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Involvement of a chromatin modifier in response to mono-(2-ethylhexyl) phthalate (MEHP)-induced Sertoli cell injury: Probably an indirect action via the regulation of NFκB/FasL circuitry

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ABSTRACT

The Fas/FasL signaling pathway, controlled by nuclear factor-κB (NFκB) at the transcriptional level, is critical for triggering germ cell apoptosis in response to mono-(2-ethylhexyl) phthalate (MEHP)-induced Sertoli cell (SC) injury, but the exact regulation mechanism remain unknown. Here, we discovered that expression level of Metastasis associated protein 1 (MTA1), a component of the Mi-2/nucleosome remodeling and deacetylase complex, was upregulated in SCs during the early recovery after MEHP exposure. This expression change was in line with the dynamic changes in germ cell apoptosis in response to MEHP treatment. Furthermore, a knockdown of MTA1 by RNAi in SCs was found to impair the MEHP-induced early activation of NFκB pathway and abolish the recruitment of NFκB onto *FasL* promoter, which consequently diminished the MEHP-triggered FasL induction. Considering that Fas/FasL is a well characterized apoptosis initiating signaling during SCs injury, our results point to a potential "switch on" effect of MTA1, which may govern the activation of NFκB/FasL cascade in MEHP-insulted SCs. Overall, the MTA1/NFκB/FasL circuit may serve as an important defensive/repairing mechanism to help to control the germ cell quality after SCs injury.

1. Introduction

Phthalic acid esters are widespread in the environment due to their use as plasticizers to produce plastic wraps, toys, and bags, and have been frequently shown to adversely affect spermatogenesis [1]. Among phthalic acid esters, di-(2-ethylhexyl) phthalate (DEHP) is widely dispersed throughout the environment due to its increased commercial use. Mono-(2-ethylhexyl) phthalate (MEHP), one of the active metabolites of DEHP, is a well known Sertoli cell (SC) toxicant [2]. The testicular toxicity is characterized with detachment and sloughing of spermatogenic cells due to increased germ cell apoptosis and atrophy in seminiferous tubules [3].

The FasL/Fas signaling pathway has been established to operate as an essential mechanism to regulate germ cell apoptosis in the testis as part of a physiologic pathway to match germ cell numbers to the SCs supportive capacity [4]. Recent studies indicate that the induction of FasL protein expression in germ cells by MEHP treatment is critical for initiating massive germ cell elimination by apoptosis after SCs injury [5]. MEHP-induced FasL expression requires the transcriptional regulation of transcription factors such as nuclear factor-κB (NFκB) as well as specificity protein-1 (Sp-1) [6]. However, the exact regulation of Sertoli cell NFκB/FasL expression in response to MEHP remains to be fully delineated.

Regulation of fundamental germ cell apoptosis demands dynamic coordinated participation of transcription factors and their coregulators at the target gene chromatin [7]. Metastasis associated protein 1 (MTA1), a component of the Mi-2/nucleosome remodeling and deacetylase complex, plays a central role in the regulation of divergent cellular pathways by associating and modifying the acetylation status of the target gene chromatin [8]. In testis, MTA1 is predominantly expressed in pachytene spermatocytes and weakly expressed in SCs [9–11]. Data from this lab have shown that MTA1 might operate as an indispensable modulator in the maintenance of the proper apoptotic balance of meiotic sper-

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matocytes under certain pathologic conditions [12,13]. In addition, SC-expressing MTA1 is crucial to maintain the germ cell nursery function and normal anchoring junction formation in SCs [14]. Although MTA1 has been linked intimately with spermatogenesis and is widely regarded as a potential master coregulator, the role of MTA1 in SCs injury remains unrecognized and delineated here. For the first time, we provide evidence that NFκB controlled FasL expression after MEHP exposure is governed by MTA1 originated from SCs. Our results reiterate the importance of paracrine interactions between SCs and germ cells during SCs injury.

2. Materials and methods

2.1. Animal treatment

Adult male C57BL/6 mice, obtained from the Animal Research Center of our university, were given a single dose of MEHP (1 g/kg) (Sigma) by oral gavage. Control animals ($n = 5$) received a similar volume of vehicle (corn oil). Mice were then sacrificed at different time-points ($n = 5$ for each time-point) by CO_2 inhalation. All procedures involving animals were approved by the local ethical committee.

2.2. Cell preparation and treatment

Sertoli cells (SCs) were prepared as described elsewhere [14]. SCs Cultures were hypotonically treated with 20 mM Tris (pH 7.4) for 2.5 min to lyse residual germ cells. The purity was monitored by RT-PCR analyses using primer sets specific to marker genes. To evaluate the effect of MEHP-triggered SCs injury on MTA1 expression, cells were dosed with 200 μM MEHP diluted in Me_2SO for various time periods as indicated in Figure legends. In another experimental setting, SCs were incubated with 5 μg/ml actinomycin D (ActD, Sigma) and 200 μM MEHP together for 12 h before being subjected to the following biochemical analyses of MTA1 expression level.

To determine the effect of MTA1 knockdown on NFκB signaling in response to MEHP treatment, SCs receiving siRNA treatment were dosed with 200 μM MEHP diluted in Me_2SO for 12 h before being harvested for further analysis.

2.3. Detection of apoptosis by in situ end labeling of fragmented DNA (TUNEL)

TUNEL assay was done using In Situ Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany) following instructions of the manufacturer. To assess apoptosis in testicular cells, 100 seminiferous tubules were observed in each section at a magnification of 400×. A histogram of the number of TUNEL-positive germ cells per seminiferous tubule and the percentages of the number of seminiferous tubules containing TUNEL-positive germ cells were analyzed.

2.4. RT-PCR and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from SCs using RNeasy Mini Kit (QIAGEN Inc., Valencia, CA, USA). For RT-PCR, first-strand cDNA was synthesized with Superscript III (Rnase H-Reverse Transcriptase; Invitrogen) and PCR was set up according to Promega's protocol. The primers used for detection *MTA1, Rhox5, Sycp3, Dbil5* and *Gapdh* were chosen according to the previous report [14]. Amplification of *Gapdh* was served as the internal control. PCR products were quantified by SYBR green intercalation using the MiniOpticon™ system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). *Gapdh* was used to obtain the ΔΔCt values for the calculation of fold increases.

2.5. Western blotting

Western blotting was carried out as described previously [11,13]. Briefly, protein samples were prepared in ice-cold RIPA buffer (Tris–HCl 50 mM, NaCl 150 mM, Triton X-100 1% vol/vol, sodium deoxycholate 1% wt/vol, and SDS 0.1% wt/vol pH 7.5) supplemented with complete proteinase-inhibitor cocktail tablets (Roche Diagnostic, Mannheim, Germany). 20 μg of protein sample were separated by SDS/PAGE and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). Membranes were then incubated with different primary antibodies including goat anti-MTA1, rabbit anti-FasL, rabbit anti-β-actin and rabbit anti-goat IgG-HRP secondary antibody (Santa Cruz Biotechnology, Inc.); rabbit anti-pho-Iκκ α/β, rabbit anti-Iκκ α/β, rabbit anti-pho-p65 and rabbit anti-p65 (Cell Signaling Technology) and goat anti-rabbit IgG-HRP secondary antibody (Abcam). β-actin serves as an internal control. Immunostained bands were finally detected by using an ECL kit (Amersham Biosciences, Buckinghamshire, UK).

2.6. In vitro siRNA treatment

We designed and synthesized chemically two siRNA sequences targeting MTA1 (GenBank accession number: AF463504.1) (Ruibo Co., Shanghai, China). The oligo sequences used were as follows: si-1 (sense: 5′-AGUAGAAGAAGAAAUCCUCACdTdT-3′, antisense: 3′-dTdT GAGGAUUUCUUCUUCUACUCU-5′); si-2 (sense: 5′- AAUAU GUCCAACAAGAUGCGCdTdT-3′; antisense: 3′-dTdT GCAUCUU-GUUGGACAUAUUGG-5′). MTA1-siRNAs or a non-silencing scrambled control siRNA (Altogen Biosystems, NY) were transfected into SCs using Lipofectamine 2000. 48 h after transfection, cells were collected and subjected to other experiments.

2.7. NFκB activity monitored by NFκB filter assay

48 h after siRNA treatment, SCs were dosed with 200 μM MEHP diluted in Me_2SO for 12 h. Subsequent nuclear extraction was carried out using a commercial kit from Signosis (Zhongzhi Biotech Development Co., Ltd. Wuhan, China). Signosis' NFκB filter assay was performed to evaluate the activity of NFκB pathway as instructed by the manufacturer. The bound NFkB probe was finally measured with luminescence using GloMax™ 20/20 Luminometer (Promega).

2.8. Chromatin immunoprecipitation (ChIP)

48 h after siRNA treatment, SCs were dosed with 200 μM MEHP diluted in Me_2SO for 12 h. Subsequent ChIP assays were carried out as described elsewhere by using a kit from Upstate Biotechnologies (Lake Placid, NY) [15]. Briefly, SCs were cross-linked with 1% formaldehyde and sonicated at 30% output setting (Misonix Sonicator Q700, OpticsPlanet, Inc., IL) followed by centrifugation for 15 min at 16,000×*g* at 4 °C. Supernatant (50 μl) was taken as an input control and the rest of the sample was immunoprecipitated with antibodies against NFκB and normal rabbit-IgG (both from Santa Cruz Biotechnology Inc.). After overnight incubation at 4 °C, precipitates were eluted and incubated at 65 °C overnight to reverse formaldehyde cross-linking. DNA was purified by phenol/chloroform extraction and ethanol precipitation. The chromatin fragments were amplified by PCR using primers flanking the NFκB binding sites of murine *FasL* promoter as follows: (forward) ACAGGCCTCTCAGG-ACACAC and (reverse) TAAGGTTTCCGCAGTCAAGG [16].

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