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Tyrosine phosphorylation of dihydrolipoamide dehydrogenase as a potential cadmium target and its inhibitory role in regulating mouse sperm motility

Xinhong Li*, Lirui Wang, Yuhua Li, Jieli Fu, Linqing Zhen, Qiangzhen Yang, Sisi Li, Yukun Zhang

Shanghai Key Lab of Veterinary Biotechnology, School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai 200240, China

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ABSTRACT

Cadmium (Cd) is reported to reduce sperm motility and functions. However, the molecular mechanisms of Cd-induced toxicity remain largely unknown, presenting a major knowledge gap in research on reproductive toxicology. In the present study, we identified a candidate protein, dihydrolipoamide dehydrogenase (DLD), which is a post-pyruvate metabolic enzyme, exhibiting tyrosine phosphorylation in mouse sperm exposed to Cd both in vivo and in vitro. Immunoprecipitation assay demonstrated DLD was phosphorylated in tyrosine residues without altered expression after Cd treatment, which further confirmed our identified result. However, the tyrosine phosphorylation of DLD did not participate in mouse sperm capacitation and Bovine Serum Albumin (BSA) effectively prevented the tyrosine phosphorylation of DLD. Moreover, Cd-induced tyrosine phosphorylation of DLD lowered its dehydrogenase activity and meanwhile, Nicotinamide Adenine Dinucleotide Hydrogen (NADH) content, Adenosine Triphosphate (ATP) production and sperm motility were all inhibited by Cd. Interestingly, when the tyrosine phosphorylation of DLD was blocked by BSA, the decrease of DLD activity, NADH and ATP content as well as sperm motility was also suppressed simultaneously. These results suggested that Cd-induced tyrosine phosphorylation of DLD inhibited its activity and thus suppressed the tricarboxylic acid (TCA) cycle, which resulted in the reduction of NADH and hence the ATP production generated through oxidative phosphorylation (OPHOXS). Taken together, our results revealed that Cd induced DLD tyrosine phosphorylation, in response to regulate TCA metabolic pathway, which reduced ATP levels and these negative effects led to decreased sperm motility. This study provided new understanding of the mechanisms contributing to the harmful effects of Cd on the motility and function of spermatozoa.

1. Introduction

Emerging evidence indicates that Cd is an acute toxicant to disrupt male reproductive system, related to the poor semen quality and male infertility. In the testis, effects of Cd range from disruption of the blood-testis barrier to oxidative stress as well as necrosis at higher experimental dosage (Thompson and Bannigan, 2008). Moreover, pubertal Cd exposure was reported to destroy testicular development and spermatogenesis by disrupting the synthesis of testosterone in adult mice (Ji et al., 2010). Consequently, Cd caused a remarkable drop in weight of testes and epididymis, sperm concentration, motility, and synchronously an elevation in dead and abnormal sperm (El-Demerdash et al., 2004). One in vivo study even observed the male infertility caused by Cd (Monsefi et al., 2010). Our earlier study also discovered that apoptosis rates of the germ cells in testis and epididymis elevated gradually with Cd doses increasing (Li et al., 2010). Therefore, Cd-induced germ cell apoptosis, loss of daily sperm production, and decreased sperm motility might be responsible for the decline of male fertility (Meeker et al., 2008; Oliveira et al., 2009). However, the detailed molecular metabolisms have not been clearly elucidated.

Numerous studies reported that mitochondria were the key intracellular targets for Cd. For example, the previous study by Zhang et al. reported that DLD was significantly down-regulated in Leydig cells with Cd exposure, which indicated that DLD might be involved in the cytotoxic effects (Zhang et al., 2011). Also, DLD is associated with steroid biosynthesis in Leydig cells exposed to heavy metals including Cd (Ji et al., 2015). Actually, DLD is an NAD^+-dependent oxidoreductase and also a part of pyruvate dehydrogenase complex, α-ketoglutarate dehydrogenase complex, and branched chain keto

* Corresponding author.
E-mail address: lixinhong7172@sjtu.edu.cn (X. Li).

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acid dehydrogenase complex (Patel and Roche, 1990). Therefore, DLD is a house-keeping protein and badly, its knockout has been proven to be fatal (Johnson et al., 1997). However, whether DLD is affected and involved in cytotoxic effects of mouse sperm administered by Cd is unknown.

Kasturi et al. found that novel tyrosine-phosphorylated DLD involved in capacitation of hamster spermatozoa and that DLD is indispensable for hyperactivation and also for acrosome reaction, as both the events were suppressed by specific DLD inhibitor, MICA (Mitra and Shivaji, 2004). Furthermore, a tyrosine-phosphorylated protein, located in the mid-piece (housing mitochondria) of mouse spermatozoa, has been linked with fertilization (Urner et al., 2001). Because spermatozoa are relatively silent in transcription and translation, posttranslational modifications play a pivotal role in regulating their functions in such cells. Therefore, the objective of the present study was to investigate whether mitochondrial DLD would respond to Cd and if so, whether such a response could be linked to the decreased sperm motility and male fertility. Based on the Cd doses used in the previous studies by Roychoudhury et al. (2010) and Oliveira et al. (2009), here we adopted a series of Cd concentrations (0.1, 0.5, 1, 5, 10, 50 μM) in vitro and a similar injection dosage (1.2 mg/kg BW) in vivo in the present study. Significantly, we found Cd promoted tyrosine phosphorylation of DLD, inhibited its dehydrogenase activity, and thus decreased ATP production and sperm motility. This study revealed a novel regulation of DLD activity and provided some new insights into the molecular mechanisms contributing to the harmful effects of Cd on sperm motility and functions.

2. Materials and methods

2.1. Reagents and antibodies

Cadmium chloride (Cd^{2+}, $CdCl_2$) was purchased from National Medicines (China). Bovine serum albumin (BSA) and PVDF membranes were purchased from Millipore (Billerica, MA). Molecular weight markers, acrylamide (40%) and β-mercaptoethanol were obtained from Bio-Rad (USA). Anti-phosphotyrosine (anti-PY) monoclonal antibody was obtained from BD Biosciences. Anti-mouse IgG HRP-conjugated secondary antibody were purchased from Cell Signaling Technology (Danvers, MA). Anti-GAPDH antibody was bought from Gene Tex. Anti-DLD was obtained from Santa cruz. The chemiluminescence detection kit (ECL) was obtained from GE Healthcare. Alexa 555-conjugated anti-mouse antibody and 4′,6-diamidino-2-phenylindole (DAPI) were from Molecular Probes (Invitrogen). Other chemical products were acquired from Sigma-Aldrich (St. Louis, MO).

2.2. Media

The basal medium (non-capacitating medium, C) was modified Krebs-Ringer medium (Whitten's HEPES-buffered medium) (Moore et al., 1994). Briefly, this medium included the following compounds: NaCl (100 mM), KCl (4.4 mM), KH_2PO4 (1.2 mM), $MgSO_4$ (1.2 mM), Glucose (5.4 mM), Calcium Lactic (2.4 mM), HEPES (20 mM) and sodium pyruvate (Pv-Na) (0.8 mM). The basal medium does not support capacitation of mouse spermatozoa. Non-capacitating medium, supplemented with 20 mM $NaHCO_3$ plus 5 mg/ml BSA was designated the capacitating medium (Cap) (Arcelay et al., 2008). In all cases, pH was adjusted to 7.3 and the medium was maintained at 37 °C bath water before use.

2.3. Mouse sperm preparation in vitro

Caudal epididymal spermatozoa were collected from *Kunming* retired breeder males sacrificed according to institutional guidelines for ethics in animal experimentation (Rule number 86/609/EEC-24/11/86). Each epididymis was placed in 1 ml of non-capacitating medium. During a 10 min incubation at 37 °C, spermatozoa released into the medium. Then epididymis was cast and the suspension was adjusted to a final concentration of 1–2×10^7 cells/ml with basal medium before dilution in the experimentally designed medium. To study the effects of Cd on mouse sperm *in vitro*, spermatozoa were incubated at 37 °C bath water for 2 h in media containing a series of Cd concentrations (0.1, 0.5, 1, 5, 10, 50 μM). During incubation, the tubes were shaken gently every quarter of an hour to prevent precipitation.

2.4. Mouse sperm preparation in vivo

KunMing mice were obtained from Shanghai laboratory animal center of Chinese academy of sciences. The mice were maintained in a controlled temperature (20–25 °C)and humidity (50 ± 5%) environment with a 12-h light/dark cycle, allowed free access to food and water all the time. Animal experiments were conducted in accordance with the institutional guidelines for ethics described above. After a week of quarantine and acclimation, the male mice mated with the females (age, >8 weeks) in every cage. Two weeks later, mouse pups were born and the females were reserved for the next mating experiment, while the male ones were randomly divided into two groups each cage. In order to distinguish the two groups, we cropped a small part of the right ear of half of the male mouse at random and designated them as the Cd Group. In Cd group, mice were intraperitoneally injected with $CdCl_2$ (1.2 mg/kg BW) once every 5 days from postnatal day (PND) 5 to PND 60. In the control group, mice were injected with the same volume of normal saline (NS). The mice were sacrificed at PND 60. Then epididymides from 12 mice each group were excised, dissected and placed in 1 ml non-capacitated medium to release motile sperm. After 10 min, epididymal tissues were removed and sperm were washed with PBS.

2.5. SDS-PAGE and immunoblotting

After incubation, sperm were collected by centrifugation, washed in 1 ml of phosphate buffer solution (PBS) (8 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 0.2 g KCl, and DDH_2O to a total of 1 L) and centrifuged (12,500 *g*, 6 min) more than three times, resuspended in 10 μL of 5 × sample buffer (1.67 ml DDH_2O, 0.5 mM, pH 6.8, 5.83 ml Tris-base, 2.5 ml glycerol, 833 mg SDS, 1 mg Br-phenol blue, total 10 ml), boiled for 4 min at 100 °C and centrifuged (13,500 *g*, 10 min). Supernatants were then supplemented 10% β-mercaptoethanol and boiled again for 3 min. The protein samples were either used instantly or stored at −80 °C fridge.

Protein extracts acquired from 1 to 2×10^6 sperm were loaded per lane and subjected to SDS-PAGE using 10% mini-gels. Then proteins were electro-transfered to PVDF membranes at 90 V for 2 h on ice. Immunoblotting with anti-PY was carried out as previously described (Krapf et al., 2010; Zhen et al., 2016). PVDF membranes were blocked with 1% BSA in T-TBS (30 mM Tris-base, 0.8% (w/v) NaCl, 0.1% Tween 20, pH 7.5) and anti-PY diluted at 1: 10,000 final concentration. Corresponding secondary antibody was diluted in T-TBS (1:10,000). An enhanced chemiluminescence ECL plus kit (GE Health) and a ChemiScope 3300mini integrated chemiluminescence imaging system (Clinx, China) were used for detection. When necessary, PVDF membranes were stripped for 1 h in stripping buffer, subsequently re-immunoblotted with anti-GAPDH. Image analysis was conducted using the freeware Image J.