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Cigarette smoke affects posttranslational modifications and inhibits capacitation-induced changes in human sperm proteins

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ABSTRACT

Sperm are highly dependent on posttranslational modifications of proteins. Massive phosphorylation on tyrosine residue is required for sperm capacitation. Sumoylation has also been recently implicated in spermatogenesis and sperm functions. Cigarette smoke is known to cause oxidative stress in different tissues, and several studies suggest that it causes oxidative stress in sperm. Whether tobacco affects posttranslational modifications in human sperm is currently unknown. In this study, we show that a short exposure of human sperm to physiological concentrations of cigarette smoke extract (CSE) causes the partial de-sumoylation of many sperm proteins. Furthermore, the presence of a low concentration of CSE in the human tubal fluid during an induction of in vitro capacitation inhibits the capacitation-associated increase in protein phosphorylation. Collectively, changes in posttranslational modifications may be one of the mechanisms through which exposure to tobacco can negatively affect sperm functions and cause fertility problems.

1. Introduction

Human sperm are produced in the testis through spermatogenesis, followed by their additional maturation in the epididymis. Before fertilization, sperm undergo a final activation in the female reproductive tract, known as capacitation, which involves the reorganization of the sperm plasma membranes and changes in the beating patterns of the flagella [1,2]. Capacitation is followed by an acrosome reaction and is a prerequisite for successful fertilization. At the molecular level, capacitation is hallmarked by the massive phosphorylation of sperm proteins on tyrosine residues; this process can be successfully induced and monitored in vitro. Beyond phosphorylation, various other posttranslational modifications have been detected in sperm, including nitrosilation, acetylation, and ubiquitination, but their role in sperm activity is less understood [3–5]. We have recently shown that numerous human sperm proteins are also modified by sumoylation (a covalent modification by small ubiquitin-like modifiers [SUMO proteins]). The identified targets of SUMO corresponded to flagella proteins, heat shock proteins, metabolic enzymes and the proteins involved in sperm maturation and differentiation [6]. Overall, a precisely regulated interplay between different posttranslational modifications should be very important for sperm because their transcription machinery is inactive.

It has been demonstrated that cigarette smoke can adversely affect spermatogenesis and sperm functions. Sperm from smokers show higher level of DNA damage, apoptosis, and markers of oxidative stress (e.g., 8dg, lipid peroxidation) [7–11]. Tobacco severely affects sperm motility, the ability to undergo hyperactivation and acrosome reaction, bind zona pellucida and fertilize the egg, however the underlying mechanism is not well understood [7–17]. Studies in other tissues have shown that oxidative stress can affect the posttranslational modifications of proteins, such as sumoylation and phosphorylation; these changes in turn may play a role in stress-induced pathways [18]. We have recently shown that the in vivo exposure of mice to tobacco smoke can cause oxidation and de-sumoylation of proteins in testicular cells [19]. Whether tobacco affects posttranslational modifications in human sperm, including those required for sperm activation and capacitation, is not known.

In this report, we show that a short exposure of human sperm to a physiological concentration of cigarette smoke extract (CSE) causes the partial de-sumoylation of sperm proteins. Furthermore,

Abbreviations: HTF, human tubal fluid; CSE, cigarette smoke extract; HSA, human serum albumin.

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the presence of a low concentration of CSE in the human tubal during in vitro capacitation inhibits the capacitation-associated increase in protein phosphorylation. Collectively, changes in post-translational modifications can be in part responsible for the negative effect of tobacco on sperm functions.

2. Materials and methods

2.1. Reagents and antibodies

Rabbit polyclonal antibodies against SUMO2/3 (ab3742) and ubiquitin (ab7780) were purchased from Abcam (Cambridge, MA, USA). The mouse G10 Platinum anti-phosphotyrosine antibody was obtained from Millipore (05-1050; Temecula, CA, USA). All remaining reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise noted.

2.2. Human sperm

Sperm samples were obtained from the male fertility clinic at the Weill Cornell College of Medicine of Cornell University in New York, NY. Informed consent was obtained from all patients, in accordance with the protocol approved by the Institutional Review Board (IRB) of the Weill Cornell Medical College. Semen samples were obtained by masturbation after 3–5 days of sexual abstinence and were subjected to a routine seminal analysis of volume, sperm concentration, total sperm number per ejaculate, motility, vitality and normal morphology, according to the World Health Organization criteria (WHO, 1999). The human specimens received for the experiments did not contain any code derived from individual personal information.

The samples were separated on 40–80% PureSperm gradient (Nidacon, Mölndal, Sweden), according to the manufacturer's instructions. The fractions were pelleted and washed with 1 ml of human tubal fluid (HTF) medium (Irvine Scientific, Santa Ana, CA, USA) and centrifuged at 700 × *g* for 10 min.

2.3. Preparation of cigarette smoke extract and sperm treatment

Cigarette smoke extract (CSE) was prepared as described previously [12,20–22]. In brief, one research cigarette (3R4F: 10 mg of tar and 0.8 mg of nicotine, Tobacco Research, University of Kentucky, Lexington, KY, USA) was attached to a tube connected to a Buchner flask that contained 25 mL PBS. The smoke derived from the cigarette was drawn into the flask under a vacuum generated by a nickel-plated water aspirator. The pH of the solution was then adjusted to 7.2–7.4 with 1 N HCl and filtered through a 0.22-μm pore filter to remove bacteria and large particles. The resulting 100% CSE was diluted with PBS to achieve 1–20% concentrations and used within 30 min of preparation. In each treatment, approximately 3×10^6 cells were resuspended in 1.5 ml of PBS with or without CSE and incubated at 37 °C with 5% CO_2 for 1 h. Treatments were followed by the preparation of whole-cell protein lysates. Each experiment was repeated at least three times.

2.4. In vitro capacitation

For some experiments, motile sperm fractions were used to induce in vitro capacitation. Sperm pellets consisting of approximately 3×10^6 cells were resuspended in 1.5 ml of HTF that was supplemented with human serum albumin (HSA, 5 mg/ml final concentration) and $NaHCO_3$ (10 mM final concentration) and incubated with or without the addition of 5% CSE for 4 h at 37 °C in 5% CO_2. The control sample was incubated without the addition of HSA and $NaHCO_3$. The sperm were pelleted and washed with 1 ml of HTF at 700 g for 10 min.

2.5. Protein extraction and western blot analysis of SUMO expression

For each condition, sperm pellets of approximately 3×10^6 sperm were resuspended in 80 μL of 2× Laemmli buffer (126 mM of Tris/HCl, 20% Glycerol, 4% SDS) and boiled for 5 min at 100 °C. After boiling, the samples were centrifuged for 2 min, and the supernatants were collected in fresh Eppendorf tubes. Protein concentrations were determined via a bicinchoninic acid protein assay using bovine serum albumin (BSA) as the standard (Pierce, Rockford, IL, USA). Before running the samples, beta-mercaptoethanol was added at 5% and Bromphenol blue at 0.02% of the sample volume, and the samples were boiled again for 3 min.

Gel electrophoresis was performed under reducing conditions using NuPAGE 4–12% gradient Bis-Tris polyacrylamide gels and MOPS running buffer (Invitrogen, Carlsbad, CA, USA) at a constant 200 V. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (0.45 μm, Invitrogen, Carlsbad, CA, USA) using NuPAGE transfer buffer. Protein electrophoresis and transfer were performed with an Invitrogen XCell SureLock Mini-Cell electrophoresis system. Western blotting was performed using the ECL plus kit (GE Healthcare, Piscataway, NJ, USA), in accordance with the manufacturer's instructions. SUMO1, SUMO2/3, and ubiquitin antibodies were used at 1:500 dilutions, and anti-phosphotyrosine was used at a 1:1000 dilution in PBS containing 1% BSA and 0.1% sodium azide. Equal loading was ensured with a monoclonal anti-β-tubulin antibody (1:2000; Abcam, ab7291).

2.6. Densitometry and statistical analysis

Quantitative densitometry analyses for SUMO, ubiquitin and phosphotyrosine were performed using the Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA) and the density values were normalized to tubulin. In each experiment, controls (untreated samples) were considered as 100% and other samples were normalized to the controls. The results were expressed as the means ± standard deviation (SD). To calculate the difference between samples, Student's paired *t*-test was used. *p* values <0.05 were considered statistically significant.

3. Results and discussion

To gain insights into possible effects of CSE exposure on sumoylation in sperm, western blot analyses were performed to compare the levels of high-molecular weight SUMO conjugates in cells subjected to different concentrations of CSE. The concentrations of 1–5% CSE were chosen because the nicotine concentrations in this extract were similar to the blood concentrations measured in the blood of the smokers [12,21]. The 1–5% CSE range was used in several previously published studies [20,22]. Despite some slight variations among different sperm samples in their response to CSE, a decrease in sumoylation of most high molecular weight proteins was significant in the range of 1–5% CSE ($p < 0.05$, $n = 5$; Fig. 1A, two different samples are shown). The exception was prominent bands at around 90 kDa which showed either no change or an increase in the intensity (Fig. 1A, arrows); those bands were excluded from the densitometry analysis. Thus, similar to the results obtained with mouse germ cells in vivo, a short exposure of human sperm to CSE caused de-sumoylation of many proteins, even at concentrations as low as 1%. It has been shown in other tissues that oxidative