G Protein Mediated Hatching Regulation in the Mouse Embryo

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ABSTRACT : Hatching occurred in the time dependent manners and strictly controlled. Although, the hatching processes are under the control of muti-embryotrophic factors and the expressed G proteins of cell generate integrated activation, the knowledge which GPCRs are expressed during hatching stage embryos are very limited. In the present study, which G proteins are involved was examined during blastocyst development to the hatching stage. The early-, expanded-, and lobe-stage blastocysts were treated with various G_{α} activators and H series inhibitors, and examined developmental patterns. Pertusis toxin (PTX) improved the hatching rate of the early-stage blastocyst and lobe-formed embryos. Cholera toxin (CTX) suppressed the hatching of the early-stage blastocyst and expanded embryos. The effects of toxins on hatching and embryo development were changed by the H7 and H8. These results mean that PTX mediated GPCRs activation is signaling generator in the nick or pore formation in the ZP. In addition, PTX mediated GPCR activation induces the locomotion of trophectoderm for the escaping. CTX mediate GPCRs activation is the cause of suppression of hatching processes. Based on these data, it is suggested that various GPCRs are expressed in the periimplantation stage embryos and the integration of the multiple signals decoding of various signals in a spatial and temporal manner regulate the hatching process.

Key words : G protein, Hatching, Pertusis toxin, Cholera toxin, H series inhibitor

INTRODUCTION

The embryonic stage of hatching is various according to the species. In the case of mammal, it occurs during early embryonic stage just before implantation, in contrast to the ovoviparous and viviparous animals. In mouse, zona pellucida (ZP) is constructed with glycoproteins; mZP1, mZP2 and mZP3 (Wassarman, 2008). ZP serves as a barrier that normally allows only sperm of the same species access to the egg, initiates the acrosomal reaction, prevents any additional spermatozoa from reaching the zygote after fertilization, acts as a porous filter through which certain substances secreted by the uterine tube an reach the embryo during early cleavage, serves as an immunological barrier between the mother and the antigenically different embryo, prevents the blastomeres of the early cleaving embryo from dissociation, and facilitates the differentiation of trophoblastic cells. In addition, ZP prevents premature implantation of the cleaving embryo into the wall of the uterine tube.

As expected from the known function of ZP, shedding the ZP is the critical step in embryo development and any disruption of the hatching process is the cause of implantation failure leading to infertility (Petersen et al., 2005). Hatching occurred in the time dependent manners and have to be controlled strictly. To shedding the ZP, the early-stage embryo has to develop into the blastocyst stage. Blastocoel formation is regulated by stage specific gene expression and formed by active transport of ions and water. This transport is characteristic of trophectoderm (TE) and the polarized localization of functional protein is critical. The hatching competence includes expansion of blastocoels in mouse. The basolaterally localized alpha 1

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beta1 Na,K-ATPase isozyme appears to function uniquely to drive fluid transport (Kidder & Watson, 2005). Rho-kinase, a downstream effector of small GTP-binding protein RhoA, Na^+/H^+ exchanger-3 are also involved in blastocoel formation (Kawagishi et al., 2004a, b). Caudal-type homeobox protein 2 (Cdx2) fail maintain the trophecodermal integrity but is not cause of blastocoel formation (Strumpf et al., 2005). Also, E-cadherin-catenin cell adhesion family, the tight junction gene family, Tead4-Yap system, and aquaporin gene family are also involved in blastocoels formation (Kurotaki et al. 2007; Watson & Barcroft, 2001).

The process of hatching is different between species (Seshagiri et al., 2009). These are accomplished by the involvement of regulatory molecules, primarily embryotrophic factors, which encompass transcription factors, protease, growth factors and cytokines (Kane et al., 1997; Sechagiri et al., 2002). In hamster embryo, epidermal growth factor (EGF), heparin binding-EGF (HB-EGF), transforming growth factor-β and leukemia inhibitory factor (LIF) associated with a complete dissolution of zona, predominantly brought about by embryo-derived cysteine proteinase-like activities (Seshagiri et al., 2002). In mouse embryos, platelet activating factor (PAF), concanavalin A (Con A), LIF and SLPI enhance ZP shedding (Cheon, 2010). As expected, those embryotrophic factors improve the early-stage embryo development *in vitro*. The formation of a nick in the ZP caused by a tension exerted by the increasingly blastocoels expansion is initially observed but the expansion is not sufficient to induce embryo hatching in mouse. Completed ZP shedding is needed more processes including protease activity (Gonzales et al., 2001; Sireesha et al., 2008) and actin filaments mediated locomotion of trophectodermal cell (Cheon et al., 1999). Those are correlated with the quality of blastocyst (Pribenszky et al., 2011).

Such a cellular mechanisms are under the control of cellular signaling of muti-embryotrophic factors. So far, PI3K/Akt signaling pathway, PKC and calcium are known cellular signaling mediators. Inhibition of PI3K/Akt signal transduction pathway resulted in a significant delay in blastocyst hatching (Riley et al., 2005). PAF works through PKC activation and ConA works through free calcium fluctuation in the trophectoderm during hatching stage (Cheon, 2005; 2006). Many of them work through G protein-coupled receptors (GPCRs) mediated signal transduction. Cells coexpress a large number of different GPCRs, the activation of which generates multiple signals that are integrated (Prezeau et al., 2010). However, which GPCRs are expressed during hatching stage embryos are very limited. Besides, the hatching stage embryos are under the control of muti- embryotrophic factors. Therefore, in the present study, which G proteins are involved was examined.

MATERIALS AND METHODS

1. Reagents

Cholera toxin, a ribosylase of $G_{s\alpha}$ (Fujino & Regan, 2006), pertusis toxin, a ribosylate of G_i or G_o (Oda et al., 1999), 1-(5-isoquinolimnesulfonyl)-2-methylpiperazinme dihydrochloride (H7), a cyclic Ca^{2+} dependent protein kinase C (PKC) inhibitor (Tsubaki et al., 2007), and N-[2-(methylamino)-ethyl]-5-isoquinolinesulfonamide (H8), a moderate inhibitor of protein kinase A and G (Engh et al., 1996), were purchased from Sigma.

2. Animals and Embryo Collection

All animals involved in this study were approved by the IACUC (Institutional Animal Care and Use Committee) at Sungshin Women's University and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Six-week old outbreed CD-1 mice were purchased from Charles River Co. and hosed in animal house kept standard condition at Sungshin Women's University. Virgin CD-1 female mice were superovulated with 5 IU of pregnant mares' serum gonadotrophin (eCG, PMSG, Sigma) followed by 5 IU of human chorionic gonadotrophin (hCG, Sigma)

after 48 hours. After administration of the hCG, one female was caged overnight with one stud male, and checked the copulation plug the following morning. After 72 hr of hCG injection, the pregnant mice were sacrificed and the compacted embryos were collected with flushing from the utero-tubal junction area with Biggers-Whitten-Whittingham (BWW) medium (1971) containing 0.4% bovine serum albumin (BSA).

3. Culture of the Embryos

The healthy embryos were cultured in 10 ㎕ BWW medium containing 0.4% bovine serum albumin (BAS, Sigma, St. Louis, MO, USA) under the mineral oil which was equilibrated with BWW medium. Culture was carried out in the CO_2 incubator conditioned with 37℃, 100% humidity, 5% CO_2 and 95% air. 10 ng/㎖ pertusis toxin (PTX, Sigma) and 10 ng/㎖ cholera toxin (CTX, Sigma) were treated for 24 hr or 12 hr according to the treated-embryonic stages and observed the embryonic development. 50 μM H-7 or 100 uM H-8 was cotreated with CTX or PTX, and observed the embryonic stages under the microscope (Olympus IX70). Experiments for each group were repeated at least 7 times.

4. Statistical Analysis

For exogenous SLPI experiment and ODN experiment, developmental frequency were analyzed either by Chi-squred test. The results were considered to be statistically significant at $P<0.05$.

RESULTS

1. Effects of CTX and PTX on the Development of blastocyst

To evaluate which heterodimeric G proteins are involved in blastocyst hatching, the G_α activators were treated for 24 hr to early-stage blastocyst. The developmental rates to the hatching stage were significantly lower in CTX treated blastocyst (64/85, 76.4%) than those of the control (70/81, 86.9%) (Fig. 1). On the other hand, the hatching rates were significantly higher in PTX treated embryos (77/79, 97.9%) than those of the other groups (Fig. 1).

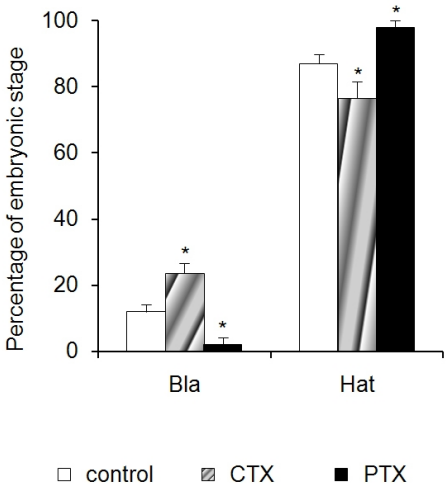

Fig. 1. Development of early blastocyst to hatching stage embryo cultured in BWW medium containing cholera-toxin (CTX, 10 ng/㎖) and pertusis toxin (PTX, 10 ng/㎖) for 24 hr. Bla; early-, middle- and late-blastocyst stage embryos, Hat; hatching stage embryos. *: $p<0.05$ versus control group.

2. Effects of CTX and PTX on the Nick or Partial Pore Formation of ZP

It is possible that the effects of CTX and PTX on the hatching were indirect because the early-stage blastocyst is not ready for hatching. Therefore, CTX and PTX were treated at expanded-stage blastocysts. Interestingly, CTX significantly suppressed the hatching of expanded embryos (29/78, 37.1%) compared with the control (63/76, 79.3%) and PTX (62/78, 77.1%). In the case of PTX, the hatching rates were not different from the control (79.3% vs 77.1%) (Fig. 2).

3. Effects of CTX and PTX on the Locomoation of TE

After formation of nick or pore in the ZP by the pressure or protease activity, the locomotion mediated by actin filaments are the essential to escaping the ZP. To

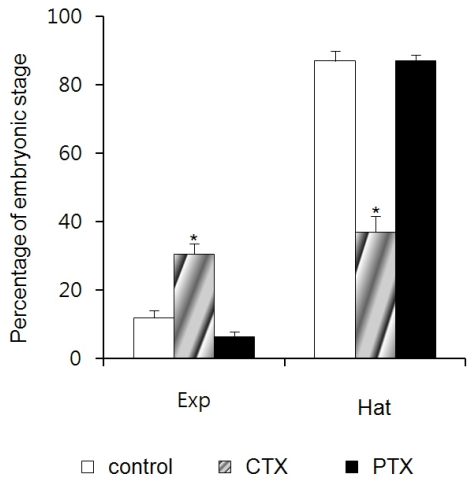

Fig. 2. Development of expanded blastocyst to hatching stage embryo cultured in BWW medium containing cholera-toxin (CTX, 10 ng/㎖) and pertusis toxin (PTX, 10 ng/㎖) for 12 hr. Exp; expanded-stage embryos, Hat; hatching stage embryos. *: $p<0.05$ versus control group.

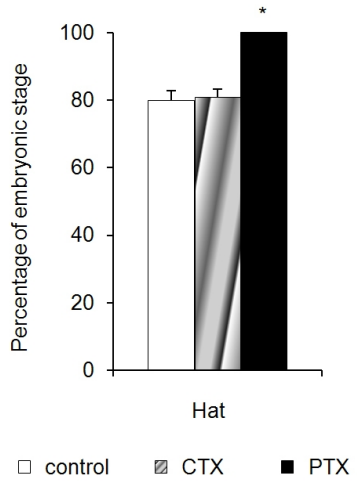

Fig. 3. Development of lobe-formed blastocyst to hatching stage embryo cultured in BWW medium containing cholera-toxin (CTX, 10 ng/㎖) and pertusis toxin (PTX, 10 ng/㎖) for 12 hr. Hat; hatching stage embryos. *: $p<0.05$ versus control group.

evaluate whether GPCRs are involved or not, CTX and PTX were treated at lobe formed blastocyst. CTX did not effect on the hatching of the lobe-formed embryos (61/74, 80.9%) compared with the control (59/74, 80%). On the other hand PTX treated lobe-formed embryos all escaped the ZP (100%). There were significant differences between PTX and other groups (Fig. 3)

4. H7 and H8 on the Effects of CTX- or PTX-mediated G Protein Activation

To examine whether modulation of PKC or PKA activity can alter the effects of CTX, the early-stage blastocysts were cotreated with H7 and H8. H7 cause of degeneration (more than 55%) of blastocyst and the hatching rate was significantly lower than that of control (86.95% (70/81) vs 22.9% (16/62)) (Fig. 4A). On the other hand, H8 significantly suppress the development of blastocyst to the hatching stage (86.9% (70/81) vs 34.16% (22/63)) (Fig. 4B).

In the case of PTX, it was stimulated the development of early stage embryos and lobe-formed embryos to the ZP escaping embryos. Cotreatment of H7 with PTX induced degeneration of early-stage blastocyst (21/71, 30.77%) compared with the control (0/71, 0 %) and significant number of the embryos were stayed at blastocyst stage compared with the control (40.6% (27/71) vs 13.1% (11/81)) (Fig. 5A). In the case of cotreatment with H8, it caused of death of all examined embryos (68/68, 100%) (Fig. 5B).

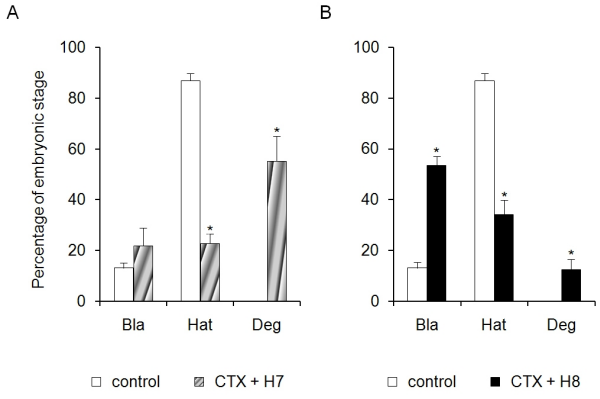

Fig. 4. Development of early blastocyst to hatching stage embryo. Embryos were cotreated with choleratoxin (CTX, 10 ng/ml) and H7 (A) or H8 (B) for 24 hr. Bla; early-, middle- and late- blastocyst stage embryos, Hat; hatching stage embryos, Deg; degraded embryo. *: $p<0.05$ versus control group.

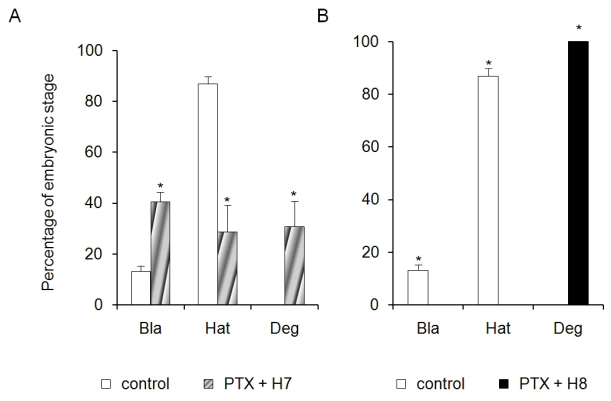

Fig. 5. Development of early blastocyst to hatching stage embryo. Embryos were cotreated with pertusis toxin (PTX, 10 ng/ml) and H7 (A) or H8 (B) for 24 hr. Bla; early-, middle- and late-blastocyst stage embryos, Hat; hatching stage embryos, Deg; degenerated embryo. *: $p<0.05$ versus control group.

DISCUSSION

Escaping the ZP is a complex process of cellular and molecular mechanisms. The cellular responses for blastocyst expansion and further stage development are under the control of communication between embryo and uterine environment. So far, few cellular signaling molecules are evaluated in hatching in mammals. According to the previous results in our laboratory, the PAF, concanavalin, and prostaglandins can improve the development of preimplantation stage embryos. It is suggested that protein kinase C and calcium mediate the cellular signal transduction of these regulators. These mean that G proteins may involve in the hatching process.

Many of the communicator work through their specific receptors which coupled with G proteins. G proteins activate enzymes, ion channels, transporters, others controlling transcription and development. The human genome encodes roughly 950 G protein-coupled receptors. Heterodimeric G proteins constructed with G_{α} and the tightly associated $G_{\beta\gamma}$ subunits. G_{α} has many classes of subunits: $G_{s\alpha}$, $G_{i\alpha}$, $G_{o\alpha}$, $G_{q/11\alpha}$ and $G_{12/13\alpha}$ (Tadevosyan et al., 2012). However, it is not known which G-proteins are involved in the hatching. Interesting, as expected, PTX which ribosylate the $G_{i\alpha}$ or $G_{o\alpha}$, improved significantly the hatching or early-stage blastocyst and lobe-formed blastocyst. On the other hand, CTX which ribosylate $G_{s\alpha}$, suppressed significantly the hatching of the embryos which were treated from early-stage blastocyst and expanded-stage embryos, but decreased the hatching rate without statistical significance in the embryos which were treated from lobe-formed stage embryos. These results clearly showed that embryotrophic factors whose receptors are coupled with G-proteins are involved in hatching process and blastocyst development.

PTX did not enhance or suppress the hatching of expanded blastocysts, although expanded stage is the time for ZP rupture. It means that PTX mediated G-protein activation is not need at the initiation time of hatching. On the other hand CTX mediated G-protein activation is need for hatching at this embryonic stage. Interestingly, after nick or lysis the ZP by pressure or protease, CTX did not effect on the hatching. The hatching rates were similar with the control. However, interestingly, all the PTX-treated lobe-formed embryos escaped the ZP. It means that PTX mediated activation of $G_{i\alpha}$ or $G_{o\alpha}$ proteins involve in actin locomotion at this stage.

Many of the embryotrophic regulators work through G protein-coupled receptors (GPCRs) mediated signal transduction. Interestingly, the effects of CTX or PTX were modulated by the cotreatement of H7 or H8. Cotreatment of PTX and H8 for 24 hr caused of cell death of all the treated early-stage blastocyst, but cotreatment of CTX and H8 for 24 hr caused developmental arrest of the treated early-stage blastocyst. In the case of cotreatment of PTX and H7, or CTX and H7, the treatment caused of cell death and developmental arrest of the treated early-stage blastocyst. Such results supported by the following suggestions: cells coexpress a number of different GPCRs, the activation of which generates multiple signals that are integrated (Hur & Kim, 2002; Prezeau et al., 2010).

In summary, PTX mediated GPCRs activation is the

signaling generator in the nick or pore formation in the ZP. Besides, PTX mediated GPCR activation induce the locomotion of trophectoderm for the escaping. CTX mediate GPCRs activation is the cause of suppression of hatching processes. The effects of GPCRs activation were changed by the H7 and H8. Based on these data, it is suggested that various GPCRs are expressed in the periimplantation stage embryos and the integration of the multiple signals decoding of various signals in a spatial and temporal manner regulate the hatching process.

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