Caffeine’s Effects On Skeletal Muscle Bioenergetics In Vitro

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ABSTRACT
The purpose of this investigation was to study the effects of caffeine on respiration rate and utilization of glucose, glutamine, and fatty acids in C2C12 mouse skeletal muscle cells. Treatment of cultured C2C12 cells with 1 mM caffeine for 24 hours significantly (p<0.05) decreased the basal and maximal oxygen consumption rate (OCR), and therefore respiratory capacity when compared to controls. In addition, caffeine decreased the extra-cellular acidification rate (ECAR), indicating a reduction in the rate of anaerobic glycolysis. Measurement of OCR through the use of a metabolic assay analyzer also revealed a significant (p<0.05) decrease in glucose oxidation and fatty acid oxidation in response to caffeine. However, caffeine induced an increase in fatty acid oxidation as a percentage of total fuel usage. The reduction in OCR and ECAR following caffeine treatment suggests that this drug likely inhibits ATP turnover or induces programmed cell death in C2C12 muscle cells.

Caffeine is the most widely used drug in the western world (Graham, 2001). Approximately 75% of consumed caffeine comes in the form of coffee and this precious liquid is second only to oil in dollar amount traded annually (Keisler and Armsey, 2006). Once ingested, caffeine is rapidly absorbed in the gastrointestinal tract (Blanchard and Sawyers, 1983) and disseminated throughout the entire body (Axelrod and Reisenthal, 1953), and it is known to cause numerous physiological and neurological effects (Ritchie et al., 2007, Davis et al., 2002, Bell et al., 1998, Daniels et al., 1998). Caffeine has been used to enhance athletic performance since the 1920s (Jacobson and Kulling, 1989) and it is an exceedingly popular mental stimulant (Wesnensten et al., 2005). However, the cellular ramifications of chronic caffeine use remain ambiguous, even though use of caffeine is approaching record highs (Keisler and Armsey, 2006, Spriet, 1995).

The effects caffeine imposes on those who ingest it are intimately tied to cellular respiration (Bracco et al., 1995, Robertson et al., 1978). During cellular respiration, oxygen, and fuel molecules such as fatty acids, glucose, and glutamine are consumed by mitochondria to produce adenosine triphosphate (ATP)—the cellular unit of energy (Aschenbach et al. 2002). If nutrient availability is not a limiting factor, cells that process oxygen at higher rates can produce more ATP. For example, the primary limiting factor during exercise is the rate at which muscle cells consume oxygen to meet the body’s elevated energy (Bassett and Howley, 2000). When energy demand is not met by aerobic respiration, anaerobic fermentation supplies the required ATP, but it is less efficient at making ATP and also produces lactic acid as a byproduct (McArdle et al. 2010). Lactic acid production from the anaerobic process of glycolysis acidifies the interior of the cells and releases free H+ ions into the extracellular fluid (Owicki and Parce, 1992).
Glycolytic activity can therefore be measured by observing alterations in the H⁺ ion concentration in the extracellular fluid immediately surrounding cells (Lardner, 2000). Together, measurement of the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) provides a useful indicator of the bioenergetics status of skeletal muscle cells (Wu et al. 2007). Therefore, this work employed a measurement technique to evaluate the effect of caffeine on aerobic and anaerobic metabolism in C2C12 cells.

Caffeine has been found to influence OCR and metabolism (Bauer et al. 2001). Long-term administration of caffeine in preterm infants is associated with an increase in oxygen consumption. Another study conducted by Chad and Quigley (1989) saw oxygen consumption and metabolic rate in female subjects increase significantly 1 hour after caffeine ingestion. Caffeine has also been shown to activate the AMP activated protein kinase (AMPK), which in turn leads to mitochondrial biogenesis, and therefore an increase in cell metabolic rate (Mathew et al., 2014). Additionally, skeletal muscle mitochondria primarily oxidize three key fuels: long chain fatty acids, glucose, and glutamine (Kelley and Mandarino, 2000). There is evidence that caffeine alters substrate utilization (Raney and Turcotte, 2008, Arciero et al., 1995). Inhibition of these fuels yields changes in respiration and therefore differences between these changes observed in cells treated and untreated with caffeine provide data on how caffeine affects oxidation of key metabolic fuels.

The present study evaluated the effects of caffeine exposure on the bioenergetic status of C2C12 skeletal muscle cells. We tested the hypotheses that 24 h of 1 mM caffeine treatment would increase the overall rate of respiration in C2C12 cells, due to its stimulatory effects on certain cell types, and that caffeine treatment would increase use of glucose as a metabolic fuel over fatty acids and glutamine. The widespread use of caffeine, including among children whose muscles are still developing, make understanding its effects on muscle metabolism an important public health issue.

**METHODS**

**Cell Culture**

C2C12 mouse skeletal muscle cells (myoblasts) were acquired from ATCC (ATCC CRL-1772, Manassas, VA, USA). Cells were grown at 37 °C in 5% CO₂ and cultured under sterile conditions in growth medium [Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum, penicillin/streptomycin]. Proliferation of the cells was monitored using a tissue culture light microscope to determine confluence. To permit passage of cells between containers, adherent cells were dissociated from the vessel in which they were cultured using the proteolytic enzyme trypsin.

**Bioenergetic Assay**

Oxygen Consumption Rate and Extracellular Acidification Rate

C2C12 myoblasts were plated on XFp microplates (Seahorse Bioscience, North Billerica, MA, USA) at a density of 10,000 cells/well in growth medium and cultured at 37 °C in 5% CO₂. Cells reached ~90% confluence after 1 day, at which time growth media was removed and replaced with differentiation media (DMEM with 2% horse serum). After 5 days of serum restriction, multinucleated myotubes formed and were ready for treatment. Fresh media was administered every 24 hours. To determine the mitochondrial and glycolytic function in C2C12 muscle cells, a cell mitochondrial stress assay was conducted using a Seahorse XFp Extracellular Metabolic Flux Analyzer. After myocytes had differentiated 5 days, cells were treated with 1 mM caffeine in differentiation media for 24 hours. After 21 hours of the incubation period, cell media was replaced with non-bicarbonate, pH 7.4 assay media (XF Assay Medium, Seahorse Bioscience), supplemented with 25 mM glucose, and 4 mM sodium pyruvate, and incubated for the final 3 hours in a non-CO₂
incubator. The same media was used as vehicle for the compounds that were injected as part of the assays. The XFp Analyzer was then used to measure the cell oxygen consumption rate (OCR) in pmol O$_2$/min and extracellular acidification rate (ECAR) in mpH/min. OCR and ECAR values measure rates of mitochondrial respiration and glycolytic activity, respectively.

Two types of assays were run to analyze OCR and ECAR data from skeletal muscle cells: the Mitochondrial Stress assay and Mitochondrial Fuel Flex assay (Seahorse Biosciences). In the case of the Mitochondrial Stress assay, the XFp analyzer sequentially injected the following compounds into the assay medium: oligomycin (1 µM final concentration), which blocks the ATP synthase and shows the OCR dedicated to ATP production; carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP: 4 µM final concentration), which permeabilizes the inner mitochondrial membrane and shows maximal OCR; and rotenone/antimycin A (1 µM final concentration for each), which blocks complex I and III showing non-mitochondrial OCR. Each assay used one Seahorse Bioscience 8-well microplate; 3 wells for each of the two treatment groups (control and 1 mM caffeine), and 2 calibration control wells that contain only assay media. Two plates of cells were analyzed for each parameter that was tested (n=6). OCR and ECAR were measured at three time points during basal respiration and after each drug injection.

**Metabolic Fuel Utilization**

The second assay type conducted was the Mitochondrial Fuel Flex assay and can be described as follows. The analyzer sequentially injected mixtures of the following compounds into the assay medium: UK5099 (2 µM final concentration), which inhibits the mitochondrial pyruvate carrier and thus the glucose oxidation pathway, BPTES (3 µM final concentration), which inhibits the enzyme glutaminase, subsequently preventing oxidation of glutamine, and etomoxir (4 µM final concentration), which inhibits carnitine palmitoyl-transferase 1A, an essential enzyme for translocating long chain fatty acids from cytosol into mitochondria for oxidation, which therefore inhibits long chain fatty acid oxidation. By selectively inhibiting the oxidation of glucose, glutamine or fatty acids while measuring the OCR, the contribution of each fuel to total OCR of cells can be determined. Therefore, one can measure the effects of caffeine treatment on cellular fuel preference. Just as with the Cell Mitochondrial Stress assay, there were 3 wells per treatment group (control and 1 mM caffeine), and three plates were used per experiment (n=9).

**Data Analysis**

All data were entered into Microsoft Excel, which was used to produce graphs and calculate mean values for OCR and ECAR. Results are reported as means ± SE. Data were analyzed using ANOVA and paired t-test in JMP Pro 12. Statistical significance was determined if p < 0.05.

**RESULTS**

**Effect of Caffeine on Mitochondrial OCR**

The effects of caffeine on skeletal muscle OCR and ECAR during a mitochondrial stress assay are shown in Figure 1. There was a significantly (p<0.05) lower mean OCR initially (basal OCR) and after addition of FCCP (maximal OCR) (Figure 1A). A summary of the data collected for OCR is presented in Figure 2, where every measure of mitochondrial function had a lower mean value in the caffeine treated cells than in the control. However, only the basal and maximal OCR and the aerobic scope (difference between maximal OCR and basal OCR) were significantly (p<0.05) lower in the caffeine treated cells. Similarly, mean ECAR was noticeably lower in the caffeine treated cells where every measurement reflected a lower mean value (Figure 1B). However, only the ECAR measured immediately after FCCP addition was significantly (p<0.05) lower in
caffeine treated cells compared to the control.

**Effects of Caffeine on Metabolic Fuel Utilization**

To understand caffeine’s effects on fuel utilization of the three major energy sources for skeletal muscle cells (glucose, glutamine, fatty acids) the OCR was measured while sequentially inhibiting each fuel. The subsequent drop in OCR following inhibition of a particular fuel is indicative of the energy obtained from oxidizing that fuel in respiration. The subsequent addition of inhibitors of the other two fuel sources caused an additional drop in OCR, and the total decrease in OCR can be used to calculate the percent contribution of each fuel to basal OCR. OCR measurements during addition of inhibitors for each fuel is shown in Figure 3, and a summary of the data for each fuel is shown in Figure 4. There was a significant (p<0.05) reduction in utilization of glucose and fatty acids in the caffeine treated cells, and mean glutamine utilization also was lower in the caffeine treated group, although not significantly (p>0.10) so (Figure 4A). The percent contribution of glucose to total fuel utilization was lower in caffeine treated cells, while fatty acid oxidation represented a significantly (p<0.05) larger contribution to total fuel use after caffeine treatment (Figure 4B). Together, the collected data showed that fatty acids were the most substantial source of cellular energy, followed by glutamine, with glucose as the least employed source of cellular energy among the three examined fuels. Caffeine caused a relative increase in dependence on fatty acids.

**DISCUSSION**

**Effect of Caffeine on Mitochondrial OCR**

Basal oxygen consumption rate in C2C12 myocytes treated with caffeine was significantly lower than that of the untreated control muscle. Caffeine’s ability to decrease calcium (Ca^{2+}) sequestering by the endoplasmic reticulum (ER) (Krizaj et al., 1999, Blinks et al., 1972, Smits et al., 1985, Schoppe et al., 1997) may have had an effect on the observed decrease in mitochondrial OCR. Excessive Ca^{2+} release and increase in cytosolic Ca^{2+} causes ER stress and could have led to the decreased OCR observed (Mekahli et al., 2011). Additionally, all OCR measurements for caffeine-treated cells exhibited lower OCR than the control, which points toward two likely conclusions. The first is that caffeine imposes a direct effect on cellular energy needs in C2C12 cells. This could have occurred by directly affecting mitochondrial function. Caffeine has been shown to hinder mitochondrial capacity to recover membrane potential (Sardão et al., 2002) and the existence of concentration gradients across mitochondrial membranes is of considerable functional importance (Clausen et al., 1991). Altering mitochondrial ability to maintain these gradients could reduce their ability to produce ATP and lead to the overall OCR drop that was observed in caffeine-treated cells compared to the control. The second possible explanation is that the caffeine treatment irreversibly altered cell metabolic function. Damaged ability to metabolize available fuels could have affected the viability of cells. According to Fernandez et al. (2003) concentrations of caffeine promote cell death in a dose-dependent manner. One investigation found that inhibiting AMP activated protein kinase in the presence of caffeine treatment significantly reduced autophagy in C2C12 cells (Mathew et al., 2014). Therefore, if the cause of decreased mean OCR in caffeine treated cells resulted from autophagy, AMPK activity likely played a part. This could have been an adverse effect of the 24-hour caffeine treatment, thus leaving fewer viable cells to produce ATP, and could explain the decrease in caffeine treated cell mean OCR. This would also explain the similar decrease in mean ECAR measured in caffeine treated cells.

**Effect of Caffeine on Metabolic Fuel Use**

C2C12 cells treated with caffeine showed a significantly increased utilization of long chain fatty acids and a significant decrease in
Figure 1: (A) Oxygen consumption rate (OCR) of caffeine treated and control muscle cells (n=6). Caffeinated cell OCR shows a significant (p<0.05) drop in both basal and maximal (after FCCP addition) OCR. (B) Extracellular acidification rate (ECAR) of caffeine treated cells also showed a decrease during maximal respiration (after FCCP addition) and lower ECAR values throughout (though not all were statistically significant).
Figure 2: Basal (A) and maximal (B) OCR were significantly (p<0.05) lower in caffeine treated cells (n=6). Aerobic scope, which is the difference between basal and maximal OCR, was also significantly (p<0.05) lower in the caffeine treated cells (C). Proton leak (D) and the amount of OCR dedicated to ATP production during basal metabolism (E) were both comparable (p>0.25) between control cells and those treated with caffeine.
Figure 3: (A) Oxidation of glucose as measured by OCR (n=9). The addition of UK 5099 inhibited glucose oxidation, then fatty acid and glutamine oxidation were inhibited by Etomoxir and BPTES. (B) Oxidation of glutamine in OCR (n=9). The addition of BPTES inhibited glutamine oxidation, then glucose and fatty acid oxidation were inhibited by UK5099 and Etomoxir. (C) Oxidation of fatty acids in OCR (n=9). The addition of Etomoxir inhibited fatty acid oxidation, then glucose and glutamine oxidation were inhibited by UK5099 and BPTES.
Figure 4: (A) Analyses of absolute glucose, glutamine, and fatty acid oxidation in caffeine treated and control cells (n=9). Caffeine induced a significant (p<0.05) decrease in the absolute rate of glucose and fatty acid oxidation. (B) % of total oxidative metabolism of each key fuel in caffeine treated and control cells (n=9). Caffeine also induced a significant (p<0.05) increase in fatty acid oxidation and a decrease in glucose oxidation as a percentage of the total fuel use.
glucose oxidation, when examined as a percentage of total fuel use (Figure 4B). Caffeine has been shown to indirectly increase glucose uptake and fatty acid utilization by activating a calcium-induced AMPK dependent pathway (Raney and Turcotte, 2008). AMPK activity has been shown to increase fatty acid oxidation in human skeletal muscle as well (Wojtaszewski et al., 2003). Similar to the pattern seen in the mitochondrial stress assay, every OCR measurement of all 3 fuel substrates was decreased (though not always significantly) in caffeine treated cells compared to the control. The results of a study by Vaughan et al. (2012) found that low caffeine doses can significantly increase skeletal muscle metabolism, while higher doses can limit this increase. Numerous studies have been done analyzing in vivo effects of caffeine on metabolic fuel utilization; however, countless factors and pathways modulate cellular caffeine responses in whole body organisms (Echeverri et al., 2010, Acheson et al., 2004, Costill, et al., 1978). Some of caffeine’s reported effects on substrate utilization in these studies reflect a similar pattern of decreased glucose and increased fatty acid utilization (Arciero et al., 1995). Thus, the findings of this study are in agreement with similar findings in primary literature, although very little research has been conducted examining the explicit effects of caffeine on mitochondrial substrate utilization in skeletal muscle in vitro. One study conducted by Ukropcova et al. (2005) which collected data in vitro and in vivo subjects found that in vivo insulin sensitivity was related to a higher in vitro capacity for lipid oxidation. However, because caffeine reacts synergistically with epinephrine (Butcher et al., 1968) and causes numerous effects on the somatic nervous system (Krizaj et al., 1999), more research should be conducted examining correlations between in vitro and in vivo studies of insulin resistance.

Future Work

Previous studies have identified caffeine as an AMPK activator and have correlated AMPK activation with increased mitochondrial formation in mammals. However, we plan to further investigate C2C12 exposure to caffeine to confirm its effects on metabolism. We are currently conducting metabolic assays to measure cellular energy allocation. Additionally, metabolic alterations can induce structural changes in cells. Therefore, evaluating the connection between environmental caffeine content and possible influence on skeletal muscle physiology may prove crucial to better understand the drug’s induced cellular effects.

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