintenance of c-Abl.

Though Tyr-15 phosphorylation of CDK5 has been shown to increase kinase activity, a recent report casts doubt on the role of Tyr-15 phosphorylation in the regulation of CDK5 activity (34). We therefore also examined the effect of CDK5 inhibition on heat sensitivity using the CDK5 inhibitor roscovitine. Given that cells exposed to 42 °C were able to recover total CDK5 and pY15-CDK5 levels when allowed to recover at 37 °C post-hy-
perthermia and had only minimal levels of active caspase-3, we hypothesized that inhibition of CDK5 with roscovitine should sensitize cells to mild hyperthermia. As anticipated, roscovitine treatment increased cytochrome c release and apoptosis in cells exposed to 42 °C, corresponding with an increased abundance of NOXA protein and a loss of MCL1.

Roscovitine is a potent inhibitor of CDK5 (IC50 of 0.2 μM) as well as CDK1 and CDK2 (IC50 of 0.7 μM) but has minimal inhibitory activity against a number of other kinases (35). Roscovitine treatment has been reported to induce apoptosis that was associated with decreased levels of MCL1 in neutrophils and multiple myeloma cells (36–38). Interestingly, Gautam et al., (38) observed an increased abundance of NOXA mRNA in roscovitine-treated neutrophils, although protein levels were not measured. We found that both hyperthermic exposure and roscovitine treatment on their own caused a significant increase in NOXA protein and that the combined treatment of hyperthermia plus roscovitine augmented this increase. This led us to speculate that CDK5 activity might play a role in regulating the half-life of NOXA such that CDK5 inhibition, by heat shock or roscovitine treatment, could result in decreased NOXA phosphorylation and a reduced rate of turnover. However, we found that neither roscovitine treatment, overexpression of CDK5-D145N or prevention of NOXA phosphorylation by a Ser-13-alanine mutation (S13A) had any effect on the turnover rate of NOXA. Interestingly treatment of cells with roscovitine or overexpression of CDK5-D145N resulted in an increased abundance of NOXA protein suggesting that CDK5 inhibition directly affects NOXA expression.

The observation that CDK5 inhibition caused an increased accumulation of NOXA protein without affecting its turnover rate led us to consider that NOXA expression was being affected at the level of mRNA expression or translation. We have recently found that NOXA expression is controlled by the microRNA miR-23a (23), whereby mir-23a binding to NOXA mRNA affects both transcript levels and translation. We have also demonstrated that hyperthermia causes a reduction in miR-23a levels, which results in an increased abundance of NOXA mRNA and protein leading to cell death (23). Consequently, we hypothesized that miR-23a levels might be regulated by CDK5. As predicted, our results demonstrate that either roscovitine treatment or expression of CDK5-D145N caused a dramatic reduction in miR-23a levels and a corresponding increase in NOXA mRNA. In the case of CDK5-D145N expression, the loss of miR-23a could be rescued by overexpression of the CDK5 activator p35 resulting in a corresponding decrease in Noxa mRNA expression. Additionally, overexpression of miR-23a provided resistance to apoptosis in cells exposed to roscovitine alone or in combination with hyperthermia most likely through depletion of NOXA mRNA.

Given that inhibition of CDK5 activity reduces miR-23a expression, we speculate that CDK5 regulates the activity of a transcription factor controlling miR-23a expression. A number of transcription factors have been implicated in the regulation of miR-23a expression including Srf, Myf6, NFATc3; c-myc and CREB (39–42), though it is currently unknown if these transcription factors are altered during hyperthermia with the exception of c-myc (43). Alternatively, both hyperthermia and roscovitine treatment are known to inhibit RNA polymerase II, which could potentially affect miR-23a levels. MacCallum et al., (36) suggest that roscovitine induces apoptosis through inhibition of RNA polymerase II dependent transcription, which would result in the selective loss of short-lived proteins such as MCL1. MiR-23a is transcribed as part of a pri-miRNA cluster.

**Figure 8. Overexpression of miR-23a prevents NOXA expression in cells treated with mild heat shock or roscovitine.** A, RT-qPCR analysis of miR-23a and NOXA mRNA levels in PEER cells that stably overexpress a control miRNA (C-miR) or miR-23a (mean ± S.E., n = 3). Results of semi-quantitative RT-PCR are shown on the right. B, control miRNA and miR-23a-overexpressing cells were incubated with 0 or 10 μM roscovitine and incubated at either 37 °C for 7 h or exposed to 42 °C for 1 h and then incubated at 37 °C for 6 h. NOXA and active caspase-3 (p17) levels were measured by Western blotting. C, measurement of apoptosis by Annexin-V staining of samples treated as described in B (mean ± S.E., n = 3, * indicates a significant difference between non-treated and roscovitine-treated cells p < 0.05).
by RNA polymerase II (44) and therefore hyperthermia or roscovitine treatment could potentially reduce miR-23a levels by directly inhibiting RNA polymerase II, thereby causing an increase in NOXA mRNA and protein abundance. However, NOXA, like MCL1, also has a short protein half-life and therefore it is unlikely that inhibition of RNA polymerase II would cause an increase in NOXA protein levels, suggesting that inhibition of CDK5 activity and the resulting reduced expression of miR-23a are the definitive factors controlling NOXA expression during hyperthermia.

MicroRNAs are important regulators of cell survival and not surprisingly their expression is often deregulated in a number of diseases including cancer (45). MiR-23a is transcribed as part of the miR-23a~27a~24~2 cluster, which is overexpressed in and is implicated in the pathology of cancer and cardiac hypertrophy (46). It acts as a pro-survival factor by suppressing the expression of the pro-apoptotic regulators APAF1, caspase-7 and NOXA (23, 47, 48). There are only a few reports on the effects of hyperthermia on microRNA expression (49). We have previously shown that hyperthermia causes miR-23a levels to decrease and that this results in the increased expression of NOXA leading to apoptosis (23). The results presented here suggest that the decreased abundance of miR-23a in heat-shocked cells is due to CDK5 inhibition. HSP70 overexpression prevents the heat-induced loss of miR-23a (23), which we suggest could be attributed to the ability of HSP70 to prevent the heat-induced insolubilization of total CDK5 and loss of pY15-CDK5.

CDK5 plays a critical role in regulating a number of vital cellular processes including cell, survival and migration in a variety of cell types, including lymphoid cells (1, 5–7). Deregulation of CDK5 activity is associated with the pathology of cancer, inflammation, diabetes and protein folding diseases including Alzheimer disease, amyotrophic lateral sclerosis, and Parkinson disease. Our results suggest that cellular stress could contribute to disease pathology through suppression of CDK5 activity and the subsequent effects on miR-23a and NOXA expression. CDK5 activity is often up-regulated in many cancers, potentially leading to decreased NOXA expression and protection against stress-induced apoptosis. Currently there are multiple CDK5 inhibitors that are being investigated both in vitro and in clinical trials that have shown efficacy against CDK5 (50–53).

A major finding of this study is that the cytoprotective properties of HSP70 can be attributed in part to its ability to prevent CDK5 inactivation in stressed cells. HSP70 is often overexpressed in tumor cells where it acts as an inhibitor of apoptosis and contributes to the process of tumorigenesis (16). We hypothesize that HSP70 might allow cancer cells to endure the oxidative and proteotoxic tumor microenvironment by helping to maintain the activity of CDK5. In support of this we have demonstrated the cytoprotective effect of HSP70 overexpression on both CDK5 solubility and Tyr-15 phosphorylation during hyperthermia, which ultimately results in reduced NOXA protein expression and protection against hyperthermia-induced apoptosis. Overall our results suggest that a combination therapy of hyperthermia with CDK5 inhibitors could be effective in the treatment of cancers that have elevated levels of HSP70.

Acknowledgment—We thank Jing Zhang from the University of Guelph Genomics Facility for assistance with the qRT-PCR analysis.

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