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Inhibiting c-Jun N-terminal kinase partially attenuates caffeine-dependent cell death without alleviating the caffeine-induced reduction in mitochondrial respiration in C2C12 skeletal myotubes

R.M. Downs^a, M.A. Hughes^a, S.T. Kinsey^b, M.C. Johnson^b, and B.L. Baumgarner^{a,*}

^aDivision of Natural Sciences and Engineering, University of South Carolina Upstate, Spartanburg, SC, USA

^bDepartment of Biology and Marine Biology, University of North Carolina Wilmington, Wilmington, NC, USA

Abstract

Caffeine is a widely consumed stimulant that has previously been shown to promote cytotoxic stress and even cell death in numerous mammalian cell lines. Thus far there is little information available regarding the toxicity of caffeine in skeletal muscle cells. Our preliminary data revealed that treating C2C12 myotubes with 5 mM caffeine for 6 h increased nuclear fragmentation and reduced basal and maximal oxygen consumption rate (OCR) in skeletal myotubes. The purpose of this study was to further elucidate the pathways by which caffeine increased cell death and reduced mitochondrial respiration. We specifically examined the role of c-Jun N-terminal kinase (JNK), which has previously been shown to simultaneously increase caspase-dependent cell death and reduce mitochondrial respiration in other mammalian cell lines. We found that caffeine promoted a dose-dependent increase in cell death in multinucleated myotubes but did not in mononucleated myoblasts. The addition of 10 μ M Z-DEVD-FMK, a specific inhibitor of executioner caspases, completely inhibited caffeine-dependent cell death. Further, the addition of 400 μ M dantrolene, a specific ryanodine receptor (RYR) inhibitor, prevented the caffeine-dependent increase in cell death and the reduction in basal and maximal OCR. We also discovered that caffeine treatment significantly increased the phosphorylation of JNK and that the addition of 30 μ M SP600125 (JNKi), a specific JNK inhibitor, partially attenuated caffeine-induced cell death without preventing the caffeine-dependent reduction in basal and maximal OCR. Our results suggest that JNK partially mediates the increase in caspase-dependent cell death but does not contribute to reduced mitochondrial respiration in caffeine-treated skeletal muscle cells. We conclude that caffeine increased cell death and reduced mitochondrial respiration in a calcium-dependent manner by activating the RYR and promoting reticular calcium release.

*Corresponding author. Division of Natural Sciences and Engineering, University of South Carolina Upstate, Spartanburg, SC 29303, USA. bbaumgar@uscupstate.edu (B.L. Baumgarner).

Conflict of interest

None.

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Keywords

Caffeine; Skeletal muscle; Cell death; c-Jun N-terminal kinase; Mitochondrial respiration

1. Introduction

Caffeine is perhaps the world's most widely consumed dietary stimulant. Caffeine has been shown to have beneficial effects in skeletal muscle cells, such as promoting increased glucose and fatty acid uptake and utilization [1,2]. However, there are concerns regarding the increased consumption of caffeine as it has also been shown to have side effects that could negatively impact skeletal muscle growth and development [3,4]. Caffeine has previously been shown to promote programmed cell death (apoptosis) in numerous mammalian cell lines when administered at higher doses (2–25 mM) [5–13]. In addition, caffeine has been shown to significantly reduce mitochondrial respiration when administered in higher doses [14]. Thus far, however, there is little information available regarding the potential negative effects of caffeine in mammalian skeletal muscle cells.

The endoplasmic reticulum (ER) is the primary location for folding of newly synthesized proteins and also serves as the largest storage vesicle for endogenous calcium stores [15]. Prolonged or frequent increases in intracellular calcium concentrations can lead to ER stress in skeletal muscle cells [15,16]. The induction of ER stress is characterized by reduced calcium concentrations in the ER lumen, which can interfere with proper protein folding and thus result in the accumulation of unfolded proteins in the ER [16]. Caffeine increases the calcium-sensitivity of the luminal domain of the reticular ryanodine receptor (RyR), which ultimately results in reduced luminal calcium concentrations in the ER [17]. It remains unclear, however, if caffeine can promote ER stress in mammalian skeletal muscle cells.

To combat the accumulation of unfolded proteins due to ER stress, eukaryotic cells initiate the unfolded protein response (UPR) [18]. The initiation of the UPR is characterized by the increased expression of the molecular chaperone 78 kDa glucose-regulated protein (GRP78) and other ER stress markers including the activating transcription factor-6 (ATF-6) and ATF-4 [19]. Prolonged or severe ER stress can result in the increased expression of C/EBP homologous protein (CHOP), a primary mediator of ER stress-dependent cell death [20]. In addition, ER stress has been shown to increase the phosphorylation of the stress-activated serine/threonine kinase c-Jun N-terminal kinase (JNK), thus increasing JNK activity [20]. Activated JNK directly phosphorylates the outer mitochondrial membrane protein Sab (SH3 homology associated BTK binding protein), which directly inhibits mitochondrial respiration and promotes caspase-dependent cell death [21]. Previous investigations have revealed that cytotoxic stress induced by ER stress-promoting agents, certain pharmaceuticals, and even saturated fatty acids can promote a JNK-dependent increase in cell death and reduction in mitochondrial respiration [21–25]. However, it remains unclear if JNK can promote a concomitant increase in caspase-dependent cell death and reduced mitochondrial respiration in mammalian skeletal muscle cells.

Our preliminary experiments revealed that treating multinucleated C2C12 myotubes with 5 mM caffeine for 6 h significantly increased the fragmentation of Hoechst stained nuclei

(unpublished data). In addition, we observed a significant reduction in mitochondrial respiration in myotubes treated with 5 mM caffeine for 6 h vs. control cells (unpublished data). Therefore, the purpose of this study was to investigate the mechanism by which caffeine promotes cell death and reduces mitochondrial respiration in mammalian skeletal muscle cells. We specifically examined the role of JNK in mediating increasing cell death and reducing mitochondrial respiration in skeletal myotubes. Our results suggest that JNK partially mediated the increase in caspase-dependent cell death but did not contribute to reduced mitochondrial respiration in caffeine-treated skeletal myotubes.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), horse serum (HS), penicillin/streptomycin (pen/strep), and Dulbecco's modified Eagle's medium (DMEM) were purchased from Life Technologies (Grand Island, NY, USA). Primary antibodies for GRP78, ATF6, ATF4 and CHOP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies for P-JNK, JNK and caspase-3 were purchased from Cell Signaling Technology (Beverly, MA, USA). The inhibitors Z-DEVD-FMK and SP600125 (JNKi) were purchased from Santa Cruz Biotechnology. Dantrolene was purchased from EMD Millipore (Darmstadt, Germany). All secondary antibodies were purchased from Vector Laboratories (Burlingame, CA, USA).

2.2. Cell culture

C2C12 myoblasts were purchased from ATCC (Manassas, VA, USA). Myoblasts were grown to confluence in normal growth media (DMEM plus 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin) at 37 °C in a water-saturated atmosphere of 5% CO₂. To promote differentiation of mono-nucleated myoblasts into multi-nucleated myotubes the media was switched to differentiation media (DMEM plus 2% HS, 100 U/ml penicillin, 100 µg/ml streptomycin). Fully differentiated myotubes were normally present upon 5–6 d incubation in differentiation media.

2.3. TUNEL assay

Myoblasts were seeded on 35 mm dishes that contained a 1.5 mm thick collagen coated cover-slips (MatTek Corporation, Ashland, MA, USA) in normal growth media. Triplicate groups of myoblasts or myotubes either received vehicle or 5 mM caffeine for 6 h. A terminal transferase dUTP nick end-labeling (TUNEL) assay was conducted to evaluate DNA fragmentation according to the manufacturer's protocol (Roche Molecular Biochemicals, Pleasanton, CA, USA). Nuclei were stained with 2 µl/ml of Hoechst 3334 for 30 min at 37 °C. All samples were washed in TBS and fixed with 4% paraformaldehyde for 20 min. Samples were analyzed using a Nikon Eclipse TS100-F microscope with a 40× fluorite objective and NIS Elements Br software (Nikon Instruments, Melville, NY, USA). Cell death was calculated as the percentage of TUNEL positive nuclei compared to total number of Hoechst stained nuclei.

2.4. Cell death ELISA

Myoblasts were plated on 6-well collagen coated plastic plates (1×10^5 cells/well) in normal growth media and grown to approximately 75% confluence. Upon confluence, all cells were switched to differentiation media for 5–6 d submitted to vehicle or caffeine treatment for 6 h. At the conclusion of each respective trial all cells were subjected to subcellular fractionation according to the manufacturer's protocol (Roche Life Sciences). Cytoplasmic fractions ($n = 6$) were analyzed for the presence of nucleosomes using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Roche Life Sciences).

2.5. Subcellular fractionation

The mitochondrial and cytoplasmic fractions of vehicle and 5 mM caffeine treated myotubes ($n = 6$) were isolated using a subcellular fractionation kit according to the manufacturer's instructions (Thermo Fisher Scientific, Grand Island, NY). Mitochondrial and cytoplasmic fractions were prepared and analyzed using quantitative western blotting as described below.

2.6. Western blotting

Cell lysates were collected in lysis buffer ($1 \times$ TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, 2 mM PMSF, 1 mM sodium orthovanadate, 10 μ l protease inhibitor cocktail), immediately snap frozen in liquid nitrogen, and stored at -20° . All samples were vortexed and centrifuged at 15,000 g for 15 min at 4°C and the supernatants were collected. The total protein concentration of each sample was determined using a Bradford assay. Western blot analysis was conducted using a previously described protocol [26]. Blots were developed using standard ECL detection and images were acquired on a FluorChem E System imager (ProteinSimple, Santa Clara, CA, USA). Digital images were analyzed using ImageJ 1.47v (National Institutes of Health, Bethesda, MD, USA).

2.7. Seahorse metabolic analyzer analysis

Cells were cultured and subjected to treatments as described above in a Seahorse XFp 8-well microplate cartridge. Two of the wells were calibration controls that contained only media, and the remaining wells contained cells. The cellular oxygen consumption rate (OCR) was measured in a Seahorse XFp metabolic flux analyzer (Seahorse Bioscience, North Billerica, MA, USA). To evaluate mitochondrial function, OCR was measured in the presence of (1) only DM to measure basal rates, (2) 1 μ M oligomycin to inhibit the ATP synthase and measure OCR from the combination of mitochondrial proton leak and non-mitochondrial oxygen consumption, (3) 2 μ M FCCP to uncouple mitochondria and measure maximal OCR, and (4) 0.5 μ M rotenone/antimycin A to inhibit the electron transport system and measure only non-mitochondrial OCR. Only the basal and maximal rates are reported for the present study.

2.8. Statistical analysis

Each experiment was repeated at least three times. Statistical analysis was conducted using a student's t-test or a one-way ANOVA with subsequent post-hoc analysis, as appropriate. A P-value <0.05 was considered significant.

3. Results and discussion

3.1. Caffeine promoted cell death in a dose-dependent manner in multinucleated skeletal muscle cells

First we sought to determine if caffeine could promote cell death in mononucleated myoblasts, as well as multinucleated myotubes. Myoblasts and myotubes were cultured on glass coverslips, treated with vehicle or 5.0 mM caffeine for 6 h and analyzed using a TUNEL assay. Our results confirmed that 5.0 mM caffeine significantly increased cell death as the level of nuclear DNA fragmentation increased by approximately 65% in caffeine vs. control myotubes (Fig. 1A). However, caffeine treatment did not significantly affect the level of nuclear DNA fragmentation in myoblasts. These results strongly suggest that undifferentiated myoblasts may be more resistant to caffeine-induced stress. This conclusion is in agreement with a previous study by Yi et al. [27], which found that C2C12 myoblasts were largely unresponsive to caffeine due to the low level of endogenous RYR expression in myoblasts prior to differentiation. Yi et al. [27] found a significant increase in RYR expression upon myoblast differentiation, which correlated to increased caffeine sensitivity in fully differentiated myotubes. The findings of Yi et al. [27] likely explain why, in the present study, caffeine treatment was only able to significantly increase cell death in myotubes. Our results suggest that caffeine-dependent cell death may be linked to the strong influence of caffeine on the luminal calcium sensitivity of the RYR in skeletal muscle cells. Together these results strongly suggest that caffeine promotes a dose-dependent increase in cell death in multinucleated, but not mononucleated, skeletal muscle cells.

Next, we examine the dose-dependent effect of caffeine in promoting cell death in fully differentiated myotubes, after which the cytoplasmic fractions were isolated and analyzed for the presence of nucleosomes using a cell death ELISA. Our results indicate that treating myotubes with 1.0, 2.5 or 5.0 mM caffeine resulted in an approximate 3-fold increase in cell death vs. vehicle treated cells (Fig. 1B). Our results are similar to those of previous investigations, which found that treating cells with higher doses of caffeine (2–25 mM) significantly increased cell death [5–13].

3.2. Caspase inhibition prevented cell death but failed to rescue mitochondrial respiration in caffeine treated myotubes

Previous investigations have demonstrated that caffeine can increased type I cell death in various mammalian cell lines by promoting the loss of mitochondrial membrane potential, cytochrome c release, apoptosome formation and a resultant increase in executioner caspase expression (cleavage) and activity [8,10]. To determine if caffeine promoted type I cell death in skeletal muscle myotubes in we first examined the level of cytochrome c in the cytoplasmic fractions of control and caffeine treated myotubes. We discovered a 40% increase in cytoplasmic cytochrome c levels in caffeine vs. vehicle (control) myotubes (Fig.

2A). In addition, we discovered a 65% increase in cleaved caspase-3 levels in caffeine vs. vehicle treated cells (Fig. 2B). To further examine the role of caspase-3 in mediating caffeine-dependent cell death we utilized the specific peptide inhibitor of executioner caspases Z-DEVD. Western blot analysis was used to verify that 10 μ M Z-DEVD was effective in inhibiting the caffeine-dependent increase in cleaved caspase-3 (Fig. 2C). The addition of 10 μ M Z-DEVD completely inhibited the caffeine-dependent increase in cell death as assessed by cell death ELISA (Fig. 2D). To determine if caffeine-dependent cell death was due to increased RYR sensitivity we treated myotubes with 400 μ M dantrolene, a specific inhibitor of RyR-mediated calcium release. This specific dose of dantrolene was chosen based on previous studies that employed dantrolene to inhibit caffeine-dependent calcium release in C2C12 skeletal myotubes [28,29]. Cell death ELISA analysis revealed that the addition of dantrolene completely inhibited caffeine-dependent cell death in myotubes (Fig. 2D). Our results strongly suggest that caffeine promoted a calcium-dependent increase in type I cell death by increasing the luminal calcium sensitivity of the RYR in skeletal myotubes.

Our next objective was to determine if the caffeine dependent reduction in mitochondrial respiration was due, at least in part, to the significant increase in cell death. A mitochondrial stress test revealed a 20% reduction in basal and 33% reduction in maximal OCR (Fig. 2E–F). The addition of 10 μ M Z-DEVD did not prevent the caffeine-dependent reduction in basal and maximal OCR (Fig. 2E–F). These data suggests that the caffeine-dependent reduction in mitochondrial respiration was not merely due to increased cell death. However, the addition of 400 μ M dantrolene completely inhibited the caffeine-dependent reduction in OCR (Fig. 2E–F). Previous research demonstrated that 1–2 mM caffeine promoted mitochondrial permeability transition MTP by increasing mitochondrial calcium uptake, which significantly increased mitochondrial depolarization and swelling [14]. These events can lead to increased cytochrome c release and caspase activation while simultaneously leading to reduced mitochondrial respiration [14,30]. Therefore, we conclude that the increased cell death and reduced mitochondrial respiration observed in the current study most likely due to increased luminal calcium sensitivity of RYR, which resulted in increased reticular calcium release and mitochondrial calcium uptake in skeletal myotubes.

3.3. Caffeine significantly increased the expression of select ER stress markers in skeletal muscle cells

To determine if caffeine treatment promoted ER stress in skeletal muscle cells we examined the expression of specific markers of ER stress. Western blot analysis revealed a dose-dependent increase in GRP78, ATF-6 and ATF-4 protein expression (Fig. 3A–C). However, we found that caffeine treatment did not significantly increase the expression of the ER-stress cell death mediators CHOP and caspase-12 (Fig. 3D–E). A previous investigation by Hosoi et al. [31] found that pretreating human neuroblastoma (SH-SY5Y) cells with 0.1–3 mM caffeine for 1 h significantly reduced the expression of ER stress makers PERK and inositol-requiring enzyme 1 (IRE1) upon 4 h incubation in 1 μ g/ml tunicamycin. Similarly, Zheng et al. [32] found that feeding zebrafish larvae a high-fat diet supplemented with 5% (w/w) caffeine significantly reduced hepatic PERK, IRE1, ATF-6 and GRP78 mRNA expression compared to larvae fed a high-fat diet alone. Our results strongly contrast those

of Hosoi et al. [31] and Zheng et al. [32] as 2.5–5 mM caffeine significantly increased ER stress in skeletal myotubes. To the best of our knowledge, the present study is the first to document a caffeine-dependent increase in ER stress in mammalian skeletal myotubes.

3.4. JNK inhibition partially attenuated cell death but did not prevent reduced mitochondrial respiration in caffeine treated myotubes

To investigate the role of JNK in mediating caffeine-dependent cell death and mitochondrial respiration we conducted a series of experiments in which myotubes were treated with vehicle or 5 mM caffeine in the presence or absence of 30 μ M JNKi. Western blot analysis revealed that caffeine treatment significantly increased JNK phosphorylation, which was completely inhibited by the addition of JNKi (Fig. 4A). Further, the addition of JNKi partially attenuated the increase in caffeine-dependent cell death as assessed by cell death ELISA (Fig. 4B). However, the addition of JNKi did not prevent the caffeine-dependent reduction in basal and maximal OCR (Fig. 4C–D). These results are somewhat similar to those of Win et al. [24], which demonstrated that even though tunicamycin increased the activation of the canonical ER stress cell death pathway, cell death was completely dependent upon increased JNK phosphorylation. In contrast to Win et al. [24], we found that the addition of JNKi did not prevent the caffeine-dependent reduction in basal and maximal OCR. We believe the absence of increased CHOP or cleaved capase-12 expression and the fact that JNK can contribute to calcium-dependent cell death in an ER stress independent manner [33] provides additional evidence that increased cell death and reduced mitochondrial respiration were most likely due to a caffeine-dependent increase in mitochondrial calcium uptake. Further research is needed to confirm the role of mitochondrial calcium uptake in mediating cell death and respiration in caffeine treated skeletal myotubes.

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Fig. 1.

Caffeine promoted cell death in a dose-dependent manner in multinucleated skeletal muscle cells. (a) C2C12 myoblasts and fully differentiated myotubes were treated with either vehicle (control) or 5 mM caffeine for 6 h ($n = 3$). A terminal transferase dUTP nick end-labeling (TUNEL) assay was conducted to examine the effect of caffeine on nuclear DNA fragmentation (TUNEL positive nuclei). Treating myotubes with caffeine promoted a 65% increase ($P < 0.05$) in TUNEL positive nuclei when compared to controls. However, caffeine treatment did not significantly increase the level of TUNEL positive nuclei in myoblasts. (b) Fully differentiated C2C12 myotubes were either treated with 0, 0.25, 0.5, 1.0, 2.5 or 5.0 mM caffeine for 6 h ($n = 6$). Cytoplasmic fractions were isolated and analyzed for the presence of nucleosomes using a cell death ELISA. Caffeine significantly increased cell death in 1.0, 2.5 and 5.0 mM caffeine treated myotubes. (* $P < 0.05$).

Fig. 2.

Caffeine promoted cell death in a caspase-dependent manner and reduced mitochondrial respiration in a caspase-independent manner in skeletal myotubes. Fully differentiated C2C12 skeletal muscle cells were either treated with vehicle (DMSO, control) or 5 mM caffeine for 6 h (n = 6). (a–b) Western blot analysis revealed a 40% increase in cytoplasmic cytochrome c levels and a 65% increase in cleaved caspase-3 expression in caffeine vs. vehicle (control) cells. (c) The addition of 10 μ M Z-DEVD-FMK (Z-DEVD, executioner caspase inhibitor) inhibited the caffeine-dependent increase in cleaved caspase-3. (d) Cell death ELISA analysis revealed that the addition of either Z-DEVD or 400 μ M dantrolene completely inhibited the caffeine-dependent increase in cell death. (e–f) Assessment of mitochondrial function following 6 h of caffeine incubation revealed a significant caffeine-induced reduction in basal and maximal oxygen consumption rate (OCR) that was prevented by the addition of dantrolene, but not Z-DEVD. (*P < 0.05).

Fig. 3.

Caffeine significantly increased the expression of select ER stress markers in skeletal myotubes. Fully differentiated C2C12 skeletal muscle cells were either treated with vehicle (DMSO, control) or 5 mM caffeine for 6 h (n = 6). **(a–c)** Western blot analysis revealed that caffeine treatment promoted a dose dependent increase in 78 kDa glucose-regulated protein (GRP78), activating transcription factor-6 (ATF-6) and ATF-4. **(d–e)** However, caffeine treatment did not significantly increase the expression of C/EBP homologous protein (CHOP) or cleaved caspase-12. (*P < 0.05).

Fig. 4.

Inhibiting c-Jun N-terminal kinase (JNK) partially attenuated cell death but did not prevent reduced mitochondrial respiration in caffeine treated skeletal muscle cells. Fully differentiated C2C12 skeletal muscle cells were either treated with vehicle (DMSO, control) or 5 mM caffeine for 6 h (n = 6). **(a)** Western blot analysis revealed that caffeine treatment significantly increased the phosphorylation of JNK (P-JNK) and that adding 30 μ M of the specific JNK inhibitor SP600125 (JNKi) completely inhibited the caffeine-induced increase in P-JNK. **(b)** Cell death ELISA analysis revealed that the addition of JNKi partially inhibited the caffeine-dependent increase in cell death. **(c–d)** Seahorse metabolic analysis revealed that adding JNKi did not prevent the caffeine-induced reduction in basal and maximal oxygen consumption rate (OCR). ^{a, b, c} Indicate significant difference between treatments with out same letters (*P < 0.05).