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Peroxisome proliferator-activated receptor δ improves porcine blastocyst hatching via the regulation of fatty acid oxidation

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A B S T R A C T

Peroxisome proliferator-activated receptor δ (*Pparδ*) is a nuclear receptor that plays critical roles in lipid metabolism, glucose metabolism, and cell growth and differentiation. Several recent studies have shown that *Pparδ* promotes blastocyst hatching *in vitro*. However, the mechanism by which it promotes preimplantation embryonic development *in vitro* remains unclear. In this study, oocytes and parthenotes were treated with a specific agonist of PPARδ, GW501516. The activation of PPARδ had no effect on oocyte maturation for 1 μM and 10 μM GW501516 compared with the control group. Additionally, the PPARδ agonist did not affect blastocyst formation (77.79 ± 3.59% [10 μM], 79.00 ± 5.53% [50 μM], and 79.64 ± 6.00% [100 μM] *vs.* 81.69 ± 2.61% [control]). However, the blastocyst hatching rate was significantly greater for parthenotes treated with 10 and 50 μM agonist, and did not differ between those treated with 100 μM agonist and the control group (61.80 ± 3.03% [10 μM], 65.10 ± 5.25% [50 μM], and 38.85 ± 7.45% [100 μM] *vs.* 41.77 ± 10.88% [0 μM]). Activation of PPARδ also increased blastocyst quality and cell number, as well as ATP production. There were no clear differences in mitochondrial membrane potential, mitochondrion copy number, or glucose consumption between the treatment and control groups. However, PPARδ activation enhanced lipid accumulation via *Fabp3* and *Fabp5*. Fatty acid oxidation also increased in response to treatment with the agonist via the rate-limiting gene *Cpt2*. Reactive oxygen species were modified and REDOX maintenance-related gene expression increased significantly in GW501516-exposed blastocysts. In addition, the activation of PPARδ resulted in changes in miRNA content. After treatment with the PPARδ agonist, miR-99 increased and miR-32 decreased. These data showed that PPARδ has a positive impact on blastocyst hatching via the regulation of lipid metabolism.
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1. Introduction

Peroxisome proliferator-activated receptor (PPAR) has three known isoforms, *Pparα*, *Pparδ*, and *Pparγ*. *Pparα* and *Pparγ* have been studied extensively; they are initially expressed during late embryonic development [1]. *Pparγ* has been detected in the trophectoderm and inner cell mass of intact and hatched blastocysts [2]. Additionally, *Pparγ* knockout in oocytes and granulosa cells leads to reduced fertility [3,4]. In contrast, *Pparδ* is ubiquitously expressed and appears to play a general role in development [5]. It functions as a transcription factor in the regulation of genes involved in many biological processes, such as metabolism, cellular differentiation, and inflammation. Several recent studies have demonstrated that the prostacyclin (PGI_2)/PPARδ axis increases blastocyst hatching and implantation *in vitro* [6,7], and PGI_2 is a potential agonist of PPARδ [8]. PPARδ activation by PGI_2 or a synthetic agonist increases both the rate and quality of blastocyst implantation sites [9]. PGI_2-induced PPARδ activation accelerates blastocyst hatching, however the deletion of *Pparδ* results in delayed blastocyst hatching, but not in impaired blastocyst development [6].

Fatty acid β-oxidation (FAO) is a critical and efficient energy source for oocyte and embryonic development [10–13]. The oxidation of a single fatty acid, such as palmitate, can produce up to 106 ATP molecules [14]. The utilization of FAO is dependent on the

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transport of fatty acids into mitochondria and catalysis by carnitine palmitoyl transferase 1 (*Cpt1*). Carnitine is then removed by *Cpt2*, and the fatty acid undergoes β-oxidation to produce ATP [15]. FAO is essential to oocyte nuclear maturation in mouse, bovine, and porcine oocytes [10]. The stimulation of FAO in cumulus oocyte complexes (COCs) has a positive effect on embryonic development, while reduced FAO negatively affects mouse oocyte quality [16]. The inhibition of FAO can cause a reduction in the number of trophectoderm and inner cell mass cells in mouse blastocysts [17]. Carnitine-induced stimulation of FAO during embryo culture has a positive effect on embryonic development [18].

Several studies have confirmed that the activation of PPARδ enhances FAO. Overexpression of *Pparδ* in adipose tissues increases the expression of genes involved in fatty acid oxidation [19]. In addition, the activation of PPARδ by an agonist affects the regulation of genes involved in fatty acid transport, FAO, and mitochondrial respiration in skeletal muscle [20]. A similar effect has been observed for a PPARδ agonist in a line of mouse skeletal muscle cells, which display an increase in the expression of genes involved in FAO and lipid utilization [21].

Recent evidence suggests that *Pparδ* is important for embryonic development. The PGI_2/PPARδ axis could control blastocyst hatching [6,9]. However, the mechanism by which *Pparδ* influences blastocyst hatching is unclear. Thus, in the current study, we examined whether *Pparδ* regulates blastocyst hatching via the ATP production pathway. We also determined whether ATP production is regulated by glucose metabolism or lipid accumulation in porcine blastocysts during the hatching process.

2. Materials and methods

All chemicals used in this study were purchased from Sigma–Aldrich (St.Louis, MO, USA), unless otherwise indicated.

2.1. Oocyte collection, in vitro maturation, and embryo culture

Ovaries from prepubertal gilts were obtained from a local slaughterhouse, maintained in saline at 37 °C, and transported to the laboratory. Follicles that were 3 to 6 mm in diameter were aspirated. Cumulus-oocyte complexes (COCs) that were surrounded by more than three layers of cumulus cells were selected for culture [22]. In total approximately 500 COCs were isolated from follicles and washed three times in TL-HEPES. COCs were cultured in tissue culture medium 199 (TCM 199) supplemented with 10% porcine follicular fluid, 0.1 g/L sodium pyruvate, 0.6 mM L-cysteine, 10 ng/mL epidermal growth factor, 10 IU/mL luteinizing hormone, and 10 IU/mL follicle stimulating hormone at 38.5 °C for 44 h in a humidified atmosphere of 5% CO_2 and 95% air. The PPARδ agonist GW501516 (Merck Millipore, Darmstadt, Germany) was added to the IVM medium at concentrations of 1 and 10 μM. After maturation, cumulus cells were removed by treatment with 0.1% hyaluronidase for 2 to 3 min and repeated pipetting. For parthenogenetic activation, oocytes with polar bodies were selected and activated by two direct current pulses of 1.1 kV/cm for 60 μs and then incubated in porcine zygote medium (PZM-5 or UCSU37) containing 7.5 μg/mL cytochalasin B for 3 h. Finally, in total approximately 420 embryos were cultured in PZM-5 or UCSU37 medium for 8 d at 38.5 °C in a humidified atmosphere of 5% CO_2 and 95% air. On the 5th day, fetal bovine serum was added to the medium at a concentration of 4%. To observe the effect of *Pparδ* on early porcine embryonic development, the PPARδ agonist GW501516 was added to the medium after activation at final concentrations of 10, 50, or 100 μM ($n = 20$ per treatment). A concentration of 10 μM was used in subsequent experiments because it was the minimum concentration that had an effect on blastocyst hatching.

2.2. ATP content assay

The ATP Determination Kit (Invitrogen, Carlsbad, CA, USA) was used to measure ATP concentrations in blastocysts exposed to 0 or 10 μM GW501516 (10 blastocysts per group × 3 replicates). Briefly, samples were washed three times with PBS and then transferred individually into 1-mL tubes on ice. Media were removed and blastocysts were frozen and thawed for lysis. Approximately 100 μL of ice-cold somatic cell reagent (FL-SAR) was added to each tube, and samples were incubated in an ice-water bath for 5 min. Thereafter, 100 μL of ice-cold assay buffer (diluted 1:25 with ATP assay buffer, FL-AAB) was added and the tubes were maintained at room temperature for 5 min under limited light conditions. The ATP concentration was measured using a luminometer (Berthold, Wildbad, Germany) with a sensitivity of 0.01 pmol. The ATP concentration in the control group was arbitrarily set to 1.

2.3. Glucose consumption assay

The glucose concentration was determined using the Glucose (HK) Kit (GAHK20; Sigma). Briefly, UCSU37 medium was collected after an 8-day culture of embryos in the presence or absence of GW501516. The medium and glucose assay reagent were pipetted into the tubes, mixed, and incubated for 15 min at room temperature. The glucose concentration was examined by measuring absorbance at 340 nm. The glucose concentration in the control group was arbitrarily set at 1. Three separate experiments were performed with 10 blastocysts per group examined in each experiment.

2.4. Membrane potential assay and mitochondrial copy number analysis

Day-8 blastocysts were washed three times with PBS and incubated in culture medium containing 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1) (Invitrogen) at a concentration of 1 mM at 37 °C in 5% CO_2 for 30 min. Membrane potential was calculated as the ratio of red florescence, which corresponded to activated mitochondria (J-aggregates), to green fluorescence, which corresponded to less-activated mitochondria (J-monomers) [23]. Fluorescence was visualized using an epifluorescence microscope (Nikon Corp., Tokyo, Japan). The fluorescence intensity in the control group was arbitrarily set at 1, and the fluorescence intensity in the treatment group was measured and expressed as relative values with respect to that of the control group. Three separate experiments were performed with 10 blastocysts per group examined in each experiment.

Total DNA was isolated from pooled 10 blastocysts using the Puregene DNA Isolation Kit (Invitrogen) according to the manufacturer's instructions. Blastocyst DNA samples were then used for real-time polymerase chain reaction (PCR) experiments. The primers for *Ndufaf3* and *Gapdh* were described in a previous study [24]. The reactions were performed as follows: 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s, and a final extension at 72 °C for 5 min. The relative quantification of mitochondrial copy number was performed using the $2^{-\Delta\Delta Ct}$ method. Three separate experiments were performed with 10 blastocysts per group examined in each experiment.

2.5. Lipid content measurements

Lipid content was measured in Day-8 blastocysts cultured in the presence or absence of GW501516. Blastocysts (10 blastocysts per

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