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Original article

The NF-κB signaling system is required for blastocyst hatching in the golden hamster: Mediated by the expression of hatching-promoting cathepsins

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ABSTRACT

Background: Blastocyst hatching is a prerequisite for successful implantation. Knowledge on this phenomenon is scarce in humans and the available information stems from studies on rodents. In hamsters, we earlier showed that embryo-derived cathepsin (Cat) proteases (Cat-L, -B and -P) are involved in blastocyst hatching and it is governed by a few molecular regulators. Here, we assessed the involvement of NF-κB signaling in blastocyst development and hatching.
Methods: Hamster embryos, recovered from super-ovulated hamsters, were cultured in the absence or presence of NF-κB inhibitors (BAY-11-7082 or JSH-23). Development through peri-hatching stages and hatching rates were scored. Embryonic mRNA and protein expressions analyzed for NF-κB and Cats (-L, -B, -P); their levels correlated with hatching rates; their cellular immuno-localizations were examined.
Results: Transcript and protein expression of NF-κB components (IKK, Rel-A and IκB-b) were observed in 8-cell embryos through hatched-blastocysts. The NF-κB inhibitors (BAY-11-7082 and JSH-23) inhibited blastocyst hatching in a dose-dependent manner; percentages being $37.8 \pm 3\%$ and $36.5 \pm 2.8\%$, respectively; >90% hatching in untreated-controls. NF-κB inhibition lead to a peri-hatching inflated-state of blastocysts, in contrast to deflated-state observed with control blastocysts. Also, NF-κB signaling inhibition-mediated reduction in hatching rates were accompanied by significant reductions in transcript and protein levels of Cat-L, -B and -P.
Conclusion: We conclude that NF-κB signaling components are expressed during peri-hatching blastocyst development and that it is important for blastocyst hatching. Our results provide the first evidence for the involvement of NF-κB transcription-factor in mammalian blastocyst hatching phenomenon.

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1. Introduction

During early mammalian development, hatching of blastocyst is a prerequisite for successful implantation and establishment of pregnancy. In most species studies, including humans, blastocyst hatching occurs in an inflated state with the initiation of a 'nick' in the zona pellucid. Post-hatching, zona remains intact and blastocyst persists in an inflated state.[1–5] *In vivo*, blastocyst hatching is seldom accompanied by a fully inflated state and the intact zona is normally not observed in the uterine flushates. Interestingly, in the golden hamster, cultured blastocysts undergo inflation-deflation events and hatching occurs in a deflated state, accompanied by a complete dissolution of zona.[6–9] Besides, hatched blastocysts remain deflated and exhibit spatially restricted cellular extensions *viz.*, trophectodermal projections (TEPs).[6–9] Characteristics of hamster hatching occurring *in vitro* are apparently similar to those observed *in vivo*.[6] We earlier showed that the blastocyst hatching is brought about by embryonically-expressed cathepsins (Cat), for example, Cat-L, -B and -P.[7,10] Besides, TEPS enrich hatching-associated Cats and their potential molecular regulators such as LIF, HB-EGF and COX-2.[8–9] These interesting observations make the hamster model an interesting early developmental paradigm to study molecular regulation of blastocyst hatching phenomenon in mammals.

Among molecular regulators of embryonic cell proliferation and differentiation, transcription factors are supreme. One such factor

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is NF-κB and its signaling system.[11–14] Numerous studies have shown the pleotropic role and the functional significance of NF-κB signaling system in a few reproductive events[15–16] and during implantation and early pregnancy.[17–19] However, the NF-κB system in preimplantation development is less studied.[20–21] Importantly, its possible role in blastocyst thatching is virtually unknown. Interestingly, we earlier reported the critical need for COX-2 in hamster blastocyst development and hatching.[9] Because, one of the major transcriptional regulator of COX-2[18,22] is NF-κB and because COX-2 derived prostanoids are critical for blastocyst hatching and implantation,[8,9,23] we considered important to investigate the role of NF-κB signaling module in hamster blastocyst hatching phenomenon. For this, we examined IkB (inhibitor of IK-kinase) and RelA (a transcription factor) which are critical effectors of the NF-κB pathway. We used the two specific inhibitors (BAY11-7082 and JSH-23 082) that act *via* two different pathways, in order to examine whether or not both lead to similar blastocyst development and hatching phenotypes. While BAY11-7082 suppression of IkB compromises downstream effects, JSH-23 prevents nuclear localization of Rel-A, thereby suppressing the expression of NF-κB pathway target genes. Data pertaining to these set of experiments is described in this article.

2. Material and methods

2.1. Animals

Sexually mature golden hamsters (*Mesocricetus auratus*) were maintained on a 14 h light:10 h dark lighting schedule at ambient temperatures and provided with pelleted feed and water *ad libitum*. Six- to eight-week-old female hamsters were used as embryo donors. Females were mated overnight with proven stud males. Vaginal smears were checked on the following day for presence of sperm and assigned as Day 1 of pregnancy. The Institutional Animal Ethics Committee approved procedures for animal handling and experimentation, in accordance to guidelines on Use of Laboratory Animals for Research (INSA, New Delhi).

2.2. Recovery and culture of embryos

Female hamsters were superovulated with an i.p. injection of 30 IU pregnant mares' serum gonadotrophin on the day of post-estrous discharge and mated 3 days later. In the evening of day 3 of pregnancy, *in vivo* developed 8-cell embryos were collected by flushing the excised uterine horns with 0.5 ml of equilibrated HECM-2m.[8–10,24] Well-formed, non-compact 8-cell embryos were used for all experiments. Embryos were cultured in 50 μl of HECM-2 h at 37 °C in an atmosphere of 5% CO_2 in air and monitored microscopically every 12 h for a total of 72 h for development and hatching. Blastocysts with completely lysed zonae were considered fully hatched. Cultured embryos at different stages were used for RNA isolation or stored at −70 °C or fixed in 4% paraformaldehyde (PFA) for immuno-staining purposes. All reagents were procured from Sigma (St Louis, MO, USA) unless specified otherwise.

2.3. Embryonic mRNA isolation, Real Time PCR analysis

Embryos were collected at 72 h of culture and embryonic polyA+-RNA was isolated using Dyna beads-Oligo dT25 (Dynal Biotech, Oslo, Norway, now a part of Life-Technologies). Briefly, embryos ($n = 20$) were lysed in 100 mM Tris buffer (pH 7.6) containing 1% SDS, 500 mM LiCl and 10 mM EDTA (pH 8.0). The lysate was loaded onto 20 μl of oligodT25 beads using Dynal Magnetic Particle Concentrator. The beads were then washed in wash-buffer I (10 mM Tris, pH 7.6, with 150 mM LiCl, 1 mM EDTA, pH 8.0 and 0.1% SDS) and then in wash-buffer II (10 mM Tris, pH 7.6 containing 150 mM LiCl and 1 mM EDTA, pH 8.0). The Poly A+ RNAs were eluted into double distilled water by heating the RNA containing beads at 65 °C for 90 s followed by snap freezing. The isolated poly A+ enriched mRNA, dissolved in water, was used for setting up reverse transcription reactions in 20 μl of reaction mixture with random hexamers and MMLV-RT (Fermentas, now a part of Thermo Scientific, Ontario, Canada,) and appropriate controls were used. One embryo equivalent cDNA were used in a final volume of 20 μl for both RT-PCR and qPCR reactions. QPCR reactions were carried out using SYBR green qPCR Mastermix and Real-time PCR detection system, according to the manufacturer's instructions (Applied Biosystems, US). For an internal control, the β-actin gene was used and expression is used for normalization.

Primers used for hamster cathepsin L were: F: 5′-TGCAATGGTG-GCCTGATGG-3′, R: 5′-GAACCCCATGGTCAAGGTC-3′ (Acc No-AF479267), for hamster cathepsin P: F: 5′-ACTGGCAACCTGACCAC-TCTAAG-3′, R:5′-TGGGAAGCATCAACCGCAG-3′ (AccNo-AF479266) and β-actin F: 5′-TGAACCCTAAGGCCAACCGT-3′, R: 5′-GCTCA-TAGCTCTTCTCCAGG-3′ (Acc no.-AF014363). Three identical real-time qPCR assays were performed, and in each assay, the transcript levels of each of the zonalytic proteases were measured in duplicate and Ct value for each transcript was determined. Relative quantification of gene expression was analyzed by the $2^{-\Delta\Delta}$Ct method.[25]

2.4. Indirect immunocytochemistry, nuclear staining and protein quantitation

Embryos were fixed for 45 min at room temperature in 4% PFA (pH 7.4) in phosphate buffered saline (PBS). Fixed embryos were washed in PBS-PVA, permeabilized with 0.2% Triton X-100 for 30 min and blocked in 3% BSA for 2 h at room temperature. Embryos were rinsed in PBS-PVA, and then incubated with primary antibodies. All primary antibodies used were at 1:100 dilutions in blocking solution overnight at 4 °C. The following primary antibodies were used: rabbit anti-IκB polyclonal antibody (SC-371), rabbit anti-RelA polyclonal antibody (SC-372), all from Santacruz Biotech., USA; rabbit-anti-phospho IKK α/β monoclonal antibody (CST-2697), rabbit anti-phospho RelA monoclonal antibody (CST-3033) and rabbit anti-polyclonal antibody (CST-3253), all from Cell Signaling Technology, USA; mouse anti-cathepsin (cts)-L monoclonal antibody (Serotec Ltd., Oxford, UK), and rabbit anti-cts-P polyclonal antibody.[7,26] Control embryos were treated with appropriate non-immune IgG which acted as negative controls.

After a thorough washing in PBS-PVA, embryos were incubated in appropriate secondary antibodies for 2 h. Nuclear staining was accomplished by slightly modifying an earlier nuclear staining procedure used[29] by incubating embryos in 100 μg/ml RNAse (Fermentas, Canada) for 15 min followed by incubation in 0.05 μg/μl Propidium Iodide for 30 min. All embryos were washed for 10 min, two times, then mounted using Vectashield anti-fade solution (Vector Laboratories, USA) and viewed under Carl Zeiss LSM Meta laser-scanning confocal microscope (Carl Zeiss Micro-imaging, GmBH, Germany) for fluorescence signal.

2.5. Influence of NF-κB inhibitors on hatching

Effect of two highly specific inhibitors *viz.*, JSH-23 and BAY-11-7082, of NF-κB signaling[27,28] was tested on blastocyst hatching. These were sourced from Cayman Chem. (Ann Arbor, MI, USA). Individual inhibitors were dissolved in DMSO (Sigma, St Louis, MO, USA). In control experiments, embryos were treated with similar concentrations of appropriate diluents. Freshly recovered 8-cell

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