Modeling the Effect of Cigarette Smoke on Hexose Utilization in Spermatocytes

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Abstract

We set out to determine whether the addition of an aryl hydrocarbon receptor (AHR) antagonist has an effect on glucose/fructose utilization in the spermatocyte when exposed to cigarette smoke condensate (CSC). We exposed male germ cells to 5 and 40 µg/mL of CSC ± 10 µmol/L of AHR antagonist at various time points. Immunoblot expression of specific glucose/fructose transporters was compared to control. Radiolabeled uptake of 2-deoxyglucose (2-DG) and fructose was also performed. Spermatocytes utilized fructose nearly 50-fold more than 2-DG. Uptake of 2-DG decreased after CSC + AHR antagonist exposure. Glucose transporters (GLUTs) 9a and 12 declined after CSC + AHR antagonist exposure. Synergy between CSC and the AHR antagonist in spermatocytes may disrupt the metabolic profile in vitro. Toxic exposures alter energy homeostasis in early stages of male germ cell development, which could contribute to later effects explaining decreases in sperm motility in smokers.

Keywords

cigarette smoke, spermatocytes, fructose, GLUT9a, GLUT12

Introduction

A growing body of evidence shows that polycyclic aromatic hydrocarbons (PAHs) are toxic to the testis, resulting in impaired spermatogenesis and subfertility.¹⁻⁵ Although it is clear that one such PAH, 2,3,7,8 tetrachlorodibenzo-p-dioxin (ie, dioxins), acts through the aryl hydrocarbon receptor (AHR) to mediate toxicity, the specific mechanisms by which PAHs exert their toxic effect remain uncertain.^{3,6-8}

Cigarette smoke, composed of over 4000 chemicals including known carcinogens such as PAHs,⁹ has been linked to aberrant spermatogenesis and semen parameters associated with male factor infertility.¹⁰⁻¹³ Furthermore, cigarette smoke is known to induce biological changes that can lead to abnormalities in a smoker's progeny.^{14,15}

Cigarette smoke consists of 2 phases: a gas phase and a particulate phase known as cigarette smoke condensate (CSC). Upon inhalation, both forms are absorbed into the systemic circulation and accumulate in seminal plasma in the male reproductive tract, either by diffusion or by active transport. This results in alterations in the concentration, motility, and morphology of sperm, changes in the testicular hormonal environment,¹⁶ and DNA damage.^{13,17-21}

Aryl hydrocarbon receptor agonists have been shown to disrupt hexose metabolism in various tissues both in vivo and in vitro.^{8,22-25} Mammalian testes and spermatozoa are known to utilize various glucose transporters (GLUTs)²⁶⁻²⁹ that serve

to transport glucose and/or fructose for critical sperm cell metabolism and function.^{27,28,30,31}

We have recently shown that CSC induces oxidative stress, DNA damage, and seminiferous tubule cell apoptosis in mice, ultimately leading to altered sperm motility. We also examined the effect of CSC exposure on a spermatocyte cell line, which represents the premeiotic stage of spermatogenesis (eg, primary spermatocytes). These cells exhibited oxidative damage and upregulation of several antioxidant genes.¹⁻⁵ Disruption of spermatocyte energy utilization may be the mechanism responsible for the abnormal semen phenotypes seen in chronic male smokers.

Identifying the mechanism of toxic injury to male germ cells will lead to a better understanding of how toxicants contribute to infertility in men. Therefore, we hypothesized that 1 reason for the damage observed in spermatocytes might be that CSC

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exposure results in disruptions of energy homeostasis. Here we have assessed that possibility by measuring uptake of glucose and fructose in CSC-treated spermatocytes. Additionally, we have examined the effect of CSC on expression of several hexose transporters (called GLUTs) expressed in the spermatocytes. We examine the role that blockage of the AHR receptor has on energy utilization in the spermatocyte. Finally, we provide direct observation of CSC effects on adenosine triphosphate (ATP) production in spermatocytes.

Materials and Methods

Cell Culture and In Vitro CSC Treatment

The murine spermatocyte cell line GC-2spd(ts), American Type Culture Collection (ATCC), Manassas, Virginia,^{32,33} was grown to 70% confluence in ATCC-formulated Dulbecco Modified Eagle Medium (DMEM) plus 10% fetal bovine serum (BSA; ATCC), 100 U/mL penicillin, and 100 U/mL streptomycin (Lonza, Maryland) in an air atmosphere of 5% CO₂ at 37°C. The cells were amplified in complete medium per the manufacturer's instructions and used between passages 5 and 11. Previous cell viability assessments using the trypan blue exclusion test demonstrated no noticeable cytotoxicity under these conditions.⁵ The GC-2spd(ts) cells were then grown to 70% confluence and then incubated with serum-deficient media for 24 hours to avoid any influence of growth factors.

Cigarette smoke condensate (Murty Pharmaceuticals Inc, Lexington, Kentucky) was dissolved in 100% dimethyl sulfoxide (DMSO) to obtain a stock concentration of 40 mg/mL. Spermatocyte treatment with DMSO (0.1%), 5 mg/mL or 40 µg/mL CSC for 18 and 24 hours, was performed based on studies showing CSC effects on AHR at these time points.⁵ Cigarette smoke condensate was purchased commercially. The CSC is prepared using a Phipps-Bird 20-channel smoking machine designed for Federal Trade Commission testing. The particulate matter from Kentucky standard cigarettes (1R3F; University of Kentucky, Lexington, Kentucky) is collected on Cambridge glass fiber filters, and the amount of CSC obtained is determined by increase in weight of the filter. The average yield of CSC is 26.1 mg/cigarette.

Cigarette Smoke Condensate and AHR Antagonist Treatment

Cells were treated with 10 µmol/L of the AHR antagonist CH223191, 2-methyl-2H-pyrazole-3-carboxylic acid-(2-methyl-4-o-tolyl-azophenyl)-amide (EMD Chemicals, Gibbstown, New Jersey), for 1 hour, followed by 5 or 40 µg/mL of CSC for 18 or 24 hours. Unlike other AHR antagonists, such as resveratrol and flavones, which might have weak AHR agonist properties at high concentrations, CH223191 is a pure AHR antagonist.³⁴⁻³⁶

Fructose and Glucose Uptake Assays

The GC-2spd(ts) cells were split into 12-well culture dishes and allowed to attain epithelial morphology with intercellular

contacts. The GC-2spd(ts) cells were then treated as mentioned earlier with CSC ± AHR antagonist for a maximum of 24 hours, immediately after which time uptake of 2-deoxyglucose (2-DG) and fructose was measured. Radiolabeled fructose uptake in GC-2spd(ts) cells was measured according to established methods with the following adaptations.³⁷ Cells were hexose deprived for 10 minutes in Hanks Balanced Salt Solution (HBSS) in a 37°C, 5% CO₂ humidified incubator. The medium was changed to Ringer's phosphate buffer containing 2 mol/L [¹⁴C]-D-fructose or [¹⁴C]-2D-glucose (American Radiochemicals, Inc, St Louis, Missouri) for 2.5 minutes at 37°C. The reaction was quenched in ice-cold HBSS, and cultures were washed in Ringer before lysis in 0.1 N NaOH and 0.1% sodium dodecyl sulfate solution. A portion (70%) of each lysate was counted in Econosafe liquid scintillation fluid (Fisher Scientific, Waltham, Massachusetts). We normalized uptake to cell number (4000 cells per well). Each substrate was assayed in triplicate 3 unique times.

Western Blot

After the treatment regimens, the cells were gently washed with ice-cold phosphate-buffered saline and then scraped into radioimmunoprecipitation assay buffer containing 0.1 mol/L phenylmethylsulfonyl fluoride (Sigma-Aldrich, St Louis, Missouri), 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, and 1× miniprotease inhibitor cocktail (Roche Applied Science, Indianapolis, Indiana) for protein extraction. Cells were lysed by sonication on ice with three 10-s bursts. Whole cell lysates were clarified by centrifugation at 10 000g for 10 minutes at 4°C, and the protein concentration was determined by the bicinchoninic acid assay. Aliquots of 30 µg of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membrane. The blots were probed with rabbit anti-GLUT5 (Abnova, Taiwan), anti-GLUT8,³⁸ anti-GLUT9a,³⁹ and anti-GLUT12 antibodies²⁶ (1:1000) followed by incubation with goat anti-rabbit secondary antibody (1:20 000; SantaCruz Biotech, Santa Cruz, California). Enhanced chemiluminescence reagent (Amersham, Piscataway, New Jersey) was used for detection, and the blots were reprobed with an anti-β-actin antibody for normalization. Fold changes are reported relative to DMSO control. Image J (US National Institutes of Health, Bethesda, Maryland) was used to quantify the density of the bands.

Intracellular ATP Assay for Spermatocytes, GC-2spd(ts)

The concentration of intracellular ATP in the CSC-exposed spermatocytes was measured based on our previously published protocol with slight modifications.⁴⁰ In brief, the spermatocyte cell line GC-2spd(ts), 1 × 10⁶ cells/volume, 10-cm plate, grown in DMEM were serum starved and exposed separately to different concentrations of CSC (5, 20, and 40 mg) and equal volume of DMSO control. The cells exposed for 24 hours were harvested using 2 mL trypsin (containing 0.25% EDTA; Sigma) and equal volume of complete medium

Figure 1. Effects of cigarette smoke condensate (CSC) $\pm$ aryl hydrocarbon receptor (AHR) antagonist on hexose uptake. A, 2-Deoxyglucose (2-DG) uptake by spermatocytes treated with the dimethyl sulfoxide (DMSO; vehicle control), CSC (40 μ g/mL), AHR antagonist, or CSC + AHR antagonist, as indicated. N = 2 experiments in triplicate. B, Fructose uptake by spermatocytes treated with the DMSO (vehicle control), CSC, AHR antagonist, or CSC + AHR antagonist, as indicated. ** indicates $P = .01$ (analysis of variance [ANOVA] compared to DMSO). N = 2 experiments in triplicate. All values are expressed as mean $\pm$ standard error of the mean (SEM).

followed by centrifugation for 2 minutes at 1500 rpm. Supernatant was discarded, the pellet was washed with 1 mL cell dissociation solution (Sigma), and then extracted in 100 μ L of 0.1 N NaOH by homogenization followed by incubation at 80°C for 20 minutes. The homogenate (100 μ L) was neutralized with 50 μ L mixture of 0.15 N HCl and 0.1 mol/L Tris-HCl (pH 6.6) to form 34 mmol/L Tris-HCl (pH 8.1). The extract was used for ATP measurement by enzymatic reaction and the values were normalized to the amount of total protein in the cell lysate.

The assay was carried out using 20 microL of cell extract. Blank, ATP standards, and cell extracts were added to 50 μ L of ATP reagent (60 mmol/L Tris-HCl pH 8.1, 0.03% BSA, 1.5 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, 450 μ mol/L D-glucose, 100 mmol/L nicotinamide adenine dinucleotide phosphate ($NADP^+$), 3 μ g/mL hexokinase without $(NH_4)_2SO_4$, and 1 μ g/mL glucose-6-phosphate dehydrogenase without $(NH_4)_2SO_4$), and the reaction was terminated after 20 minutes at 60°C for 20 minutes by adding 10 mL of 0.5 N NaOH. After adding a 1 mL mixture of 6 N NaOH, 10 mmol/L imidazole, and 0.01% H_2O_2 were heated for 20 minutes and fluorescence was measured using a fluorometer at 340 nm. The enhanced fluorescence of $NADP^+$ is indicative of ATP levels.

Statistical Analysis

One-way analysis of variance followed by the Fisher least significant difference test was used to compare substrate uptake and GLUT expression between experimental treatments and DMSO control. For analysis of the equality of continuous distribution of samples, a Kolmogorov-Smirnov test was used. Statistical significance was defined as P value $< .05$.

Results

Effects of CSC on Fructose and Glucose Uptake

Spermatocytes took up 50-fold more fructose than 2-DG. Exposure to CSC for 24 hours slightly decreased 2-DG uptake while slightly increasing fructose uptake, but the differences were not statistically significant. The addition of an AHR antagonist alone did not significantly alter fructose or 2-DG uptake, but uptake of 2-DG was significantly ($P = .01$) lower and uptake of fructose trended toward a decrease in cells exposed to both CSC and AHR antagonist (Figure 1).

Effects of CSC on Expression of GLUTs

We have previously demonstrated that the GLUT9a and GLUT8 are expressed in mouse spermatocytes *in vivo*.^{27,41} Others have also shown that GLUT5 is a fructose transporter present in spermatocytes *in vivo*.⁴² We thus first confirmed that these GLUTs are all expressed at the protein level in the GC-2spd(ts) cells *in vitro* (Figure 2A). Furthermore, as GLUT12 expression has been shown to be dependent on GLUT8 expression in other systems of fructose transport,³⁷ we examined expression of this protein as well and found its expression in spermatocytes *in vitro*.

Levels of GLUT9a did not change with exposure to 40 μ g/mL CSC; however, the addition of the AHR antagonist decreased protein levels by 51%, a change that approached statistical significance ($P = .08$). The GLUT12 is present on murine spermatocytes, though, its expression did not change significantly when exposed to 40 μ g/mL CSC. The addition of the AHR antagonist reduced the level of expression by 53%, approaching statistical significance ($P = .07$). Expression of GLUT5 and GLUT8 did not change with exposure to 40 μ g/mL CSC $\pm$ AHR antagonist (Figure 2B).

Figure 2. Glucose transporter (GLUT) expression profile of spermatocytes. A, Western blot of previously (GLUT 5, 8, and 9a) and newly (GLUT 12) described GLUT transporters present in murine spermatocytes. Cigarette smoke condensate (CSC) alone does not affect whole cell protein expression among GLUT 5, 8, 9a, and 12; however, GLUT 9a and GLUT 12 begin to decrease with concurrent exposure to AHR antagonist. All Western immunoblots represent multiple determinations (>3). B, Quantification of fold change (expressed as standard error of the mean [SEM]) among GLUT 5, 8, 9a, and 12 normalized to actin. Comparisons between control (dimethyl sulfoxide [DMSO]) and CSC + antagonist for 24 hours for GLUT 9a and GLUT 12 approach significance.

In order to determine whether this effect of CSC was dose dependent, the above-mentioned experiments were repeated in triplicate with 5 µg/mL. No significant change in expression of GLUT9a or GLUT12 was seen with this lower concentration (data not shown). As a result, the effect appears to be dependent on the dose of CSC added.

Cigarette Smoke Condensate Lowers ATP Production in Spermatocytes

Intracellular ATP production was measured after exposure to varying concentrations of CSC (5 µg/mL, 20 µg/mL, and 40 µg/mL) compared to control. At 20 and 40 µg/mL of CSC exposure, ATP production was attenuated 18% and 12%, respectively, from baseline suggesting that there is a downstream effect of CSC on energy production in the spermatocyte. (Figure 3)

Figure 3. Effect of cigarette smoke condensate (CSC) on adenosine triphosphate (ATP) production in spermatocytes. When examining the percentage change in ATP production in CSC-exposed spermatocytes compared to control (dimethyl sulfoxide [DMSO]), there is a significant dose-dependent effect. With both 20 and 40 µg/mL CSC, the spermatocyte ATP production decreased significantly ($P < .05$) compared to 5 µg/mL.

Discussion

Here, we have shown that fructose is utilized more than 2-DG in spermatocytes and that chronic CSC exposure alone does not influence uptake of substrate. However, when combined with the AHR antagonist, 2-DG uptake is significantly decreased. Furthermore, protein expression of GLUT9a and GLUT12 decreases from control after the pure AHR antagonist (CH223191) is added with 40 µg/mL CSC but not 5 µg/mL. Finally, we show that ATP production is decreased suggesting that CSC has both direct and indirect effects on cellular metabolism.

Previously, we showed that accumulation of reactive oxygen species and messenger RNA expression of antioxidant genes (*Hsp90*, *Nrf2*, and *Cyp11a1*) peak at 6 hours.⁵ When examining protein expression levels, peak expression occurred between 20 and 24 hours after exposure. Addition of the AHR antagonist mitigated this expression; however, when examining CSC and AHR antagonist exposure together, the effect on spermatocyte energy utilization appears to be different.

Environmental exposures ranging from diesel exhaust^{43,44} to cigarette smoke¹³ have been implicated in reductions in sperm count, motility, viability, and oxidative stress leading to DNA damage, but many of these observations have been made in the latter stages of spermatogenesis. Furthermore, evidence suggests that once spermatozoa escape the Sertoli cell environment, toxic exposures and resulting oxidative stress can lead to worsening DNA damage.⁴⁶

Spermatogenesis is the process by which male germ cells develop. Spermiogenesis is the process by which spermatids undergo morphologic changes to package their DNA, thus acquiring their characteristic shape. Spermiation is the process

Figure 4. Spermatogenesis. Type A spermatogonia undergoing mitosis and either replenishing pool of germ cells or differentiating into type B spermatogonia (basal compartment of seminiferous tubule). Meiosis I and II: preleptotene spermatocytes cross blood testis barrier (BTB) into adluminal compartment where meiosis II produces haploid round spermatids. Spermiogenesis and spermiation: haploid round spermatids undergo spermiogenesis, which is defined by the formation of the acrosome, and are released from the seminiferous epithelium into tubules. Reprinted from Lie PPY, Cheng Y, Mruk, DD. Coordinating cellular events during spermatogenesis: a biochemical model. *Trends in Biochemical Sciences*. 2009;34:366-373 with permission from Elsevier.⁴⁵

by which spermatozoa are released into the lumen of the seminiferous tubule and then travel to the epididymis where they acquire their motility (Figure 4).

A body of evidence^{4,47-49} suggests that toxic exposures in premeiotic stages of male germ cell development may be responsible for adverse phenotypes in latter stages of spermatogenesis affecting mature sperm's motility, viability, impaired acrosome reaction, and subsequent fertilization suggesting that early exposures lead to late effects.^{48,49}

Previously, we have used an in vitro spermatocyte cell line to model how environmental toxins potentiate increases in oxidative stress,^{5,50} providing further explanation for the mechanism behind abnormal semen parameters and perhaps subfertility seen in male smokers. Here, under similar conditions, we show that cigarette smoke can influence hexose utilization, perhaps leading to a preference for fructose among spermatocytes in vitro.

Glucose transporter 5 is abundantly expressed in human spermatozoa and is known to transport fructose with high affinity.^{42,51} The relationship between AHR and energy

utilization and transport has been described in other tissues.^{8,22,52} Specifically, activation of cytochrome p450 1A1 (CYP1A1) leads to a profile of increased expression of GLUT1/GLUT3 and concomitant increases in glucose utilization in CaCo₂ cells.⁵² When CYP1A1 is no longer detectable, expression of GLUT5 is increased and fructose uptake increases while glucose consumption decreases.⁵² This reciprocal relationship between different hexose preference and AHR activation parallels findings in this study.

Furthermore, constitutively activated AHR promotes transformed growth factor β (TGF- β) signaling, which is responsible for cell proliferation. In glioma cells, the AHR antagonist (CH223191) is known to reduce TGF- β and cellular proliferation.⁵³

In our model, CSC simulates an activated AHR state, after the addition of the antagonist, 2-DG uptake decreases and fructose uptake trends downward (Figure 1). Although sperm's primary substrate is glucose, we measured 2-DG uptake instead, since it is phosphorylated by hexokinases and is trapped in the cell, which allows for an accurate measurement of uptake.

Furthermore GLUT5, a dominant fructose transporter, expression remains constant with addition of the antagonist while GLUT9a and GLUT12, 2 hexose transporters that have specificity for both glucose and fructose, decrease (Figure 2B).

These findings suggest a role for GLUT9a and GLUT12 as a newly evolved, redundant transport system designed to maintain energy substrate in times of stress. As the stress increases with the addition of the AHR antagonist, secondary GLUTs like 9a and 12 are the first to downregulate and thus decrease the ability of the cells to transport critical energy substrates resulting in a decrease in ATP production and a loss of cellular function. In mature spermatids, for example, hypoexpression of GLUT9a is associated with low motility and fertilization rate.^{27,54}

Fructose uptake was several orders of magnitude higher than 2-DG uptake (50-fold), suggesting a metabolic profile favoring fructose utilization. This is possibly due to the fact that GLUT5, a dominant fructose transporter, is stable throughout the experiment's time points allowing influx of fructose. Alternatively, this could also be due to the fact that 2-DG has a lower affinity for the GLUTs than glucose.⁵⁵

In vitro, spermatocyte exposure to CSC, or other environmental toxins like dioxins, results in an AHR-dependent upregulation of downstream antioxidant genes like *Cyp1a1*.⁵⁶ The AHR antagonists have recently been shown to mitigate the toxic effects of cigarette smoke in both male and female germ cells.^{5,57} When examining this relationship from the perspective of hexose utilization in the spermatocyte, it appears that the AHR antagonist in combination with the CSC alters the transporter profile of the cell with changes in hexose uptake.

Although spermatocytes exposed to CSC alone did not result in significant changes in whole cell expression of most GLUTs, the addition of an AHR antagonist resulted in a decrease in GLUT9a ($P = .08$) and GLUT12 ($P = .07$). Although GLUT1, GLUT2, GLUT3, and GLUT5 are primarily responsible for fructose transport, secondary transporters, perhaps ultimately for the needs of the mature spermatid, are involved in fine-tuning the metabolic needs of the cell.²⁷

Cigarette smoke and other environmental contaminants have been linked to abnormal semen parameters and subfertility in men, but a biological mechanism of toxicity remains elusive.^{18,58} Altered hexose utilization appears to disrupt cellular respiration in spermatocytes during exposure to CSC, which, in turn, is responsible for a decrease in ATP production and resultant oxidative stress and cell death seen previously. Finally, our work highlights a growing interest in the role of GLUTs in the functional aspects of the male gamete, and further investigation into the energetics of male germ cells could lead to a better understanding of cigarette smoke effect on sperm motility.

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